# INVESTIGATION ON IMMUNOPROTECTIVITY OF RECOMBINANT VACCINE CANDIDATES GLUTAMINE-BINDING PERIPLASMIC PROTEIN AND PUTATIVE PEPTIDOGLYCAN BINDING-PROTEIN IN MOUSE MODEL

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## ABSTRACT

### INVESTIGATION ON IMMUNOPROTECTIVITY OF RECOMBINANT VACCINE CANDIDATES GLUTAMINE-BINDING PERIPLASMIC PROTEIN AND PUTATIVE PEPTIDOGLYCAN-BINDING PROTEIN IN MOUSE MODEL

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*Bordetella pertussis* is a gram negative coccobacillus that causes pertussis known as whooping cough. After mass-vaccination started in 1940s, incidence of the disease has decreased. However, *B. pertussis* circulation in population has not been prevented completely. Starting from the first vaccination, development of several vaccines have been performed. These whole cell (Pw) and acellular pertussis (Pa) vaccines are not completely effective in terms of sustained, lifelong immunity and thus failure in eliminating subclinical infections poses a threat for both unimmunized infants and adults. The requirements for more effective acellular pertussis vaccines with protective proteins have raised the number of the studies to develop vaccines with high immune protective capacities.

Recently, a surface antigen, namely glutamine-binding periplasmic protein GnlH was found to be among novel immunogenic proteins as shown by our immunoproteome group. Besides; another surface antigen putative peptidoglycan binding protein BP0020 was shown to include Lysm domain which is found in some of protective immunogens. These proteins were chosen as novel recombinant vaccine candidates. In the present study these proteins were tested for their immunoprotective capacity in mouse model. In order to stimulate humoral and cellular responses against *B. pertussiss* infection, the genes coding for gnlH and BP0020 proteins were amplified from the genomic DNA of *B. pertussis* strains and cloned into pGEM®-T Easy vector and sequenced. For their expression in *Escherichia coli* BL21(DE3) cells, expression vector pET-28a (+) was used together with IPTG induction system. After the expression of the desired proteins His-tag affinity chromatography together with dialysis was used for the purification of the proteins. Following Western blot analysis, 10 μg of both GlnlH and BP0020 proteins were used to immunize BALB/c mice (16-18 g) at days 0 and 21. *B. pertussis* Saadet live cells were then administered intranasally to challenge mice. Bacterial colonization in mice was determined after the removal of the lungs at days 5 and 8. When compared to the control groups, bacterial colonization were found to be decreased in the lungs of the mice immunized with 10 μg recombinant GlnH and BP0020 proteins. ELISA for serum-specific IgG levels was performed after the collection of sera. The results showed that IgG levels were significantly higher in immunized mice. In addition; serum IFN-γ levels were found to be higher in vaccinated mice in comparision to control groups.

**Keywords:** Whooping cough, *Bordetella pertussis*, Subunit vaccines, Immune response, glutamine-binding periplasmic protein GnlH, putative peptidoglycan-binding protein BP0020

# ÖZ

## REKOMBİNANT AŞI ADAYLARI GLUTAMİN-BAĞLANMA PROTEİNİ GlnH VE PÜTATİF PERİPLAZMİK-BAĞLANMA PROTEİNİ BP0020'İN İMMÜN KORUYUCULUĞUNUN FARE MODELLERİNDE ARAŞTIRILMASI

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*Bordetella pertussis* boğmaca (pertussis) hastalığına neden olan gram negative, kokobasil bir bakteri türüdür. 1940'lı yıllarda gerçekleştirilen yoğun aşılama çalışmaları hastalığın insidansını yüksek oranda düşürmüştür ancak *B. pertussis*'in insan popülasyonu içerisindeki dolaşımı tam olarak ortadan kaldırılamamıştır. İlk asiların geliştirilmesinin ardından yeni aşıların üretilmesi yönündeki çalışmalar hız kazanmıştır. Söz konusu tam hücre (Pw) ve aselüler (Pa) pertussis aşıları hayat boyu süren bir koruyuculuk sağlama açısından yetersizdirler. Bu nedenle subklinik enfeksiyonlar engellenemektedir ve bağışıklık geliştirememiş yeni doğanlar ile erişkinler açısından risk devam etmektedir. Koruyucu proteinler içeren ve yüksek immün koruyuculuk kapasitesine sahip olan daha etkin aselüler aşıların geliştirilmesi yönündeki çalışmalar günümüzde giderek hız kazanmaktadır.

Periplazmik glutamin–bağlanma proteini GlnH'nin immünojenik özellikler taşıdığı grubumuz tarafından daha önce gerçekleştirilen immünoproteom çalışmalarında gösterilmişti. Buna ek olarak; varsayımsal peptidoglikan bağlayıcı protein (BP0020) daha önce yapılan çalışmalarda immün-koruyucu olduğu gösterilen proteinlerde bulunan Lysm bölgesini içermektedir. Şimdiki çalışmada söz konusu proteinlerin immün koruyuculukları fare modeli ile tespit edilmeye çalışılmıştır. GlnH ve BP0020 proteinlerini kodlayan genler *B. pertussis* genomik DNA'sı kullanılarak PCR yöntemi ile çoğaltılmış ve pGEM®-T Easy vektörüne klonlanıp sekanslanmıştır. İlgili genlerin *Escherichia coli* BL21(DE3) hücrelerinde anlatımlarının gerçekleştirilmesi için pET-28a (+) ekspresyon vektörüne yerleştirilmiştir. IPTG indüksiyon yöntemi uygulanarak rekombinant anlatımları sağlanmıştır. His-tag afinite kromatografi ve diyaliz yöntemlerinin uygulanması ile rekombinant proteinler saflaştırılıp Western blot ile doğrulukları test edildikten sonra aşılamaya hazır hale getirilmiştir. BALB/c fareleri (16-18 gr) GlnH ve BP0020 proteinlerinin her birinden 10 µg içeren kombine aşı ile 0 ve 21. günlerde aşılanmış, ardından *B. pertussis* Saadet hücrelerinin (2.5 x $10^9$) kullanımı ile intranasal yolla enfekte edilmişlerdir. Farelerin *B. pertussis* Saadet canlı hücreleri ile enfekte edilmesini takip eden 5 ve 8. günlerde akciğer alımları gerçekleştirilmiş ve bakteriyel kolonizasyon incelenmiştir. GlnH ve BP0020 proteinlerinin her birinden 10 µg içeren kombine aşı ile immünize edilmiş farelerin akciğerlerindeki bakteri kolonizasyonu kontrol grupları ile karşılaştırıldığında oldukça düşük çıkmıştır.Seruma özgü IgG seviyelerinin ölçümü için ELISA yöntemi kullanılmıştır. ELISA sonuçlarına göre; immünize edilen farelerin IgG seviyesinde kaydadeğer bir artış gözlenmiştir. Ayrıca kontrol grupları ile ile karşılaştırıldığında aşılanmış farelerin serum IFN-γ seviyelerinin de yükselmiş olduğu gözlenmiştir.

**Anahtar kelimeler:** Boğmaca, *Bordetella pertussis*, Altbirim aşı, Periplazmik glutamin–bağlanma proteinl, Pütatif peptidoglikan-bağlanma proteini BP0020, İmmün yanıt

***To my family***

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## LIST OF ABBREVIATIONS

| | |
|---|---|
| bp(s) | : Base pair(s) |
| *B. pertussis* | : *Bordetella pertussis* |
| DC | : Dendritic cell |
| DTPw | : Diphteria, Tetanus, whole cell Pertussis vaccine |
| DTPa | : Diphteria, Tetanus, acellular Pertussis vaccine |
| *E. coli* | : *Escherichia coli* |
| IPTG | : Isopropyl-b D-thiogalactopyranoside |
| i.p. | : Intraperitoneal |
| IFN-γ | : Interferon gamma |
| IL | : Interleukin |
| IgE | : Immunogobulin E |
| IgG | : Immunoglobulin G |
| kDa | : Kilodalton |
| LPS | : Lipopolysaccharide |
| NCBI | : National Center for Biotechnology Information |
| OD | : Optical Density |
| SDS-PAGE | : Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis |
| Th | : T helper cell |
| TNF | : Tumor necrosis factor |

# CHAPTER 1

# INTRODUCTION

## 1.1. Whooping Cough (Pertussis)

*Bordetella pertussis*, which is the major causative agent of highly infectious respiratory system disease "whooping cough", is a non-motile, small, fastidious gram-negative coccobacillus and an obligate human pathogen (Belcher *et al*., 2000; Mattoo and Cherry, 2005). The disease results in some potentially mortal complicatons such as; convulsions, encephalitis, bronchopneumonia, encephalopathy and persistent brain damage (Cherry *et al*., 1988; Wortis *et al*., 1996). Despite susceptibility of all age categories to infection whooping cough is predominant in infants, as recommended vaccination schedules do not begin until 2 months of age, (Kerr and Matthews, 2000). Incidence rate of pertussis was decreased dramatically after introduction of a whole-cell vaccine in 1940s, but the disease attracted a renewed attention because of its resurgence in populations that have high vaccine coverage (Mooi *et al*., 2007; Van der Ark *et al*., 2012). This resurgence could be associated with multiple factors that include increased awareness of disease, antigenic diversity between vaccine strains and clinical isolates as well as low efficiency of current vaccines (Cherry and Olin, 1999; Mattoo and Cherry, 2005; He and Mertsola, 2008).

### 1.1.1. History of Whooping Cough

Unlike other severe contagiouss diseases of human (i.e., measles and smallpox) whooping cough does not have an anchient history (Holmes, 1940). In 1540, the

whooping was first mentioned as a disease by Moulton's The Mirror of Health (Lapin, 1943). However the history of whooping cough has began after an epidemic in Paris, which was reported by Guillaume de Baillou (Cone, 1970). It was Thomas Sydenham, an English physician who had used the term “pertussis” (meaning intense cough) for the first time, in 1679 (Mattoo and Cherry, 2005). Jules Bordet and Octave Gengou examined a 5 month-old child’s sputum and observed the etiologic agent of the disease under microscope in 1900. Thus, they reported the methods for the isolation and cultivation of the organism in 1906 and this discovery was a milestone for development of whole-cell vaccines against whooping cough (Bordet and Gengou, 1906).

### 1.1.2 Pathogenesis of Whooping Cough

The transmission of whooping cough is presumed to be airbone. Severe coughing of an infected person results in aerosol droplets carrying *B. pertussis* which afterwards attaches to respiratory epithelium of the new host (Tiwari *et al*., 2005). Interaction between ciliated epithelial cells and the bacterium occurs with the help of filamentous hemagglutinin and pertussis toxin of the bacterium (Figure 1). It is thought that combined effect of pertussis toxin, filamentous hemaglutinin and other virulence factors results in paralysis of the cilia, death of ciliated cells, escape of bacteria from host defence and local tissue damage.

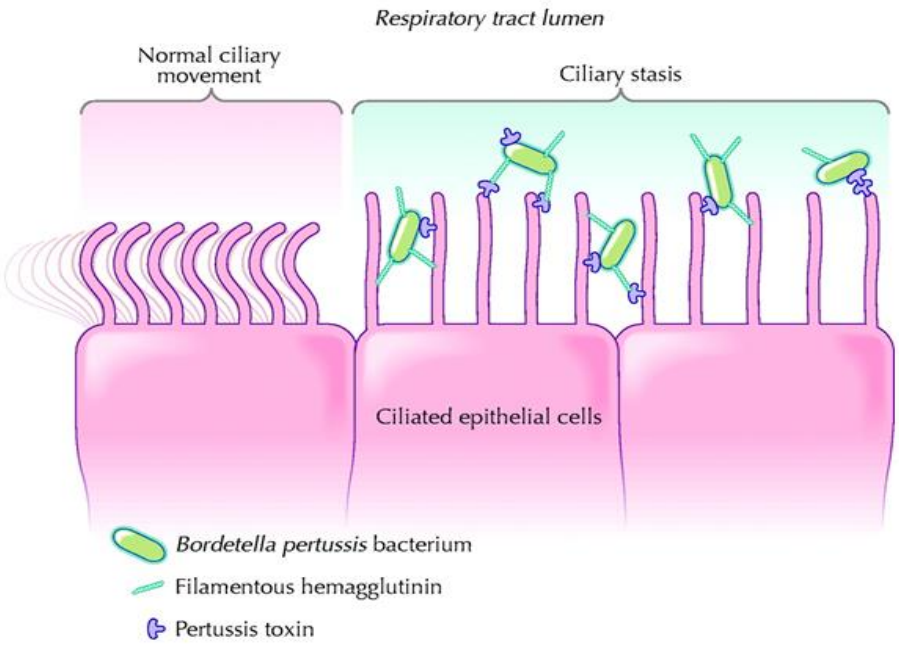


**Figure 1** Synergistic effect of virulence factors in attachment to epithelial cells of respiratory tract (Tozzi et al., 2005).

World Health Organization defined three stages of typical pertussis, namely; catarrhal, paroxismal and convalescent (Table 1). After an incubation period of 5 to 20 days catarrhal stage manifests itself with sypmtoms of a mild common cold (Cherry, 1999). These non-specific symptoms include rhinorrhoea, conjunctivitis, non-productive cough, low-grade fewer and sneezing. Since bacteria can spread easily during catarrhal stage, it is quite possible to recover *B. pertussis* from patients' nasopharyngeal specimen during this stage (Weiss and Hewlett, 1986).

At the end of the first stage, which lasts for 1-2 weeks, mild cough turns into paroxysmal cough with mucous production which marks the beginning of the paroxysmal stage (Olson, 1975). During this acute stage of the disease, due to the blockage of the lung airways with mucous, episodes of coughing end with classical deep inspiration (whooping) associated with the disease. Initially, the paroxysmal

coughs are more common at night but after a while they become frequent also during the day (Manclark and Cowell, 1984). This stage of the disease lasts for 1-2 weeks or longer.

The transition from paroxysmal stage to convalescent stage is usually gradual. The third stage of the disease is characterized by a decrease in the frequency of paroxysmal coughing coupled with a decrease of the disease severity. In case of a superimposed respiratory disease during the third stage, frequency of paroxysms can increase (Weiss and Hewlett, 1986; Sinha and Heath, 2005). The convalescent stage usually lasts for 1-2 weeks but occasionally non-paroxysmal coughing may persist up to severel months (Weiss and Hewlett, 1986; Tiwari *et al*., 2005)

Factors such as age of the patient, individual immunity, antibiotic administration, strain of pathogen, time elapsed after previous vaccination may affect the clinical symptoms and consequently all patients may not exhibit similar symptoms. Studies have shown that clinical symptoms that are generally observed in newborns include pneumonia and apnea whereas prolonged coughing is more common in adults and adolescents (Riffelmann *et al*., 2008).

**Table 1** Typical symptoms of pertussis and their evolution (Tozzi et al., 2005).

| Variable | Catarrhal phase (1-2 wk) | Paroxismal phase (3-6 wk) | Convalescent phase (>6 wk) |
|---|---|---|---|
| **Symptom** | | | |
| Cough | ++ | +++ | ++ |
| Paroxysmal cough | -/+ | +++ | -/+ |
| Whooping cough | - | +++ | -/+ |
| Vomiting | - | +++ | -/+ |
| Cyanosis | - | +++ | - |
| Apnea | - | +++ | - |

Note: + present, - absent, -/+ equivocal

### 1.1.3 Diagnosis and Treatment of Whooping Cough

Differential diagnosis of *Bordetellae* infections (*Bordetella pertussis* and *Bordetella parapertussis*$_{Hu}$) from other diseases that cause similar symptoms can be made by observation of paroxsysmal cough, absolute lymphocytosis, vomiting and low-grade fewer. However, distinguishing *B. pertussis* infection from *B. parapertussis*$_{Hu}$ infection requires sensitive and specific laboratory diagnosis techniques such as efficient culturing, PCR, direct fluorescent-antibody (DFA) test and serologic-based techniques (Mattoo and Cherry, 2005). During cultivation of *B. pertusiss* sub-standart culture results can be observed because of insufficient specimen collection and transportation (Müller *et al*., 1997). PCR amplification is another diagnostic tool to differentiate *B. pertussis* infection from other diseases. Primers that are specific to adenylate cyclase gene, promoter region of genes encoding pertussis toxin or repeated insertion sequences can be used for amplification but one should be aware of false positives resulting from contaminations (Müller *et al*., 1997; Mattoo and Cherry, 2005). Although it has a low sensitivity, DFA is an also rapid and inexpensive

method for detection of *B. pertussis* infection. Together with ELISA, serological methods like monitoring the increase in agglutinating-antibody titers against FIM2/3, LPS and PRN or detection of IgA, IgG and IgM against specific antigens (i.e. FHA, FIM, PRN and PT) can also be used for differential diagnosis (Mattoo and Cherry, 2005).

Mattoo and Cherry (2005) stated in their rewiev that there are a number of antibiotics which has *in vitro* activity against pertussis. For example, erythromycin has a widespread usage against pertusiss and is particularly effective when the treatment is started during catarrhal stage of the disease since it shortens duration of symptoms and individual contagiousness (Bergquist *et al*., 1987; Güriş *et al.*, 1999, Mattoo and Cherry, 2005). The studies conducted by Lewis *et al.* (1995) and Korgenski and Daly (1997) showed that some strains of *B. pertussis* were resistant to erythromycin as contrary to the common view. The results made it possible to use trimethoprim-sulfamethoxazole in the treatments of the patients that were infected with an erythromycin-resistant strain or of the individuals having possibility to develop side effects to erythromycin (Hoppe *et al*., 1989). In addition to trimethoprim-sulfamethoxazole, the other two promising macrolides were reported as azithromycin and clarithromycin by Wood and McIntyre (2008), which show little side effects.

### 1.1.4 Epidemiology of Pertussis

Mainly *B. pertussis* and occasionally *B. parapertussis* causes whooping cough. In pertussis cases, as reported by Watanabe and Nagai (2003), incidence of *B. parapertussis* infection shows an alteration at 1% to 40% range in different countries. Pertussis is classified as an endemic disease in United States and it shows epidemic peaks with every 3 to 4 years (Mooi *et al*., 2007). Annually, highest pertussis activity is observed in summer months (from July to September) (Health Protection Reports for Pertussis, 2013). Yet, there is a significant decrease in the number of pertussis cases as a result of vaccination; the same cyclic pattern still occurs (Cherry, 2005). Pertussis is the one of the most contagious infectious diseases in the world.

Annually, 48,5 million people are affected by whooping cough around the World (Bettiol *et al*., 2010). Pertussis is highly contagious, as can be proved with secondary attack rates reaching to 80% among susceptible household contacts. Persons with pertussis are most likely to transmit the disease to susceptible new hosts during the catarrhal period and for the first 2 weeks after cough onset (American Academy of Pediatrics. Pertussis, 2009). The disease has no race discrimination (Mooi *et al*., 2007).

In the USA during the years before 1940s, pertussis was the most mortal infectious disease on children which were at the ages of less than 14 years. During this prevaccine era, incident rate of the disease was 150 cases per 100,000 population (Cherry, 1984). In England and Wales in the prevaccine era, the average annual incidence of reported pertussis was 230 per 100,000 population (Mattoo and Cherry, 2005). More than 93% of the reported cases in the U.S before the vaccines, among the average of 178,171 cases the disease affected in the children under 10 years old. After 1940s, vaccines were introduced and incidence decreased dramatically (Pertussis (Whooping Cough), 2013).

Pertussis is one of the vaccine-preventable infectious diseases which are resulting in increasing deaths in the U.S. The number of deaths increased from 4 in 1996 to 17 in 2001, almost all of which were infants under one year (Gregory, 2006). The incident rate in infants who are too young for being vaccinated (i.e younger than 5 months of age) increased 49% from 1980s to 1990s (Tanaka *et al*., 2003).

In 1990s number of deaths due to pertussis was 167.000 and this number decreased in 2010 to 81.000 (Lozano *et al*., 2012). This reduction was due to vaccination programs. Although pertussis is one of major causes of deaths world-wide 90% of all cases occur in developing countries (Pertussis, 2013).

Although predominance in the deaths caused by pertussis occurs in the infants younger than 5 months (Celentano *et al*., 2005), some studies have suggested an

alteration between infants and adults in terms of incidence of the disease (Skowronski *et al*., 2002; von Konig *et al*., 2002; Hewlett and Edwards, 2005). Because of less severe symptoms or subclinical cases, the incident rate of the disease in adults has not been reported clearly. Thus, it could be suggested that adults and adolescents are reservoir of the disease and this situation poses a treat for unvaccinated infants (Schellekens et al., 2005; von Konig *et al.,* 2002).

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

### 1.2.1 Phylogeny of the Genus

In the light of the recent studies, *Bordetella* genus is mostly related to the genus Achromobacter which belongs to the family Alcaligenaceae (Gerlach *et al*., 2001; Brenner *et al.*, 2005; Gerlach *et al*., 2004). "*B. petrii*, *B. parapertussis*, *B. holmesii*, *B. pertussis*, *B. avium*, *B. trematum*, *B. bronchiseptica*, *B. ansorpii*, and *B. hinzii"* are the species that currently listed as *Bordetella* species (Figure 2). *B. pertussis*, *B. bronchiseptica* and *B. parapertussis* are found in human niche as reported in the studies performing comparative analyses of their 16s rRNA sequences. In addition these species generate a closely related linage which is easily distinguished from other species. The result of a multilocus enzyme electrophoresis (MLEE) studies state that the genetic divergence between these three "classical" *Bordetella* species is less than other bacterial pathogens (Musser *et al*., 1986). The host range of *B. bronchiseptica* is quite large and includes many mammals. *B. bronchiseptica* seldomly causes respiratory tract infections in humans which is mainly observed in immunocompromised individuals (Woolfrey and Moody, 1991). *B.parapertussis*$_{Hu}$ and *B.parapertussis*$_{Ov}$ that are isolated from human and sheep, are two clearly distinguished linages of *B. pertussis* (Cullinane *et al*., 1987). According to the result of several studies examining the insertion sequence (IS) element polymorphism, *B. parapertussis* was the most similar species to the common ancestor among the others.

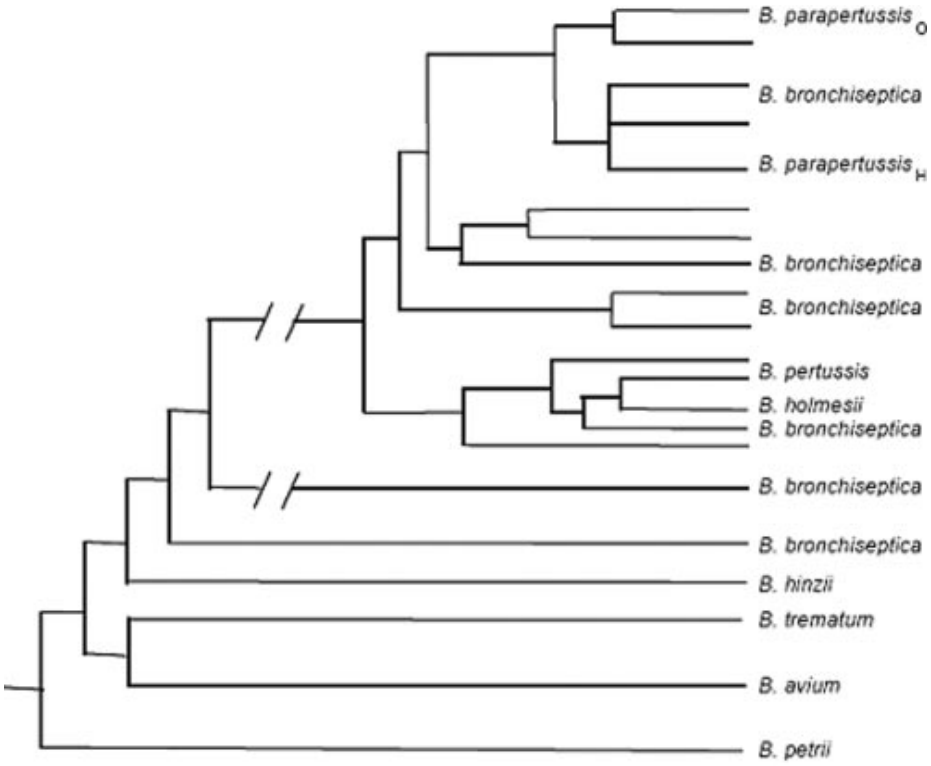


**Figure 2** Phylogenetic tree of *Bordetella* species (Mattoo and Cherry, 2005).

Although *B. pertussis* and *B. parapertussis*$_{Hu}$ have both arisen from *B. bronchiseptica* linage; they produced different lineages (van der Zee *et al*., 1997; Reischl *et al*., 2001). The close relationship of these two human-restricted species does not show any similarity in terms of immunogenicity (Khelef *et al*., 1993). Moreover, *B. parapertussis* was emerged latterly in comparison to *B. pertussis* according to the results of multilocus sequence typing (MLST), comparative genomic hybridization (CGH) (Diavatopoulos *et al*., 2005) and MLEE studies (van der Zee *et al*., 1997). The genome size and the number of functional genes of *B. bronchiseptica* are higher in comparison to the other two species. The smallest number of functional genes and the smallest size of the genome are found in *B. pertussis* (Parkhill *et al*., 2003). These

findings suggest that the decrease in the genome size as well as the rearrangements mediated by IS elements in the large genome are important in the evolution of these two human-restricted pathogens. In comparison to *B. pertussis* genome the co-linearity of *B. parapertussis* genome with that of *B. bronchiseptica* is quite higher and this shows the role of genome rearrangements (Cummings *et al*., 2004).

There are two main selection pressures which affects the evolution of *B. pertussis* and *B. parapertussis*. One is the selection pressure for a transition from chronic to acute infection and the other for recovery from existent host immunity (Bjornstad and Harvill, 2005). For instance, Pertussis toxin (PT) expression is limited to *B. pertussis* and it favors acute infection over chronic one (Kirimanjeswara *et al*., 2005). Similarly, bacterial type three secretion system (TTSS) supports the elongation of infection (Cornelis, 2000). In addition, there is a modification in lipid A structure of LPS molecules of *B. parapertussis* and *B. pertussis*. This situation results in evasion of these pathogens from host cell immune response mediated by Toll-like receptor (TLR-4) (Raetz and Whitfield, 2002; Mann *et al*., 2005).

As mentioned above, escaping from the existing immunity has an improtant role in the evolution of both *B. parapertussis* and *B. pertussis* as one of driving forces. With the exception of immunocompromised hosts and people that are in close contact with infected animals, replacement of *B. parapertussis* and *B. pertussis* infections with *B. bronchiseptica* infection caused a decrease in human infections by *B. bronchiseptica*. This replacement has been caused by the evolution which was probably caused via a selection pressure for variants which were able to avoid existing anti-*B. bronchiseptica* immunity as reported by Preston *et al*. (2007). For instance, *B. pertussis* does not have any O antigen, a highly immunogenic protein and expressed by *B. bronchiseptica* (Preston *et al*., 1999). As menioned before, *B. parapertussis* has evolved latterly and thus this brings the question whether existent anti-*B. pertussis* immunity was the main selection pressure in in human niche as reported by (Preston *et al*. (2007). Another issue is the expression of highly immunogenic trisaccharide called Band A in *B. pertussis* while it is absent in the parapertussis (Preston *et al*.,

2006).

Interestingly, because of the fact that O-antigen is being expressed by *B. parapertussis* as reported by Preston *et al*. (2007) it might be suggested that the major selection pressure role of anti *B. bronchiseptica* immunity was lost during the evolution of *B. parapertussis*.

### 1.2.2 Micro-evolution of *Bordetella pertussis*

*B. pertussis* species has low genetic diversity as a result of mass vaccination carried out since 1940s (van Loo and Mooi, 2002; Weber *et al*., 2001; Elomaa *et al*., 2005; van Loo *et al.,* 1999;). Still, genes encoding Pertussis toxin subunit-A (PtxA), Tracheal colonization factor A (TcfA), Pertactin (Prn) and fimbrial units; *ptxA, tcfa, prn* and *fim*, respectively, show polymorphisms at a level of amino acid substitions. These proteins are used in the acellular vaccines because of their immunoprotective capacities (Storsaeter *et al*., 1998; Cherry *et al*., 1998; Taranger *et al*., 2000).

One way used by pathogens to escape the immunity of host is the polymorphism in repeat sequences (Gravekamp *et al*., 1996; Franks *et al*., 2003; Delogu and Brennan, 2001). The *prn* gene coding for Prn has eleven identified alleles. (Mooi *et al*., 1998). They have two repeat sequences called R1 and R2. R1, which consists of "GGxxP" repeats, places near the specific motif that is responsible for adhesion of the host cell and called RGD (Leininger *et al*., 1992). Both of repeat sequences have protective epitope property directly related with the efficacy of the vaccine (Charles *et al*., 1991; King *et al*., 2001). Although there was a protective capacity, lacking of Prn cross-protectivity was stated in the study performed in a mouse model. In this study it was shown that immune response induced by Prn of *B. pertussis* did not has protective effect against *B. parapertussis* infection (Khelef *et al*., 1993). In most pertussis vaccines including Prn1, however, Prn2 is predominate in current *B. pertussis* populations (Mooi *et al*., 2007).

There are 5 subunits called S1-S5 or PtxA-PtxE in a Pertussis toxin. The most variable one is PtxA so it is the most immunogenic one (De Magistris *et al*., 1989; van Loo *et al*., 2002). In *B. pertussis*, there are four distinct PtxA subunutis and they are coded by six *ptxA* alleles. Current *B. pertussis* populations have PtxA1 predominantly but in the most pertussis vaccines there is PtxA2 (Mooi *et al*., 2007).

There are three fimbrial genes in *B.* pertussis called "*fimX, fim3* and *fim2"*. Predominantly, *fim3* and *fim2* are expressed (Willems *et al*., 1994; Locht *et al*., 1992; Parkhill *et al*., 2003) and as reported by Mink *et al.* (1994) they are used in determination of serelogical types 3 and 2 of *B. pertussis*. Relationship between predominating fimbrial serotypes isolated from the patients and present in the whole-cell vaccines is reciprocal and the immunoprotectivity of these fimbrial antibodies was previously shown (Preston, 1985). This property was used for testing the efficaciousness of both acellular and cellular vaccines of pertussis in several other studies (Stanbridge and Preston, 1974; Hallander *et al*., 2005). There is a polymorphism between *fim2* and *fim3*. An SNP which results in an amino acid substition causes the formation of two alleles of *fim2* (van Loo and Mooi, 2002) while three SNPs cause the formation of four alleles of *fim3*. As reported by Tsang *et al.* (2004) these are called *Fim3C, Fim3B* and *Fim3A*. There is a discrepancy in terms of frequency of alleles included in whole-cell vaccines and those in clinical isolates. This discrepancy is not limited to *fim* genes and is a reflection of the phenomenon called "vaccine-driven evolution". (Mooi *et al*., 1998; Fry *et al*., 2001; Mooi *et al*., 2001; Packard *et al*., 2004; van Amersfoorth *et al*., 2005). Also, as reported by Wendelboe *et al*. (2005) this phenomenon can be classified as one of the factors which is responsible for re-emerge of pertussis.

### 1.2.3. Virulence Factors and Molecular Pathogenesis of *Bordetella pertussis*

*B. pertussis* has variable virulence factors such as toxins, adhesins and autotransporters. The major regulators effecting the expression of these virulence factors are certain genetic modifications and phenotypic modulations occurring in

response to alterations in environmental conditions (Lacey, 1960; Stibitz *et al*., 1989). Both of these mechanisms are rooted to a two-component phosphorelay system expressed from BvgA/S locus and called "BvgAS" (Fig 3) (Uhl and Miller, 1996). Main virulence factors are regulated by this system.

#### 1.2.3.1 BvgAS System

BvgA and BvgS proteins are two main components of BvgAS system (Fig 3). Cytoplasmic membrane protein BvgS includes five domains as; linker (L), periplasmic (P), histidine phosphotransfer (HPD), receiver (R) and transmitter (T). L domain attaches P domain, which has ability of sensing changes in environmental conditions, to cytoplasmic R, T and HPD domains. H729 residue of T domain is autophosphorylated at 37°C and very low concentrations of $MgSO_4$ and nicotinic acid concentrations (Uhl and Miller, 1994; Uhl and Miller, 1996; Cotter and Dirita, 2000). This phosphoryl group is transferred to reciever (R) domain of BvgA protein passing through R and HPD domains of BvgS protein. As reported by Mattoo and Cherry (2005) BvgA protein has a helix-turn-helix (HTH) domain and phosphorylation of BvgA protein leads to the conversion of BvgA into a transcriptional activator which subsequently binds to promoters and thus transcription of genes classified as "virulence-activated genes" (*vag*) or "$Bvg^+$-phase-specific genes" are activated. BvgA activates BvgR, a transcription repressor that represses the expression of a group of genes denoted as "virulence-repressed genes" (*vrg*) or "$Bvg^-$-phase specific genes". This activation is achieved by binding of BvgA to operator sequences (Matto and Cherry, 2005). When conditions are changed, such as reduction of temperature under 37°C and at high concentrations of MgSO4 and nicotinic, signalling cascade is reversed: the BvgAS system, consequently the regulation of BvgR system, becomes inactivated which results in down-regulation of *vag* genes and derepression of *vrg* genes, respectively (Locht *et al*., 2001). However, it should be noted that there is no all-or-none phenomena about shutdowning of the BvgAS system. For example, as reported by Mattoo *et al.* (2005), the $Bvg^i$ phase, which is an intermediary phase of $Bvg^+$ and $Bvg^-$, can be observed in specific

environmental conditions such as defined range of nicotinic acid concentrations. The characterization of $Bvg^i$ phase depends on repression of *vrg* and some other genes and expression of a subset of *vag* genes (Mattoo *et al*., 2005). Frameshift mutations in *bvgAS* locus or phenotypic modulation due to environmental changes described above are two main regulators of BvgAS system (Stibitz *et al*., 1989).

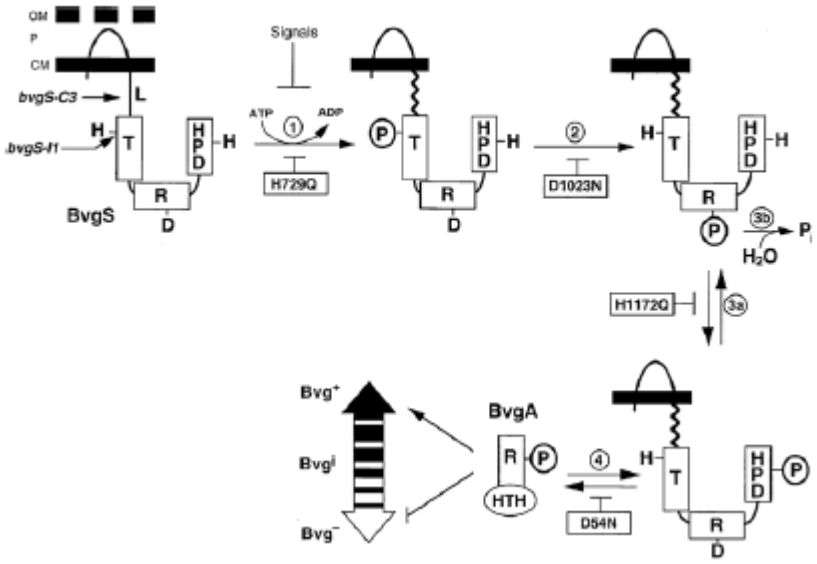


**Figure 3** BvgAS system (Cotter and Dirita, 2000).

### 1.2.3.2 Filamentous Haemagglutinin (FHA)

As reported by Loch *et al.* (2001) major adhesin of *B. pertussis* is filamentous haemagglutinin (FHA). It is translated from a virulence-activated *fhaB* gene, as a precursor with a molecular weight of 367-kDa. Mature form of this protein is a result of subsequent cleaveage of the precursor to 220-kDa hairpin-like form (Mattoo and Cherry, 2005). *fhaC* gene coding for an accesory protein helps the secretion of FHA across the outer membrane. An "FHA-specific transmembrane pore" (Fig 4) in the

outer membrane is formed by this helper protein (Jacob-Dubuisson *et al*., 1999). FHA is able to bind "VLA-5 integrin" via its specific domain called RGD (Arg-Gly-Asp). This binding allows FHA to attach to the bronchial epithelial cells, macrophages and monocytes (Mattoo *et al*., 2005; Ishibashi *et al*., 1994). FHA has also role in the binding to ciliated epithelial cells of respiratory tract and triggering "FHA-mediated hemagglutination" via its heparin binding site and carbohydrate binding domain (CRD) (Menozzi *et al*., 1991; Prasad *et al*., 1993). Moreover, various immunomodulatory actions of FHA is known. In a mouse model it was shown that *B. pertussis* infection results in IL-10 is secretion from "FHA-specific T regulatory cells" which inturn causes inhibition of protective Th1 response (McGuirk *et al*., 2002). Moreover in convalescent human serum, anti-FHA abtibodies attenuates the neutrophile-dependent phagocytosis of *B.pertussis* (Mobberley-Schuman *et al*., 2003). Another role of FHA is to prevent *B. pertussis* infected monocytes from antigen-specific $CD4^+$ T cell proliferation having role in induction (Boschwitz *et al*., 1997).

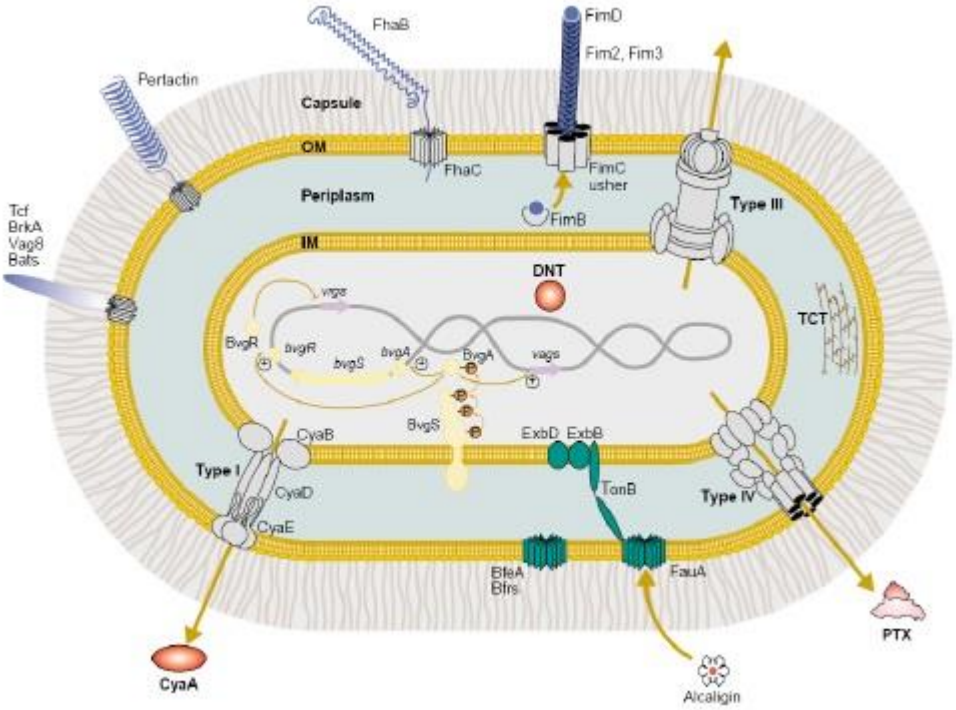


**Figure 4** *B. pertussis* virulence factors (Locht et al., 2001).

### 1.2.3.3. Fimbriae (FIM)

All *Bordetella* species including *B. pertussis* express filamentous multimeric adhesins (Fig 4) called Fimbriae (FIM) (Mattoo *et al*., 2005). The *fim3* and *fim2* genes are the sources of Fim3 and Fim2 proteins which are main fimbrial subunits used in determination of *B. pertussis* fimbrial serotypes 3 and 2 (Mooi *et al*., 1987). Fimbrial biogenesis locus fimBCD encodes a protein called FimD and this FimD subunit is accompied to Fim2 and Fim3 at their tips. Accessory proteins FimB and FimC are also encoded by the same locus with FimD and they act as a chaperon and an usher protein, respectively. FimBCD operon is located between the fhaB and fhaC loci (Willems *et al*., 1992). The expression of CR3, which is the receptor for FHA, in macrophages is induced by the binding of FimD to VL-5 integrin (Hazenbos *et al*., 1995). For the attachment and colonization of *B. pertussis* in the upper respiratory tract, Fimbriae and FHA appear to work cooperately (Mattoo *et al*., 2005).

Stimulating the early T-independent IgM, which is a Th2-mediated immune response, is the reason of the classification of Fimbrae as immunomodulatory molecules (Mattoo *et al*., 2005).

#### 1.2.3.4 Pertussis Toxin (PT)

Five different subunits denoted as S1, S2, S3, S4 and S5 and encoded by *ptxA, ptxB, ptxC, ptxD* and *ptxE* genes, respectively, form a hexameric protein of pertussis toxin (Locht and Keith, 1986). PT is secreted to outer membrane with type IV secretion system by a nonameric complex of a specific accessory protein PtI (Weiss *et al*., 1993). Two parts "A" and "B" consitute PT. Subunit B is a pentameric component and composed of two S4, S3, S5 and S2 units. As reported by Mattoo *et al.* (2005) "A" component is equal to S1. "B" component is responsible for the attachment of the toxin to the target and thus, entry of an "A" subunit to the target cell (Tamura *et al*., 1982). An ADP-Ribosyl group from NAD is transferred to trimeric G proteins by the help of A subunit in the cell. This results in blocking the activity of G proteins in the cell (Katada *et al*., 1983). G proteins are one of the key molecules in certain signalling cascades, consequently inactivation of G proteins are directly related with various systemic symptoms such as elevated insulin secretion because of lymphocytosis and hypoglycemia, histamine sensitization (Carbonetti, 2007; Mattoo and Cherry, 2005). PT has immunosupressive roles as well as immunostimulatory functions (i.e. acting as an adjuvant) (Ryan *et al*., 1998). Retardation of macrophage and neutrophil migration and delaying secreation of lysosomal enzymes from these cells, decrease in expression of "major histocompatibility complex class II" (MHC-II) on surface of monocytes and reduction of serum anti-*B. pertussis* antibody levels can be listed as exapmles of immunorepressive functions caused by PT (Meade *et al*., 1984; Brito *et al*., 1997; Shumilla *et al*., 2004; Carbonetti *et al*., 2004; Mattoo and Cherry, 2005).

### 1.2.3.5. Pertactin (PRN) and Other Autotransporters

As the name refers, autotransporters are specific proteins that are capable of transporting any thing that across outer membrane without the help of the accessory proteins (Henderson and Nataro, 2001). The effector N termial domain is allowed to transport by their conserved C terminal domain. The first autotransporter expressed in all *Bordetellae* is Pertactin (PRN) (Mattoo *et al.*, 2005). PRN has leucine-rich, proline-rich and RGC motifs which facilitate the cell attachment (Emsley *et al*., 1994). Being much more efficient the vaccines include the antigen PRN- containing dTPa. However the fact that vaccines lack this antigen is the evidence of immunostimulatory effect of anti-PRN antibodies (Gustafsson *et al*., 1996; Cherry, 1997; Cherry and Heininger, 2004). In order to resist to complement-mediated killing of *B. pertussis* cells, there is another autotransporter called BrkA. In *B. pertussis* especially TcfA is expressed and it is the only autotransporter which is not limited to the cell surface and released to the extracellular environment. However there is another autotransporter called SphB1 that has a role in C terminal maturation of FHA as reported by Coutte *et al*. (2001) and by Finn and Stevens (1995).

### 1.2.3.6 Adenylate Cyclase (CyaA)

Presentation of both adenylate cyclase and haemolytic activities makes Adenylate cyclase a bifunctional toxin (Ladant and Ullmann, 1999). In type 1 secretion system, accessory proteins CyaB,CyaD and CyaE help the toxin to be transported through the cell and outer membranes (Fig 4) (Locht *et al.*, 2001). Haemolytic activity is performed by another protein. CyaC's palmitoylation effect results in activation of C-terminal domain of the toxin. In addition, internalization of the toxin via pore formation and strengthening of N-terminal domain to the target cell surface are both directed by the same domain (Hewlett *et al*., 1988). When N terminal domain enters the cell, calmodulin activates this domain and this activation converts ATP to cAMP resulting in dramatic increase in concentration of intracellular cAMP (Confer *et al*., 1984; Wolff *et al*., 1980). This increase could be considered as immunomodulatory

effect because of its inhibitory effect on neutrophils to phagocytosis of *B. pertussis* cells and inductictive effect on macrophages' apoptosis at the beginning of infection as reported by Khelef *et al*. (1993) and Weingart *et al*. (2000).

#### 1.2.3.7 Dermonecrotic Toxin (DNT)

Injection of dermonecrotic toxin via intradermal route results in localized-necrotic lesions in mice. Intravenous injection of DNT causes the death of mice (Mattoo *et al*., 2005; Locht *et al*., 2001). There are similarities between the structures of DNT and PT. As reported by Mattoo and Cherry (2005), DNT is composed of A-B toxin with receptor binding N-terminal domain and effector C-terminal domain. Altough they have structural similarity, their intracellular locations are different. DNT is localized in the cytoplasm and is not secreted in contrast to PT (Fig. 4) (Locht *et al*., 2001). Dynamin-dependent endocytosis is used for internalization of the toxin upon the receptor binding of the toxin. Mammalian proteases activate it (Matsuzawa *et al*., 2004). This activation leads to change in amine amount via deamination or polyamination in Rho GTPase by N-terminal domain. This results in changes in certain signalling cascades due to constitutive activation of the protein (Masuda *et al*., 2000; Schmidt *et al*., 1999). As reported by Mattoo and Cherry (2005) the exact role of dermonecrotic toxin role in *Bordetella* pathogenesis is not established yet.

#### 1.2.3.8 Type III Secretion System (TTSS)

Type III Secretion System is not uniqe to *Bordetella* species. It is a complex protein structure found in many gram-negative bacteria. As reported by Galan and Collmer (1999) its role is transfering specific effector-proteins into eukaryotic cell cytoplasm. These specific effector-proteins make contribution to pathogenicity because they cause changes in the signalling pathways in the cells (Lee, 1997). *B. pertussis* has a property different than *B. bronchiseptica* and *B. parapertussis*$_{Ov}$ in the manner of causing TTSS-mediated cytotoxicity on mammalian cells in vitro (Mattoo *et al*., 2004). *B. pertussis* does not cause TTSS-mediated cytotoxicity on mammalian cells

in vitro. Differential regulation of TTSS in *B. pertussis* is thought to be result of post transcriptional modifications (Mattoo and Cherry, 2005). Locht *et al.* (2001) states that; the actual role of TTSS in *B. pertussis* pathogenesis is not clear yet.

#### 1.2.3.9 Bvg-Independent Virulence Determinants

Cell wall of gram-negative bacteria is composed of the disaccharide-tetrapeptide monomers of peptidoglycan and it is also known tracheal cytotoxin (Mattoo and Cherry, 2005). Therefore, it is needed constitutively due to cell wall metabolism and is produced by Bvg-independent pathway. In *B. pertussis* TCT is released into extracellular environment because *B. pertussis* has no AmpG-dependent mechanism which prevents the release and storage of TCT in the cell, unlike other gram-negative species (Mattoo and Cherry, 2005). In ciliated cells cytotoxic effects of TCT is observed directly. As reported by Flak and Goldman (1996), this indirect action is mainly based on the effect of TCT induction of nonciliated cells for producing more nitric oxide (NO) that diffuses into ciliated-cells. This diffusion results in damage and extrusion of mitochondria and loss of ciliated-cells from the epithelial surface. These are considered as severe consequences and lead to local tissue damage (Wilson *et al*., 1991).

Similar to other gram-negative bacteria's lipopolysaccharide (LPS) molecules, as reported by Watanabe *et al.* (1990), the LPS of *B. pertussis* also has toxic, pyrogenic and mitogenic endotoxin properties. LPS of *B. parapertussis*$_{Hu}$ and *B. bronchiseptica* contains a highly-immunogenic protein called O-antigen (Zhang *et al*., 2009; Peppler, 1984). Yet, this O-antigen is absent in LPS of *B. pertussis*. *B. pertussis* LPS layer has two components 'Band-A' and 'Band-B'. The lipid-A molecule linked to the oligosaccharide core-structure is involved in Band-B structure. Band-A incorporates Band-B with an additional trisaccharide component (Caroff *et al*., 1990). LPS layer is modified by some BvgAS regulated protein. For example, PagA protein causes palmitolylation of LPS in *B. bronchiseptica*. This situation causes speculations about probable roles of LPS in pathogenesis of *Bordetella* (van den Akker, 1998).

Contribution to resistance against death caused by antibody-dependent serum, persisting of colonization in respiratory tract and the host specificity are all determined by these modifications (Harvill *et al*., 2000; Mattoo and Cherry, 2005).

### 1.3 Pertussis Vaccines

Causative agent of pertussis was identified (Bordet and Gengou, 1906) and the first whole-cell vaccine (Pw) is produced soon after. Then in 1940s and 1950s, Tetanus and Diptheria vaccines are combined with the Pw(DTPw). Afterwards this combined vaccine become available all over the world mainly in developed countries (Storasaeter *et al*., 2007). With mainly the World Health Organisation (WHO) efforts, vaccination coverage of children with DTP worldwide increased from 5% to 78% from 1970s to mid-2000s (WHO, 2005). This mass-vaccination in 2003 confined the mortality and morbidity rates from 280,000 and $17{,}6x10^6$ respectively that would outreach to 800 000 and $50x10^6$ to according to WHO estimates. Another result of this mass vaccination is alteration in epidemiologic profile of the pertussis. Times before the vaccination, pre-school aged children had the highest incidence rate of pertussis. After vaccination, the higher incident rate shifted to infants and adolescents (Andrews *et al*., 1997; Cherry and Heininger, 2004; Edwards, 2005).

#### 1.3.1 Whole-Cell Vaccines

Main idea behind the development of whole cell vaccines is the inactivation of live *B. pertussis* cells by chemical agents (e.g treatment of formalin) or physical damage. This logic has clearly not shown any alteration from very beginning of history of Pw development. Comparing historical vaccines to current Pw, current one shows variability in reactogenicity and efficacy (Galazka, 1993). Amount of impurities (e.g liposaccharides) and methods have difference in the inactivation. The reason is the differences in composition (Relyveld *et al*., 1991; Galazka, 1993). The study that reported the DTPw vaccines' average efficacy as 78%, also showed dramatic differences in the efficacy (Jefferson *et al*., 2003). The percentage of having local

effects such as pain, redness and swelling or the percentage of systemic effects such as fever, drowsiness, anorexia, irritability in DTPw vaccinated children are more than 50% (Cody *et al*., 1981). In addition to these adverse reactions, prolonged crying, hypotonic hyporesponsive episodes (HHE), febrile convulsion, directly related with hyporesponsiveness, reduced muscle tone, cyanosis, and skin pallor could be observed as less commonly after Pw vaccinations (Howson and Fineberg, 1992; Barlow *et al*., 2001; Gold, 2002). In spite of the fact that Pa is less reactogenetive and has more specific and predictable immune responses, Pw is still popular throughout the world because of its inexpensiveness and availability (Storsaeter *et al*., 2007).

### 1.3.2 Acellular Vaccines

Acellular vaccine (Pa) development started at 1940s. At that time, first components of the vaccines were mainly based on chemicals such as trisodium phosphate treated cell extraction preparates and human erythrocyte or aluminum compound absorption (Weihl *et al*., 1963; Pillemer *et al*., 1947). Method of toxin separation from protective-antigens found in cell-extracts is used in many studies in which Pa composed of purified agents are used. This method thus decreases the reactogenicity. The first two proteins were Pertussis toxin (PT) and filamentous hemagglutin (FHA) and their protective effects were confirmed with mouse potency assays. As a result, these two proteins are used in late 1970s in Japan to formulate the first licenced Pa (Sato *et al*., 1974; Storsaeter *et al*., 2007). Serial experiments based on comparison between Pw and this Pa showed that the former is more reactogenic and less efficacious. Other proteins Fimbriae (Fim) and pertactin (Prn) which found after PT and FHA, were used in protective antigen-based acellular pertussis vaccines (Greco *et al*., 1996; Gustafsson *et al*., 1996; Brennan *et al*., 1988; Zhang *et al*., 1985).

### 1.3.3 Composition, Efficacy and Safety of Acellular Vaccines

Different entrepreneurs in 1980s and 1990s used different combinations of PT, FHA, Prn and Fim to produce two- to five- component Pa generation. Fim3 and Fim2 were found only in five or four component vaccines, yet PT toxoid were found in all Pa combinations (Table 1). A study conducted in 1996 by Gustaffon *et al.* states that two component vaccine which formulated FHA and PT only was less efficacious than five component Pa vaccine which includes FHA, PT, Prn, Fim2 and Fim3. Similarly, comparison between overall efficacies of one-or two component vaccines to three- or more-component vaccines are 67-70% to 80-84% (Jefferson *et al*., 2003). Efficacy of particular Pa is affected by antigen composition and difference in absorption capacities of components of vaccines to different aluminium salts (Denoel *et al*., 2002). Moreover, as stated by Gustafsson *et al*., (2005), even Pa shows short term protectivity, it is not guranteed that it shows continous, long-term effectiveness.

Variations in immune-responses against Pa or Pw vaccinations were observed. A conclusion of the study in which comparison between Pa or Pw vaccined infants' antigen specific Th1 and Th2 based cytokine secretions were observed was that Pw was directly related to cellular immune response due to its relation with Th1-predominated cytokine profile while Pa was directly related to cellular and humoral immune response due to stimulation of Th1/Th2 based cytokine secretion (Dirix *et al.* 2009). Inducing both cellular and humoral responses via Pa was not restricted to infants, this ability was also shown in adults and adolescents (Meyer *et al*., 2007). The same study also showed that the persistance of humoral immune response after one year from the vaccination was less appreciable than that of the cellular immune response. This means a possible role of cellular immune response in verifible protection.

Administration of Pa causes less frequent systemic and local adverse reactions listed in section 1.3.1 as well as HHE and febrile convulsions than administration of Pw according to several studies (Geier and Geier, 2004; Bernstein *et al*., 1993; Le Saux

*et al*., 2003; Schmitt-Grohe *et al*., 1997). As reported by Schmitt *et al*. (1997) and by Rennels *et al*. (2000); repetitive booster doses of Pa might cause a whole-limb swelling as an adverse reaction. According to recent studies, mainly in adults and adolescents, the risk of adverse reaction occurence could be decreased via administration of the vaccine includes reduced antigen (dTpa) as a booster rather than pediatric, conventional DTPa (Zepp *et al*., 2011; Mertsola *et al*., 2010; Zepp *et al*., 2006) As reported by Storsaeter et al. (2007); although dTpa and DTPa has no difference in their antigen compositions, DTPa has higher concentrations for each.

### 1.3.4 Combined Vaccines

As mentioned above, Pa or Pw is combined with tetanus and diphteria toxoids and this combination has been used for nearly 60-years. Combination of this vaccine with other [i.e. inactivated polio virus vaccine (IPV), conjugated Haemophilus influenza type b vaccine (Hib) and hepatitis B vaccine (HBV)] is a way of broadening the efficacy of single vaccine against these diseases (Storsaeter *et al*., 2007). With these combinations, parents and physicians are allowed to have timely and complete vaccination schedule (Dodd, 2003; Marshall *et al*., 2007). Also, combine vaccines are useful in many ways, such as less injection and less expensive. Some of the first combined vaccines included Pw are DTPw-IPV-Hib, DTPw-HBV-Hib and DTPw-HBV (Table 2) (Storsaeter *et al*., 2007). In following studies, Pa was included to combined vaccines. Safety and immunogenicity of these combined vaccines were investigated then verified by several recent studies (Kilpi *et al*., 2009; Halperin *et al*., 2009; Li *et al*., 2010; Johns and Hutter, 2010).

**Table 2** Compositions of acellular vaccines from major manufacturers (Storsaeter et al., 2007)

| Vaccine | Manufacturer | Trademark | Pertussis Antigens (μg per dose-0.5ml) PT | FHA | PRN | FIM |
|---|---|---|---|---|---|---|
| Pediatric Vaccines | | | | | | |
| DTPa | Chiron | Acelluvax | 5 | 2.5 | 2.5 | |
| | GSK | Infanrix | 25 | 25 | 8 | |
| | SP | Tripedia | 23.4 | 23.4 | | |
| | SP | Tripacel/Daptacel | 10 | 5 | 3 | 5 |
| DTPa-Hib | GSK | Infanrix/Hib | 25 | 25 | 8 | |
| | SP | Actacel | 10 | 5 | 3 | 5 |
| DTPa-HBV | GSK | Infanrix-HBV | 25 | 25 | 8 | |
| DTPa-IPV | GSK | Infanrix-IPV/Infanrix polio | 25 | 25 | 8 | |
| | SP | Tetravac | 25 | 25 | | |
| | SP | Quadracel | 20 | 20 | 3 | 5 |
| DTPa-IPV-Hib | GSK | Cinquerix | 25 | 25 | 8 | |
| | SP | Pentacel/Pediacel | 20 | 20 | 3 | 5 |
| | SP | Pentavac | 25 | 25 | | |
| DTPa-HPV-IPV | GSK | Infanrix penta/Pediarix | 25 | 25 | 8 | |
| DTPa-HBV-IPV-Hib | GSK | Infanrix hexa | 25 | 25 | 8 | |
| | SP | Hexavac | 25 | 25 | | |
| Reduced Antigen content Vaccines | | | | | | |
| dTpa | GSK | Boostrix | 8 | 8 | 2.5 | |
| | SP | Adacel/Covaxis | 2.5 | 2.5 | 5 | 3 |
| dTpa-IPV | GSK | Boostrix polio | 8 | 8 | 2.5 | |
| | SP | Repevax | 2.5 | 2.5 | 5 | 3 |

### 1.3.5 Current Issues with Pertussis Vaccines

Morbidity and mortality rates of pertussis are surely decreased by the use of whole-cell (first generation) and acellular (second generation) pertussis vaccines. But, these current vaccines still have drawbacks, such as not providing continuous-immunity, not preventing adverse-reactions in higher doses (i.e retrogenicity) and not avoiding subclinical-disease and thus not decreasing the risk of transmission to infants that are not protected (Storasaeter *et al*., 2007).

For many years, vaccine induced immunity against pertussis had a limited duration, which was a concern (Edwards, 2005). Pw-driven immunity was decreased to 4 years after beginning of vaccine administration (Jenkinson, 1988). According to another study, estimation of Pw-induced protective-immunity lasts 6 to 9 years. Pa and Pw dependent immunity durations are more or less similar with respect to another study (Torvaldsen and McIntyre, 2003). Increasing incidence rate of pertussis between adolescents who vaccinated in their childhood could inferred by inability to providing a life-long immunity of current Pa and Pw vaccines. Adolescents appear to have important role in transmission of this disease to vulnerable, unvaccinated infants (Wendelboe *et al*., 2005; Edwards, 2005).

Adolescents are potential reseviors for *B. pertussis* and this situation reflects both continous immunity issue and effective prevention of subclinical pertussis. Yet the effectiveness is to be examined. Current vaccines apperantly are not effective because adolescents have an asymptomatic disease most of the time (Edwards, 2005). In some cases, Brk-A-mediated resistance mechanism of *B. pertussis* used for enabling the pathogen to get over the bacteriacidal activity of the complement system is inactivated by antibodies (Weiss *et al*., 1999). Interfering with bacterial adherence, neutralizing toxin-activities and/or confering direct bacterial clearence are main ways used to prepare pertussis vaccines in struggling with the disease (Weiss *et al*., 1999; Kubler-Kielb *et al*., 2011). The first way is generally choosen to be used in the most of the current Pa vaccines. Among the antigens such as FHA, PT, Prn and Fim which

are mainly found in current Pa vaccines, pertactin (Prn) is the only one that stimulates the antibody production mediating bacterial clearence in a complement-dependent way (Hellwig *et al*., 2003). Beside these protein antigens, core-oligosaccharides (OS) components of *B. pertussis* LPS also cause an effective bactericidal-activity (Mountzouros *et al*., 1992; Archambault *et al*., 1991; Kubler-Kielb *et al*., 2011). Therefore, novel protein candidate investigations to enhance bacteriacidal capacity would be a proper way to develop new generation Pa vaccines (Weiss *et al*., 2004; Weingart *et al*., 2000; Weiss *et al.*, 1999).

### 1.3.5 Reverse Vaccinology and Meta-Analyses

There are two different methods in conventional vaccine development approaches; i) using several passages *in vitro* to obtain live-attenuated pathogens for being used in whole-cell vaccines, ii) identification of protective antigens for acellular vaccines (Rappuoli, R., Del Giudice, G., 1999). To identify protective antigens of a pathogen which are suitable for vaccine development *in vitro* cultivation of the pathogen and identification of components that are building the pathogen one at a time, via genetic, serological and biochemical tools, is required. Instead of being successful in many cases, this method is quite time-consuming and also not applicable for pathogens that are not suitable for *in vitro* cultivation (NIH, The Jordan Report, 2000).

The reverse-vaccinology approach takes advantage of available genome sequences of pathogens which provides a catalog of all proteins expresses by the organisms. In this approach researchers predict vaccine candidates via computer analysis of genome sequence (Rappuoli, 2000). The reverse vaccinology protocol was initially developed to predict vaccine candidates against serogroup B *Neisseria meningitidis*, the most common causative agent of bacterial meningitis worldwide (Pizza *et al.*, 2000). Chemical structure of capsular polysaccharide surrounding this serogroup is identical to an α(2–8) linked polysialic acid of human beings. Therefore, conventional vaccine development approaches could not be used as it would result in autoimmunity (Donati and Rappuoli, 2013). Pizza *et al.* (2000) have selected

potential vaccine candidates by using genomic data of the pathogen depending upon the suggestion that examples of protective antigens are generally proteins of outer membrane (Zollinger, 1997). The researchers have selected 600 genes of the pathogen and 350 of them were successfully cloned. After expression, proteins coded by candidate genes were used for immunization of mice. This process resulted with the identification of five novel vaccine candidates and soon after a new multicomponent vaccine combination was developed (Giuliani et al., 2006, Donati and Rappuoli, 2013).

Since reverse vaccinology approach described above is still labor intensive in terms of selecting protective antigens among large numbers of *in silico* predicted candidates, it is in question to find out a way to facilitate selection of promising vaccine candidates.

In a recent study, a new method named MetaVaccinology was described for vaccine candidate determination (Altındiş, 2011). In this study protein sequences of 115 different protective antigens from 23 different bacterial species (including gram-positives and gram-negatives) were used as a dataset and analysed in terms of primary sequence, domain occurrence, protein architecture and 3D structure. The results have revealed that known protective antigens have common structural and/or functional units, which were generally associated to either variable number of multiple internal repeats or to specific domains, such as Lysm, from the Pfam databases (Altındiş, 2011). The researchers have used this common features of know antigens for *in silico* prediction of new vaccine candidates against GBS and *S. aureus* and tested them in animal model. Nine antigens were tested against GBS and four of them have induced a significant protection. On the other hand, five of nine antigens tested against *S. aureus* have also induced a significant level of protection (Altındiş, 2011).

## 1.4. Aim of the Study

After the first mass-vaccination in 1940s, whole cell (Pw) and acellular pertussis (Pa) vaccines were developed. However, whooping cough has been re-observed in recent years. The disease is mainly a childhood disease. Adolescents, however, have generally an asymptomatic disease most of the time and thus current vaccines apperantly are not effective (Edwards, 2005). Several studies have shown the antigenic divergence between clinically isolated and vaccine strains. The polymorphism in filamentous hemagglutinin, fimbriae, pertactin and pertussis toxin decreases the efficiency of current vaccines. Thus, need for novel protein candidate assessments to enhance bacteriacidal capacity has been increased. Immunoproteomic studies have been increasing to find novel and effective atigens to be used in the vaccines. Immunoproteomics studies performed in our laboratory revealed several novel antigens found on the cell surface including glutamine-binding periplasmic protein GnlH (Altindis *et al.*, 2009; Tefon *et al.*, 2011). A recent study revealed that some of immunoprotective proteins had Lysm domain (Altindis, 2011). Since BP0020 also included the same domain it was in the scope of investigation.

In the present study, glutamine-binding periplasmic protein GnlH and putative peptidoglycan-binding protein BP0020 were cloned, heterogenously expresses and assessed for their immunoprotectivity in mouse model with the aim of developing new generation Pa vaccines with better protection against the disease.

# CHAPTER 2

# MATERIALS AND METHODS

## 2.1. Bacterial Strains and Plasmids

In the present study Saadet and Tohama I strains of *B. pertussis* were used (Table 2). From a baby girl having a name of Saadet in Turkey, the strain Saadet was isolated in 1948 and it is a local *B. pertussis* strain. However, the other *B. pertussis* strain, Tohama I, has been used widely all around the world as an organism in research and preparation of vaccines. In the subcloning and expression studies, two *Escherichia coli* strains were used: DH5α and *E. coli* BL21 (DE3), respectively (Table 3). The pGEM®-T Easy (Promega) vector was used for cloning, while pET-28a (+) (Novagen) vector was used for recombinant protein expression in *E. coli* BL21(DE3) (Figures 5, 6 and 7).

**Table 3** Strains used in the 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 (DEVA Holding Inc., İstanbul). |
| *E. coli* DH5α | F' ϕd*lacZ*Δ(*lacZY* 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) |

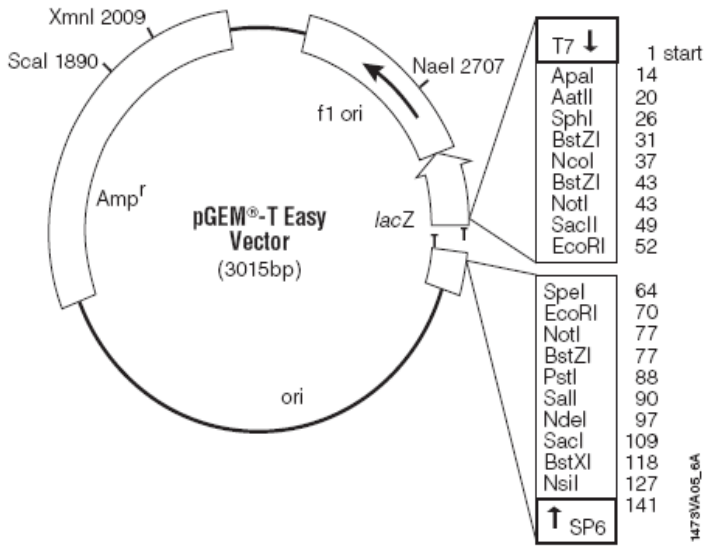


**Figure 5** The pGEM-T vector restriction map.

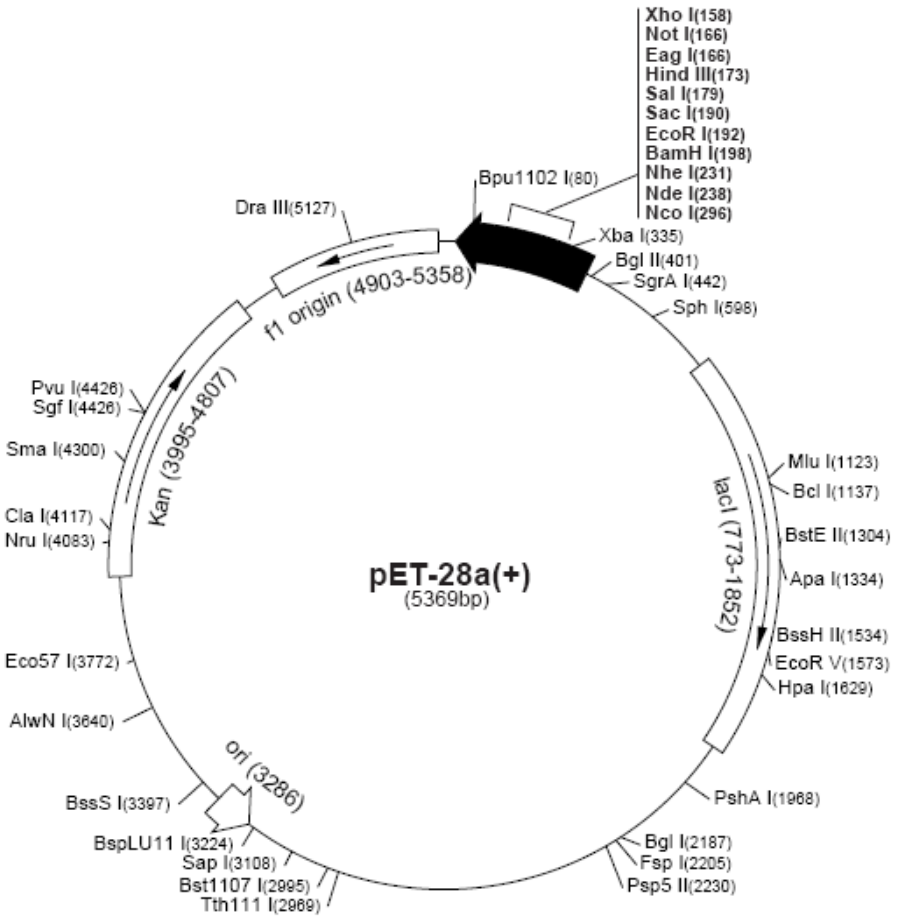


**Figure 6** The pET-28a (+) vector restriction map.

## 2.2. Culture Media

Appendix A shows the composition of the culture media as well as the preparation steps.

## 2.3. Buffers and Solutions

Appendix B shows the composition of the buffers and solutions as well as their preparation steps.

## 2.4. Chemicals and Enzymes

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

## 2.5. Maintenance of Bacterial Strains

Cohen-Wheeler agar medium (in Appendix A) was used to grow *B. pertussis* Tohama I and Saadet strains. They were grown for 72 h at $37^0$C and then stored at $4^0$C (Sato *et al*., 1972). Luria-Bertani (LB) growth medium given (in Appendix A) was used to grow *E. coli* DH5α strain. 100 µg/mL ampicillin was added to the liquid LB medium for DH5α cells containing pGEM®-T. The strain was then stored on LB agar plates containing ampicillin (Appendix A). LB liquid medium in which there was 30 µg/mL kanamycin was used to grow *E. coli* BL21(DE3) strains containing pET-28a(+). The strain was then stored on LB agar plates containing kanamycin. For the storage of the stains at -80$^o$C, 20% glycerol containing stocks were prepared.

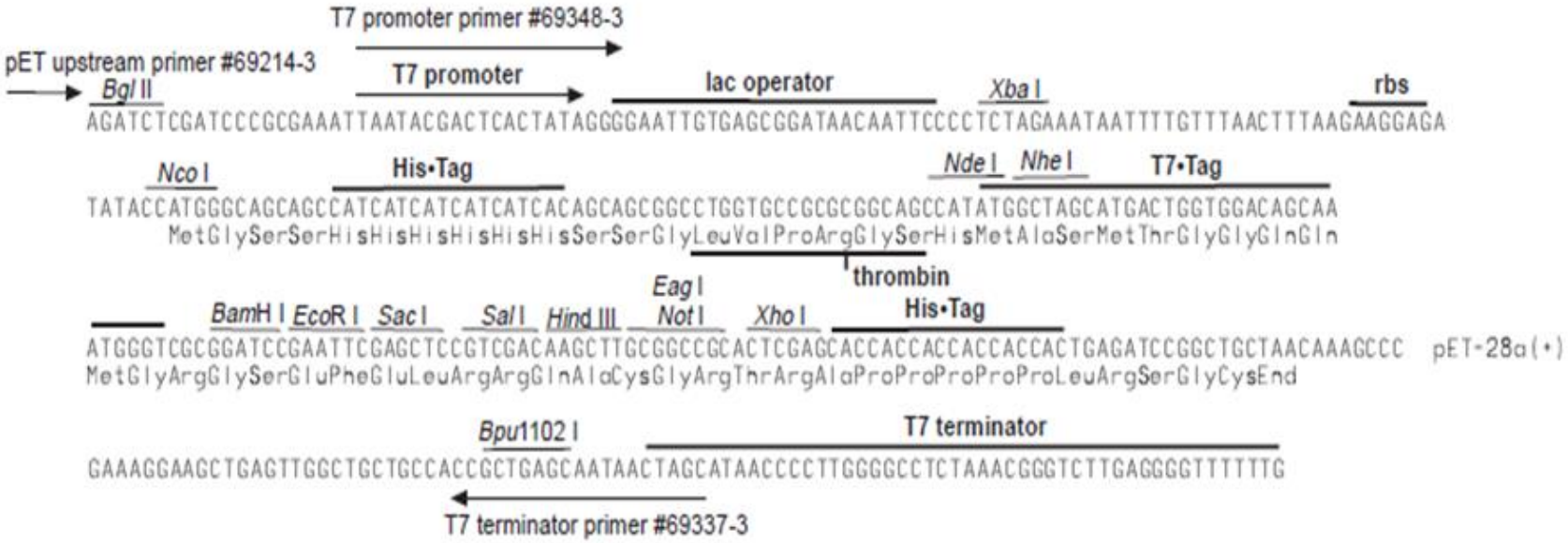


**Figure 7** Clonning region of pET-28a (+) vector

## 2.6. Genomic DNA Isolation

Chromosomal DNA isolation of *B. pertussis* was performed according to the chromosomal DNA isolation procedure by Storm *et al*. (2006). Colonies of *B. pertussis* were obtained from Cohen-Wheeler agar plates grown for 3 days at 37$^{o}$C. 200 µl TEN buffer given in the Appendix B was used to resuspend colonies and incubated at 100°C for 10 min. After centrifugation at 13,000 rpm for 2 min, the pellet was disgarded and supernatant was obtained and stored at 4°C to be used in PCR experiments.

## 2.7. Primer Design

For the amplification of BP1573 (*glnH)* and BP0020 (putative peptidoglycan binding protein) genes from *B. pertussis* Tohama I, we used the sequence information available in NCBI data base (NCBI gene IDs: 2666626 and 2664670, respectively) for primer designs (Table 4).

**Table 4** Primer sequences used in PCR-amplification. Underlined sequences show restriction enzyme recognition sites.

| Gene name | Primer | Oligonucleotide Sequence | Size of the PCR product |
|---|---|---|---|
| *glnH* | GBF | 5'- ggatccatgatcaaagcgaaagtt - 3' | 753 bp |
| *glnH* | GBR | 5'- agatcttcagggctgcttgcc - 3' | |
| BP0020 | PBF | 5'- ggatccatgattcggccgttcgcg - 3' | 1107 bp |
| BP0020 | PBR | 5'- agatctttagaattcgctgagcagcacc - 3' | |

## 2.8. Polymerase Chain Reaction (PCR)

The oligonucleotide primers given in Table 4 were purchased from SACEM, Co. (Ankara, Turkey). PCR was then carried out (10 µl 5X Phire buffer, 0.5 µg genomic NA, 2 µl of each 10 µM primers, 5% DMSO, 1 µl of 10 mM dNTP, 1 µl Phire polymerase and sterile $dH_2O$ to obtain volume as 50 µl). Table 5 shows the conditions for the amplification of the desired genes via PCR. After the amplification step, the products were loaded in 1% agarose gel and visualized upon electrophoresis.

**Table 5** PCR condition used for the amplification

| | **Temperature** | **Time** |
|---|---|---|
| Initial denaturation | 98°C | 2 min |
| Denaturation | 98 °C | 20 sec |
| Annealing | 58 °C | 35 cycles<br>20 sec |
| Extension | 72 °C | 15 |
| Final extension | 72 °C | 3 min |

## 2.9. Agarose Gel Electrophoresis

A horizontal tank was used for the electrophoresis process. As given in Appendix B, 1X TAE buffer was prepared. After mixing 6X gel loading dye and samples, the mixtures were loaded into the 1% agarose gel and run at 100 Volts for 50-60 min. Ethidium bromide staining (0.5 µg/ml) was performed immediately after the

electrophoresis. Gel Imaging System (Vilber Lourmat, Marne-la-Valle´e, France) was used to visualize the DNA bands on the gel, and photographed. In order to determine the size of the DNA samples, GeneRuler™ 100 bp plus DNA Ladder and Lambda DNA/*Pst*I Marker were used and DNA fragments at desired sizes were extracted by using Qiagen Gel Extraction Kit (Qiagen Inc., Valencia, CA).

### 2.10. Sequencing Reactions

DNA samples were sent to RefGen Inc. (Ankara, Turkey) for sequencing. Resulting sequences were analyzed, BLASTed and compared with the National Center for Biotechnology Information (NCBI) database (http://www.ncbi.nlm.nih.gov/BLAST).

### 2.11. Ligation Reactions

Theligase buffer (5 µl, 2X), 1 µl T4 ligase, 500 ng insert DNA, 1 µl pGEM®-T vector (as a subcloning vector), and sterile $dH_2O$ to complete the volume to 10 µl were mixed for ligation reaction. In order to ligate the inserts to pET-28a(+) expression vector, 2 µl pET-28a(+) vector, 1 µl 10X ligase buffer, 1 µl T4 ligase, 500 ng insert DNA were mixed and sterile $dH_2O$ was added to complete volume to 10 µl.

### 2.12. Transformation

#### 2.12.1. *E. coli* BL21(DE3) and DH5α Competent Cell Preparation

The procedure described by Sambrook *et al*. (1989) was followed to prepare the competent cells. *E. coli* from a fresh LB agar plate with slight modifications was obtained and inoculated into 50 mL LB broth in a 100 mL flask. Until $OD_{600}$ reaches to 0.6 inoculated *E. coli* cells were incubated at 37°C by shaking at 180 rpm. After the incubation, two pre-chilled, sterile 80 ml screwcap centrifuge tubes to which the cell solution was aliquoted were used and stored on ice for 10 min. Following 10 min

incubation, the cell solutions were centrifuged at 4,000 rpm for 10 min at 4°C. The supernatants were discarded and 5 ml of ice-cold 10 mM $CaCl_2$ was used to resuspend the pellets by vortexing. Resuspended pellets were re-centrifuged at 3,000 rpm for 10 min at 4°C and the new pellets were resuspended gently in 1 mL ice-cold 75 mM $CaCl_2$. Resulting competent cells were aliquoted into 100 µl aliquottes and stored at - 80°C.

### 2.12.2. Transformation of Bacteria

Competent *E. coli* cells were incubated for 15 min, on ice. After adding 10 µl of ligation-products to the cells, the cells were incubated for additional 30 min, on ice. A heat shock (42°C, 60 - 90 sec) was applied and followed by incubation on ice for 5 min. After addition of 900 µl of LB, the mixture was incubated at 37$^{o}$C for 80 min by gentle agitation at 100 rpm. Centrifugation for 10 min, at 3000 rpm was applied and 100 µl LB was used to resuspend the resulting pellets. Selective medium having desired antibiotics were used for inoculation of the transformed cells. In order to perform blue-white colony selection, LB agar media (including 20 mg/mL X-gal, 100 mg/ml IPTG and appropriate antibiotic) was used for the inoculation.

## 2.13. Plasmid DNA Isolation

For the isolation of plasmids from *E.coli* strains, Qiagen Plasmid Purification Mini and Midi Kits (Qiagen Inc., Valencia, CA) were used. The protocols described by the manufacturers were followed.

On occasion, the protocol described by Hopwood *et al*. (1985) was also used to isolate plasmids manually, with slight modifications. LB agar containing the required antibiotics was used to grow *E.coli* strains. The cells were inoculated and incubated overnight. 100 µl STE solution containing 2 mg/ml lysozyme was used to resuspend colonies obtained from the agar plates (Apendix B), and mixed by vortexing. To lyse the cells and to denature genomic DNA, cells were incubated for 10 min at room

temperature and then for 10 min, at 70°C, respectively. Following the addition of water saturated phenol-chloroform (Appendix B) upon cooling, the mixtures were centrifuged for 5 min at 13,000 rpm. Agarose gel electrophoresis was used to run 15 µl of supernatant.

### 2.14. Restriction Enzyme Digestion

The restriction enzymes diluted the in a suitable buffer to obtain 2 Units per µg of DNA. The restriction enzyme solution were added to the DNA. Following incubation for 3-5 h at 37°C, the samples were stored at -20$^{o}$C for further processing when needed.

### 2.15. Alkaline Phosphatase Treatment

A protocol described by rAPid Alkaline Phosphatase Kit (Roche) was followed for Alkaline Phosphatase treatment. Sterile distilled water was used to complete the mixture containing; 2 µl 10X rAPid Alkaline Phosphatase buffer, 1 µg vector DNA and rAPid Alkaline Phosphatase, to final volume of 20 µl. The resulting mixture was first incubated for 30 min at 37$^{o}$C and then for 2 min at 75$^{o}$C to inactivate the rAPid Alkaline Phosphatase. For future uses, the mixture was stored at -20 $^{o}$C.

### 2.16. Protein Overexpression and Purification

The procedures reported by Protino® Ni-TED 2000 protein purification system (manufacturer; Macherey-Nagel) and Ayalew *et al.* (2008) were followed for protein overexpression and protein purification. A single colony of recombinant *E. coli* BL21(DE3) strain was inoculated into 10 ml LB containing kanamycin and incubated overnight at 37°C. The 1.5 ml of culture was used to inoculate 250 ml LB containing kanamycin and further incubated at 37°C with agitation at 180 rpm until $OD_{600}$ reached to 0.6. IPTG was used in one of the cultures as an inducer at a final concentration of 1 mM, the other culture was kept as a control. Following the

induction, the cultures were incubated for 5 h. The 250 ml of the cultures were obtained and centrifuged. The 5 ml of denaturing solubilization buffer (DSB) (Appendix B) was used to resuspend the resulting pellets. Following two cycles of freeze-thaw process at – 80° C and at room temperature respectively, sonication for 5 times, 10 sec with 5 sec intervals on ice was performed. Absorbances of supernatants obtained after centrifugation at 15000 rpm for 15 min were measured at 280 nm.

Purification was performed by the His-tag columns (containing immobilized nickel ions) after equilibriation with 4 ml DSB. Supernatant were applied to the columns and upon binding of his-tagged proteins to nickel ions, untagged proteins were removed from the columns. DSB was used to wash the columns. After washing step, elution of the proteins was performed with 3 ml denaturating elution buffer (DEB) prepared as described in Appendix B. The absorbances of the eluted proteins were measured at 280 nm. Then the proteins were used to run SDS-PAGE gel.

### 2.17. Dialysis

As described in Appendix A, one liter of dialysis buffer was prepared. Before loading of the protein solution, dialysis membrane was wetted with distilled water. After overnight incubation of the samples in the buffer at 4°C, the samples were filtered through a 0.2 μm filter and the Bradford assay was used to determine the concentration.

### 2.18. Determination of Protein Concentration

The Bradford assay (Ramagli and Rodrigez, 1985) was used for the determination of total protein concentration with some modifications. 250 ml of 96% ethanol, the 500 mg Coomassie Brillant Blue G-250, and 500 ml of 85% ortho-phosphoric acid were mixed and completed to 1 liter with $dH_2O$ in order to prepare 5X Bradford reagent. After 1:5 dilution of the reagent with $dH_2O$ it was filtered three times through Whatman No. 1 filter-paper. The 1580 μl of $dH_2O$, the 20 μl of the supernatant and

the 400 µl of 1:5 diluted 5X Bradford reagent were mixed and incubated at room temperature for 10 min. The incubation was performed in the darkness. The absorbance of the solution was measured at 595 nm. Before obtaining the concentration of the protein, calibration curves were set by using Bovine Serum Albumin (BSA) as shown in Figure 8 and Table 6.

**Table 6** Calibration curve standarts for Bradford assay

| **Protein concentration** | **Volune in mixture*** | **$dH_2O$** | **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

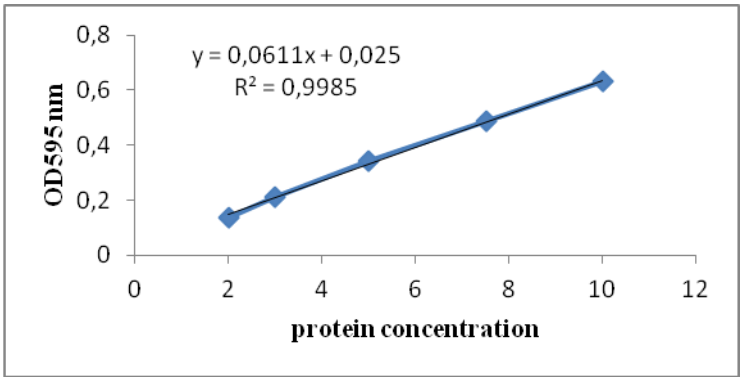


**Figure 8** Protein concentration calibration curve.

## 2.19. SDS-PAGE and Coomassie Brilliant Blue Staining

A 12% separating gel (pH 8.8) and a 4% stacking gel (pH 6.8) with an electrophoresis system (Bio-Rad, USA) were used for SDS-PAGE electrophoresis as described in Bio-Rad Cell system manufacturer's protocol (Table 7). A 16 mA constant current was applied for the electrophoresis in 1X running buffer prepared as shown in Appendix B. After completion of the electrophoresis, fixation buffer (Appendix B) was used for the treatment of the gels. Coomassie Brilliant Blue R-250 (Neuhoff *et al.*, 1988) (Appendix B) was used to stain the gels.

**Table 7** SDS-PAGE gel preparation

| | **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 |

## 2.20. Preparation of Antisera against *Bordetella pertussis*

Cohen-Wheeler agar medium was used to grow *B. pertussis* Tohama I and Saadet strains for 3 days. After the growth period, 0.85% saline solution to contain ca. $4\times10^{10}$ bacteria/ml was used to suspend the cells. Inactivation of the cells were performed for 30 min, at 56°C. Inactivated *B. pertussis* cells were used to immunize

the mice and anti-*B. pertussis* polyclonal antibodies were obtained. The 0.5 ml of cell suspension was used per animal for injection of 10 mice with two week intervals. The injection was performed twice. On day 14 after the second injection, collected Tohama (Th) and Saadet (Sa) antisera were stored at -20°C.

### 2.21. Western Blotting

In order to perform Western Blotting, the protocol (Towbin *et al*., 1979) was used with slight modifications. A semi-dry blotting system (Cleaver Scientific Ltd, UK) was used to transfer the proteins from the gel to a nitrocellulose membrane with application of 1.5 $mA/cm^2$ for 1.5 h in transfer buffer prepared as described in Appendix B at $37^oC$. A 0.5% Tween-20 in TBS was used to rinse the membrane for 10 min. After rinsing, the serum obtained from the vaccinated mice was used to incubate the membrane for 1 h. After rinsing the membrane for 10 min, 2.5% skim milk in TBS including anti-mouse IgG-alkaline phosphatase conjugate was used for the incubation of the membrane. Upon incubation TBS was used to wash the membrane. Then the membrane was treated with substrate (AP Conjugate Substrate Kit, Bio-Rad, USA).

### 2.22. Mice Experiments

In the present study, 3 weeks old BALB/c male mice (weighing between 18 and 20 g) were obtained from Ankara University, Faculty of Medicine (Ankara, Turkey). Combined vaccine of 10 µg GlnH and 10 µg BP0020 were applied to 30 mice.

#### 2.22.1. Vaccination

The 0.5 ml of combined vaccine including 10 µg of each proteins (GlnH and BP0020) absorbed to $AlOH_3$ (Alhydrogel) adjuvant was used for intraperitonel vaccination of 15 of 30 mice on day 0 and 21. For the vaccination of the other 15 mice, PBS was used as a negative control.

### 2.22.2. Challenge and Determination of Bacterial Colonization

On day 10 after the second vaccination, live cells of Saadet strain were used to challenge the mice. After collection of the bacteria, the cells were diluted in 0.85% NaCl solution (pH 7.0, 1% casamino acid). Ether was used as an anesthetic. After the application of ether, administration of 50 µl inoculum with 2.5 x $10^9$ CFU (colony forming unit) of bacteria was applied to each nostril for the mice to inhale. Bacterial colonization was determined by removing the lungs of the mice aceptically on days 5 and 8 and putting into 0.85% NaCl solution (1% casamino acid). After the homogenization of the lungs, serial dilution was performed before inoculation. Cohen-Wheeler agar medium was inoculated with 100 µl of each sample, incubated at 37$^o$C for 3-4 days and the colonies were counted.

### 2.22.3. Detection of Serum Interferon – gamma (IFN-γ) Levels

Mouse IFN-γ Minikit (Thermo Scientific, Rockford, USA) was used to determine IFN-γ secretion in accordance with the manufacturer's protocol. The 35 µl of coating antibody and 11 ml coating buffer prepared as described in Appendix B were mixed. After mixing 100 µl of the solution was put in to each well of 96-well plate. After onernight incubation of the plate at room temperature and aspiration of the coating antibody solution, 300 µl of blocking buffer (Appendix B) was added and incubated at room temperature for 1 hr. After the aspiration of the blocking solution, 1:4 and 1:8 dilutions of the serum sample were prepared. Duplication of 100 µl of each sample was added into the wells of 96-well plate. After onernight incubation of the plate at room temperature and aspiration, 300 µl/well washing buffer was used to wash the plate three times (Appendix B). Detection antibody (35 µl) and assay buffer (11 ml) prepared as described in Appendix B were mixed and the mixture was added to the wells. Upon covering the plate, 1 h incubation at room temperature was performed. Following aspiration 300 µl/well washing buffer was used to wash the wells for three times. Assay buffer was used to dilute Streptavidin-HRP at a ratio of 1/10000. The solution was added to each well and incubated at room temperature for

30 min. After aspiration step, 300 µl/well washing buffer was used to wash the wells for three times. After addition of 100 µl of TBM substrate solution, the plate was incubated in the dark for 30 min at room temperature. A 100 µl of stop solution (Appendix B) was put to the wells to stop the reaction and absorbances were measured at 490 nm.

### 2.22.4. IgG Enzyme-Linked Immunosorbent Assay (ELISA)

Two separate enzyme-linked immunosorbent assays were performed for the recombinant proteins. In one of them, wells were coated with 4 µg recombinant proteins separately and in the other one the wells contained 4 µg of each recombinant protein together.

GlnH and BP0020 proteins were diluted in carbonate/bicarbonate buffer (Appendix B) and used as ligands to coat ELISA plates, separately at a concentration of 4 µg/well. As covered with parafilm, the plate was incubated at 4$^{o}$C for overnight. Washing solution described in Appendix B was used to wash the wells for three times. Then blocking solution (100 µl) (Appendix B) was added and the plate was incubated for 1 h, at 37$^{o}$C. The 100 µl of 1:100, 1:200, 1:400, 1:800, 1:1600, 1:3200, 1:6400, 1:12800, 1:25600, 1:51200 dilutions of test sera in blocking solution were added to the wells. Followed by incubation for 1 h at 37$^{o}$C, the plate was again washed with washing solution for three times. After addition of 100 µl of alkaline phosphotase conjugated mouse-IgG (1:1000 dilution in blocking solution) and after incubation for 1 h at 37$^{o}$C, the plates were washed for four times. Following the addition of 100 µl of substrate (AP Conjugate Substrate Kit, Bio-Rad, USA) into each well and incubation of the plate at the dark for 30 min at room temperature, 50 µl 1M NaOH was added in order to stop the reaction. The absorbances were measured at 450 nm.

# CHAPTER 3

# RESULTS AND DISCUSSION

## 3.1. Cloning of Genes Encoding Glutamine-Binding Periplasmic Protein (*glnH*) and Putative Peptidoglycan-Binding Protein (BP0020) from *B. pertussis* in *E. coli*

### 3.1.1. PCR Amplification and Cloning into pGEM®-T Easy Vector

After the isolation of *B. pertussis Thoma I* genomic DNA, the genes *glnH* and BP0020 were PCR amplified (Figures 9 and 10)

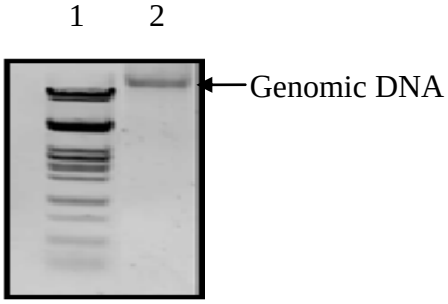


**Figure 9** Isolation of Genomic DNA. Lane 1: Marker (Lambda DNA/PstI, 24), Lane 2: Genomic DNA of *B. pertussis*.

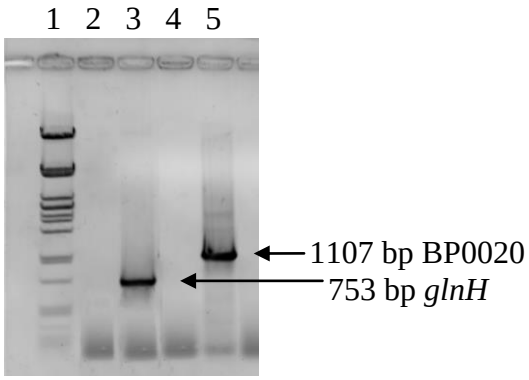


**Figure 10** *glnH* and BP0020 genes amplification from the genomic DNA of *B. pertussis*. Lane 1: Marker (Lambda DNA/PstI, 24), Lane 2: Negative control for *glnH* (no template), Lane 3: Negative control for BP0020 (no template), Lane 4: PCR product for *glnH*, Lane 5: PCR product of BP0020.

Following extraction from agarose gel the PCR products were cloned into pGEM®-T subcloning vector (Figure 5.).

### 3.1.2. Transformation of Recombinant Plasmid to *E. coli* DH5α and Cloning Verification

After the transformation of ligation products of *glnH* and BP0020 into *E. coli* DH5α competent cells, the recombinants were selected by inoculation of the cells on LA plates containing IPTG, X-gal and ampicillin. White colonies, showing the recombination, were isolated (Figure 11). In order to verify the cloning, isolated plasmids were digested with restriction enxymes, namely; *BamH*I and *Bgl*II (Figure 12).

Known sequences found in the website NCBI were compared with the sequenced products in order to further verify the sequences (Figure 13 and Figure 14). A significant homology with genes of *B. pertussis* was observed.

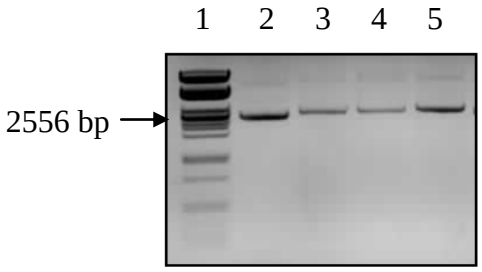


**Figure 11** Isolated plasmids from white colonies. Lane 1: Marker (Lambda DNA/PstI, 24), Lane 2: pQE60 vector as control, Lane 3-4: pGEM®-T carrying *glnH*, Lane 5: pGEM®-T carrying BP0020.

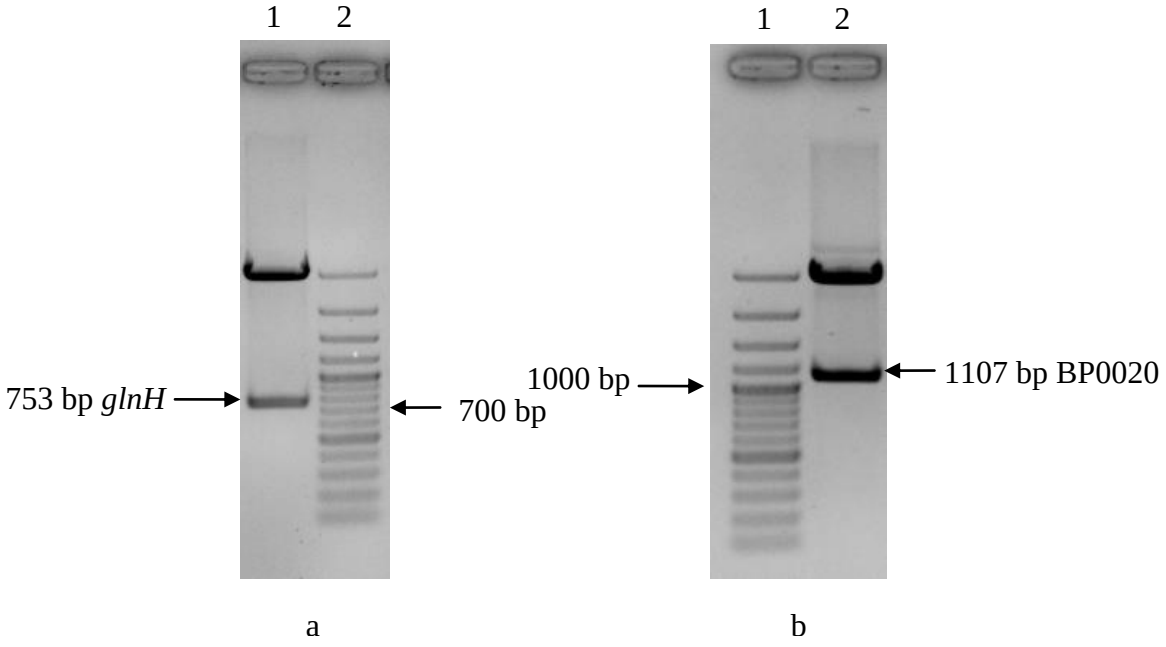


**Figure 12** Cloning verification after restriction enzyme digestion of *glnH* and BP0020 genes. a) Lane 1: BamHI and BglII digested pGEM®-T carrying *glnH*, Lane 2: Marker (O'GeneRuler™ 100 bp Plus DNA Ladder). b) Lane 1: Marker (O'GeneRuler™ 100 bp Plus DNA Ladder), Lane 2: BamHI and BglII digested pGEM®-T carrying BP0020.

>gi|33591275:1652374-1653126 Bordetella pertussis Tohama I chromosome, complete genome

ATGATCAAAGCGAAAGTTGCGGCCGTACTGATCGGCGCGTCGGCGGCCCTGCTGGGCGCCGTGTCCAC
GGCATCGGCCCAGGGCAGGGAACTGGTGGTCGCCACCGACACCGCGTTCGTACCGTTCGAGTTCAAGC
AGGGCAACACCTATACCGGCTTCGACATTGATCTGTGGGCGGCCATCGCCAAGGAGCTGAACCTGAAG
TACAAGCTGCAGCCCATGGATTTCAACGGCATCATCCCCGGCCTGCAGACCAAGAACATCGATGCGGC
GCTTGCCGGCATCACGATCCGGGACGACCGCAAGAAGGTCATCGACTTCTCCGATCCCTATTATGAGA
GCGGCCTGGCCATCCTGGTCAGCGAGAAGAACGACAGCATCAAGGCGGCCAAGGACCTCTCCGGCAAG
AGCGTGGCCGTCAAAACCGGCACCGCCACGGTCGACTTCATGAAGTCGCAGGTGCCCGACGCCAAGCT
GAAACTGTTCCCCAATATCGACAACGCCTATCTCGAACTGGCCACCGGCCGCGTCGACGCGGCGGTGC
ATGACACGCCCAACGTGCAGTACTACGCCAACACGGCGGGCAAGGGCCGCGTGAAGGTGGTCGGCACG
GTCAAGAGCGGCGACTTCTACGGCATCGCCTTCCCCAAGGGCAGCGAACTGGTGCCGCAGGTGAACAA
GGCGCTGGCCACGCTGAAAGCCAACGGCCAGTACGACGCCATCTACACCAAGTGGTTCGGCAAGCAGC
CCTGA

**Figure 13** The nucleotide sequence of *glnH* amplicon (http://www.ncbi.nlm.nih.gov).

>gi|33591275:c25459-24353 Bordetella pertussis Tohama I chromosome, complete genome

ATGATTCGGCCGTTCGCGCCAGCGTTCATGCTGGTGTGTGCCCTTGTGGCCAGCGCCGCGGTACAAGC
CCAGCCCGCAGGTAGCCTGGGAGAGAATTTTCTTTACCGAGTGCGTTCTGGCGACACGCTTATAGCGT
TAGCAAGCACTTACACCGGTAATGAATCCAACTGGTCGATCCTGCAGACGCTGAACCGGGTGGACGAC
CCCCAGCGCCTGCCGATCGCCCTCGAGCTGCGCATTCCGCTGGCCATGATCCCGGTGCAGGCGGCAGG
CGCCGAGGTGGTCCACGTCAGCGGCAACGCCACCGCCGACGGCCAGGCCCTGCGGGTCGGCACGCAGA
TCGTCGAGGGCAGCACCATCAGGACCGCGTCCGGCAGCTTCGTTACCCTGAAACTGTCCGACGGCAGC
CAGGTGACGATTCCCGAGAACGGCACGGTGGCCGCCAACCGCCTGCGCCAATTCCAGCGCGTGCCGCT
TACCGATTCGATCTTCCAGGTACAGCAAGGCGAGCTCGAATCGCGCGTGGCCCCGGGCGGCCAGGGCG
TTGGCCGCTTCGAGATCCGTACGCCGGTGGCGGTCACGGGCGTGCGCGGCACCCGCTTTCGCGTCAAG
AGCAGTCCGCGGGGCGCCAGCAGCGAAGTCCTGGAAGGCAGCGTACGCCTGCAGCCGCACGCCCCCGA
CGCCGCGCTGGCCACGCCGGTAGCGGTGTCCTCGGGCTACGGCGCCAATGTCGGCAGCGACGGCGCGT
TCTCGGGCGTGCAGGCCCTGCTGCCCGCCCCCCAACTGCAAGCCCCGGCGCGCGCCGCCTCGGGCGGC
TGGACGGTGCCGTTTGCGCCCGTATCCGGCGCTTCCGCCTATATAGTGAGGGTTTCGCGCGACGCGGA
GGGCATGCACGTGGTGGCGTCCGACCGGTTCGATACCAACGATGTACGGTTCCGCGCGCCCGGCGCCG
GAACCTACTACGTGGCGGTTCGCGCCGTGGATGCCTCCGGTTTGAACGGGCGCGAAGCCGTGCAGCCT
TTCGAAGGCGCAAACGTGCTGTCCACGCCCTATGGCCTGTCCGTAGCCACCGGCACCGGCGACCTGGT
GCTGCTCAGCGAATTCTAA

**Figure 14** The nucleotide sequence of BP0020 amplicon (http://www.ncbi.nlm.nih.gov).

### 3.1.3. Cloning of *glnH* and BP0020 Genes into pET-28a (+) Expression Vector

There were His-Tag sequences at C and N terminals of pET-28a (+) expression vector and T7 promoter controls the cloned gene expresssion. *BamH*I enzyme was used for the digestion of the vector in the subcloning step and alkaline phosphatase treatment was performed. Then ligation of the genes obtained from pGEM®-T vector were performed into pET-28a vector.

### 3.1.4. Transformation of Recombinant Plasmids into *E. coli* BL21(DE3) and Cloning Verification in *E. coli* BL21(DE3)

After the transformation of the ligation products in pET-28a (+) into *E. coli* BL21(DE3) competent cells, LB agar plates containin kanamycin were used for the inoculation of the cells. Recombinant colonies were selected and the plasmids were isolated. Cloning verification was performed by restriction enzyme digestion. *BamH*I and *EcoR*I enzymes for pET-28a (+) containing *glnH* gene and *Not*I and *BamH*I enzymes for pET-28a (+) carrying BP0020 were used for digestion (Figures 15 and 16).

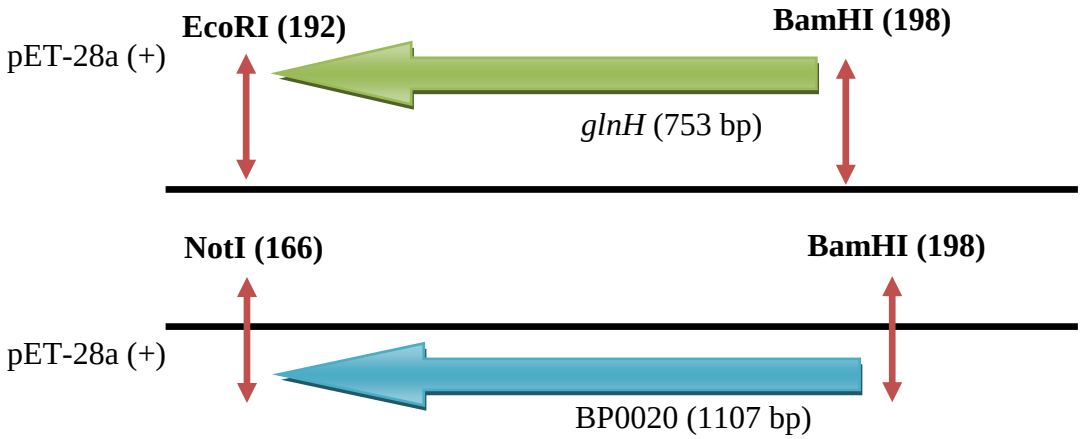


**Figure 15** Restriction digestion profile of *glnH* and BP0020 genes in pET-28a (+).

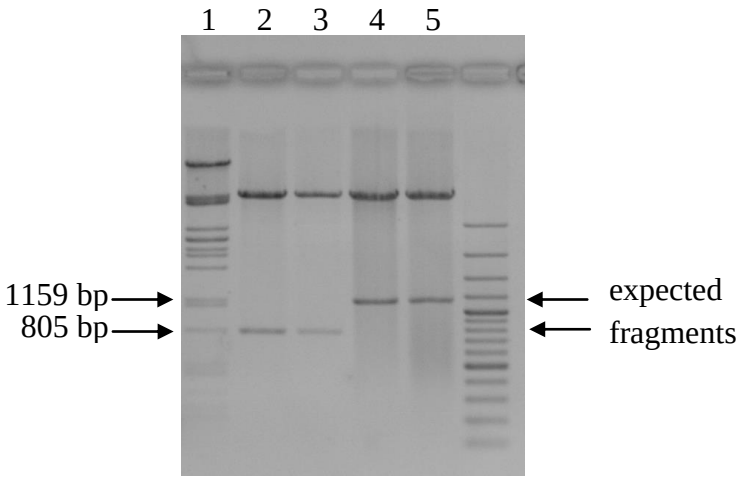


**Figure 16** Cloning verification of *glnH* and BP0020 into pET-28a (+) vector after restriction enzyme digestion. Lane 1: Marker (Lambda DNA/PstI, 24) Lane 2, 3: BamHI and EcoRI digested pET-28a (+) carrying *glnH*. Lane 4, 5: NotI and BamHI digested pET-28a (+) containing BP0020.

### 3.2. Expression of Recombinant *glnH* and BP0020 Genes in *E. coli* BL21(DE3)

*E.coli* BL21(DE3) cells transformed with pET-28a (+) carrying the gene of interest were inoculated into two flasks of LB for each sample. For the induction of recombinant His-tagged *glnH* (27.8 kDa) and BP0020 (40.9 kDa) expression addition of IPTG into one of the cultures was performed. Uninduced cultures were used as controls of over-expression (Figure 17).

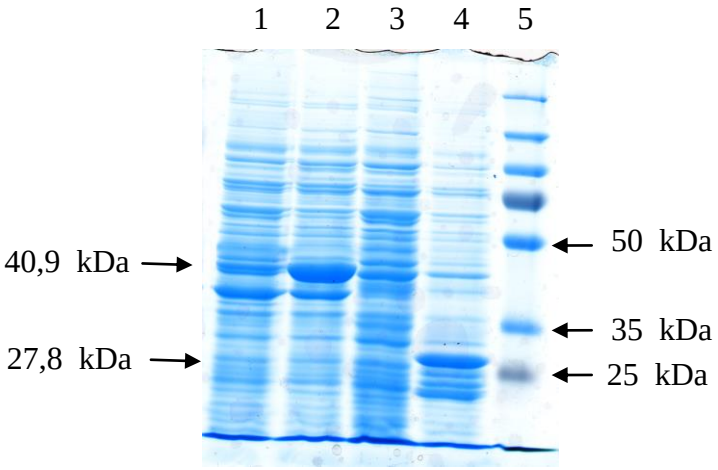


**Figure 17** Coomassie blue-staining of SDS polyacrylamide gel of expressed recombinant BP0020 and GlnH. Lane 1: Control (uninduced sample) for BP0020, Lane 2: Expressed BP0020 (IPTG-induced sample), Lane 3: Control (uninduced sample) for GlnH, Lane 4: Expressed GlnH (IPTG-induced sample), Lane 5: Marker (Pageruler$^{TM}$ Plus Unstained Protein Ladder, #26619).

### 3.3. Recombinant GlnH and BP0020 Protein Purifications by His-tag Affinity Chromatography

Purifications of recombinant, His-tagged GlnH and BP0020 proteins were performed with Protino® Ni-TED 2000 protein purification system. Following the purification step, urea and NaCl concentrations were decreased by dialysis of the proteins against a dialysis buffer given in Appendix B. Dialysis products were passed through 0.2 µm filters (Figures 18 and 19). As reported in the study performed by Constans (2002), because of their small size, less potential to interfere with protein folding and weak immunogenicity, removal of His-tag tails of the recombinant proteins was considered to be unnecessary.

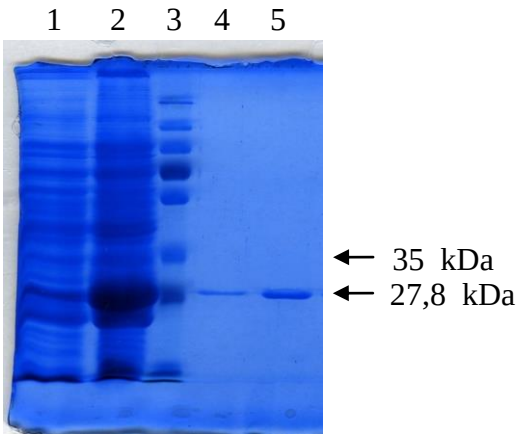


**Figure 18** Coomassie blue-staining of SDS polyacrylamide gel of purified His-tagged GlnH. Lane 1: Control (uninduced sample), Lane 2: Expressed GlnH (IPTG-induced sample), Lane 3: Marker (Pageruler$^{TM}$ Plus Unstained Protein Ladder, #26619), Lane 4, 5: Purified His-tagged GlnH.

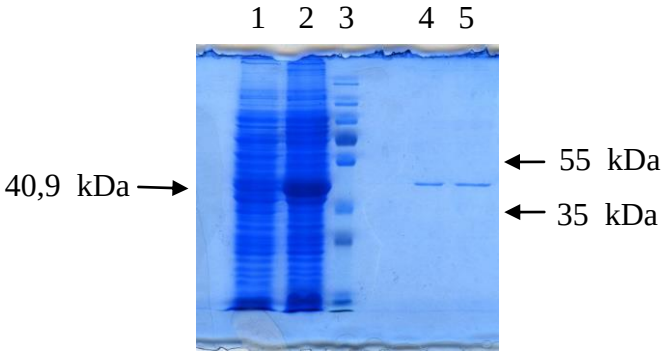


**Figure 19** Coomassie blue-staining of SDS polyacrylamide gel of purified His-tagged putative BP0020. Lane 1: Control (uninduced sample), Lane 2: Expressed BP0020 (IPTG-induced sample), Lane 3: Marker (Pageruler$^{TM}$ Plus Unstained Protein Ladder, #26619), Lane 4, 5: Purified His-tagged BP0020.

### 3.4. Western Blot Analyses of Recombinant GlnH and BP0020 Proteins

According to the study conducted by Tefon et al. (2011) in our laboratory, *B. pertussis* Tohama I and Saadet cells have GlnH protein at their surfaces and it is immunogenic. The current study was conducted in order to confirm the immunogenic property of the protein after the expression, purification and dialysis steps. The sera obtained from the mice injected were used for the Western blot analysis. As a control, uninduced whole culture lysates were used. The immunogenicity profiles of the proteins were shown in Figures 20 and 21. To further analyse the immunogenicity, colony counting and ELISA procedure are given in the next sections.

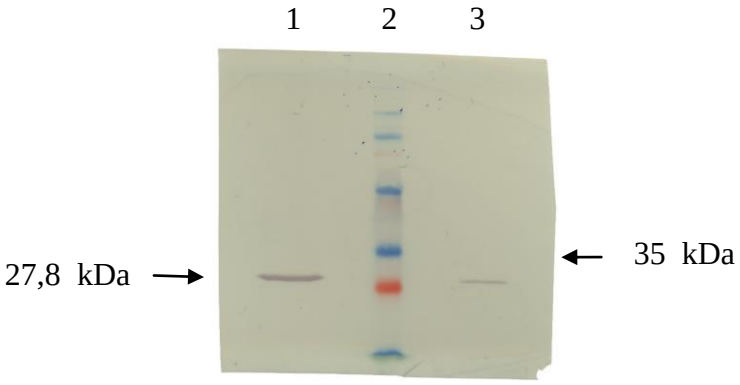


**Figure 20** Western blot analysis of purified recombinant GlnH anti-whole cell Saadet sera. Lane 1: Purified His-tagged GlnH. Lane 2: Marker (PagerulerTm Plus Prestained Protein Ladder, #26619), Lane 3: Control (uninduced sample).

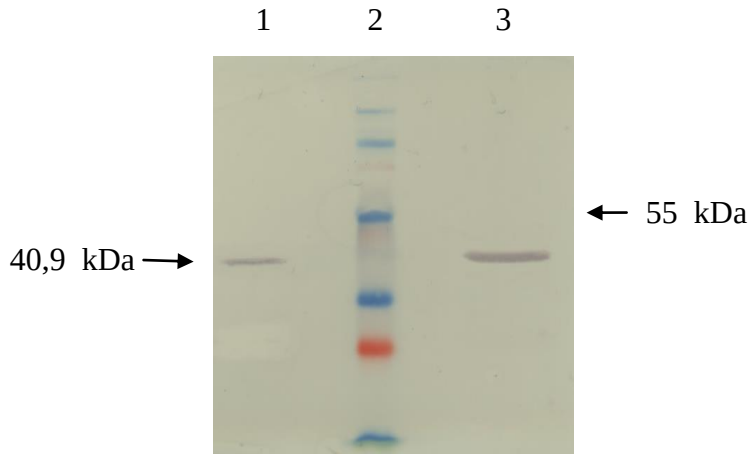


**Figure 21** Western blot analysis of purified recombinant BP0020 anti-whole cell Saadet sera. Lane 1: Control (uninduced sample), Lane 2: Marker (PagerulerTm Plus Prestained Protein Ladder, #26619), Lane 3: Purified His-tagged BP0020

### 3.5. Mice Challenge Experiments

For the immunization of BALB/c mice at days 0 and 21, 10 μg of both gnlH and BP0020 proteins were used. Intranasal challenge was performed with 2.5 x $10^9$ CFU of *B. pertussis* Saadet cells. Collection of sera was performed before the second injection and challenge. Following intranasal challenge, lungs were removed at days 5 and 8 which afterwards used to determine bacterial colonization.

Because of the similarities between respiratory *B. pertussis* infection of mice and human *B. pertussis* infection, intranasal bacterial challenge for respiratory system was used instead of intracerebral model. Postinfection symptoms like histamine sensitization and lymphocytosis are easily seen after intranasal infections, and mice are more susceptible to *B. pertussis* when they are young (Guiso *et al*., 1999).

### 3.5.1. Colonization of *B. pertussis* on the Lungs of the Mice Immunized with GlnH and BP0020

Bacterial clearance from the lung of immunized mice has been shown in earlier studies in which protective activities of *B. pertussis* antigens were determined. Studies performed on the different antigens like filamentous hemagglutinin (FHA) showed decrease in bacterial colonization in lungs of adult mouse model (Kimura *et al*., 1990). In addition, previous studies suggested that induction of the bacterial clearance was caused by the antigens used in the vaccines (Marr *et al*., 2008). Bacterial growt was lowered significantly by the antibodies against pertussis toxin, (Sato and Sato, 1990).

In the present study, the lung were removed and tested for the bacterial colonization in order to determine the immunoprotective capabilities of GlnH and BP0020 antigens. The results of the present study for bacterial clearance tests are presented in Table 8. The results showed that, bacterial colonizations were decreased in the immunized mice when compared to the control group.

**Table 8** CFU/ml calculation from the lung homogenates of the mice immunized with 10 μg of GlnH and BP0020 after intranasal challenge with live B. pertussis Saadet cells.

| **Group** | **Day 5** | **Day 8** |
|---|---|---|
| Control | $2{,}0 \times 10^2$ (±0,18928) | $5{,}0 \times 10^2$ (±0,212955) |
| Immunized | 0 (±0) | 0,53 x 10 (±1,096249) |

### 3.5.2. Serum Antibody Levels Against Recombinant GlnH and BP0020 Proteins

Before second immunization and challenge, sera from immunized and control mice were collected. The sera were used to measure serum-specific IgG levels against the recombinant proteins; GlnH and BP0020. Obtained antibody levels determined by ELISA are given in Figure 22.

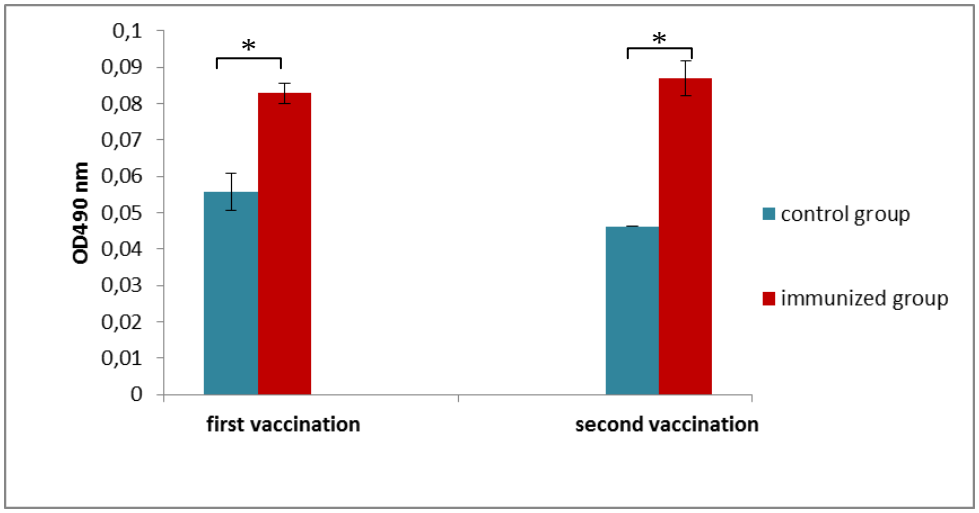


**Figure 22** Antibody levels in mice immunized with combined vaccine including 10 µg of both recombinant GlnH and BP0020 proteins (titer: 1/3200) (*$p<0.05$ between immunized and control groups).

Generally total IgG level measurement is not the most meaningful way to determine what is actually happening in the immune system. It was previously reported that there were protective activities that do not translate into antibody responses (Roberts *et al*., 1990; Shahin *et al*., 1990). In the present study it was found that there is an elevation in terms of total IgG between control and immunized mice with 10 µg GlnH and BP0020 proteins (Figure 22).

On the other hand sera were analysed to determine antigen-specific IgG levels against GlnH and BP0020 seperately. Obtained antibody levels determined by ELISA are given in Figure 23.

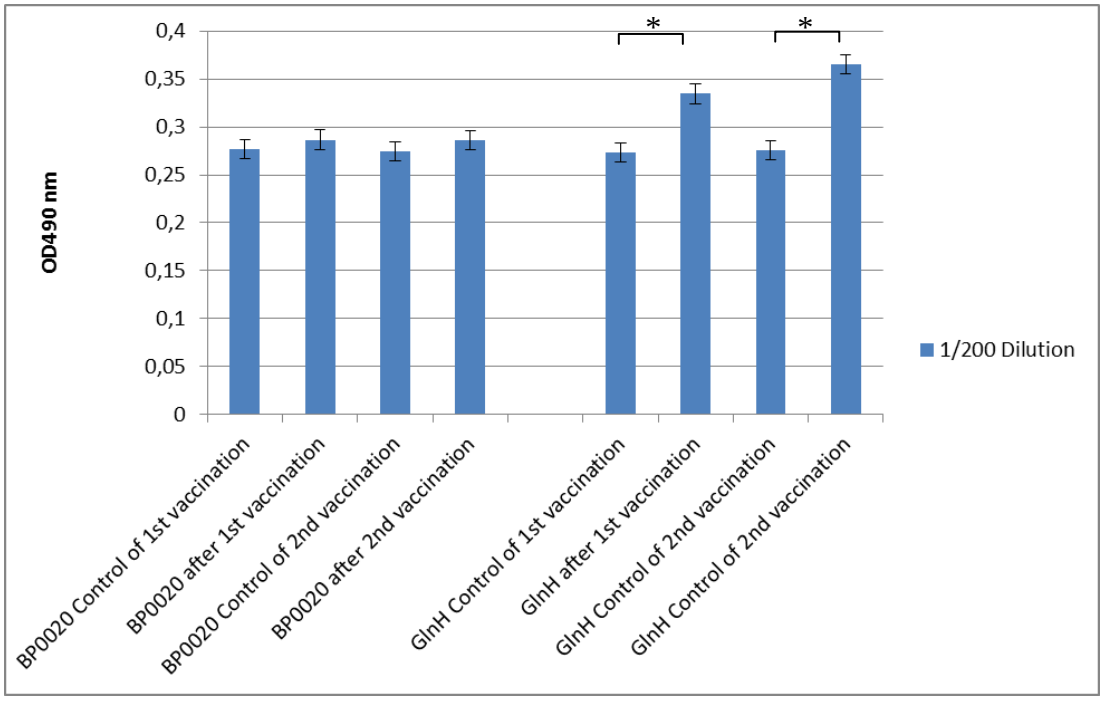


**Figure 23** Antibody levels in mice immunized with 10 µg of recombinant BP0020 and GlnH proteins (*p<0.05 between immunized and control groups).

ELISA results of separately analysed GlnH and BP0020 proteins showed an increase in antigen-specific IgG levels against both of the recombinant proteins (Figure 23). According to the results it could be claimed that specific antibody response against GlnH was stronger when compared to BP0020. The results of bacterial colonization showed the protective capabilities of the antigen and the difference in the serum IgG levels of immunized and control mice could be interpreted as compatible with colonization results.

### 3.5.3. IFN-γ Levels in Mice Vaccinated with GlnH and BP0020 Proteins

Cellular immune responses characterized as Th1- or Th2-type responses are more reliable than antibody responses. High levels of IL-4, IL-10 and IgG1 are typical characteristics of Th2-type responses, while Th1-type responses are characterized by activation of infected macrophages and increased IFN-γ, IgG2a levels to induce antibacterial mechanisms. As reported by Fisher *et al*. (1998), phagocytic macrophages having role in bacterial clearance is activated by IFN-γ. The presence of *B. pertussis* in the respiratory tract, macrophages, dendritic cells and natural killer cells which are immune system cells are activated. Bacterial antigens are presented to T cells by dendritic cells and this results in polarization of T cells to the Th1 subtype. Following the polarization Th1 cells secrete IFN-γ and thus recruit and activate neutrophils and macrophaces. The activated cells kill the bacteria by reactive oxygen intermediates or NO synthesis. The other role of IFN-γ is the stimulation of B cells. Stimulated B cells produce opsonizing and complement-fixing antibody (IgG2a in the mouse) (Mills, 2001). As reported by several previous studies, the resolution of *Burkholderia pseudomallei*, *Listeria monocytogenes*, *Bordetella bronchiseptica* and *Chlamydia trachomatis* infections caused higher IFN-γ production (Buchmeier and Schreiber, 1985, Pilione and Harvill, 2006). The lungs of the mice infected with *B. pertussis* were shown to have increased IFN-γ mRNA levels (Barbic *et al.*, 1997). The study conducted by Barnard *et al.* (1996) reported that immunized mice with antigen-specific IFN-γ production at low levels following whole cell vaccination had low survival rate. With the information about the key role of IFN- γ in *B. pertussis* infection, IFN-γ levels were determined in the present study. Serum samples were analyzed with Mouse IFN- γ Minikit (Figure 23).

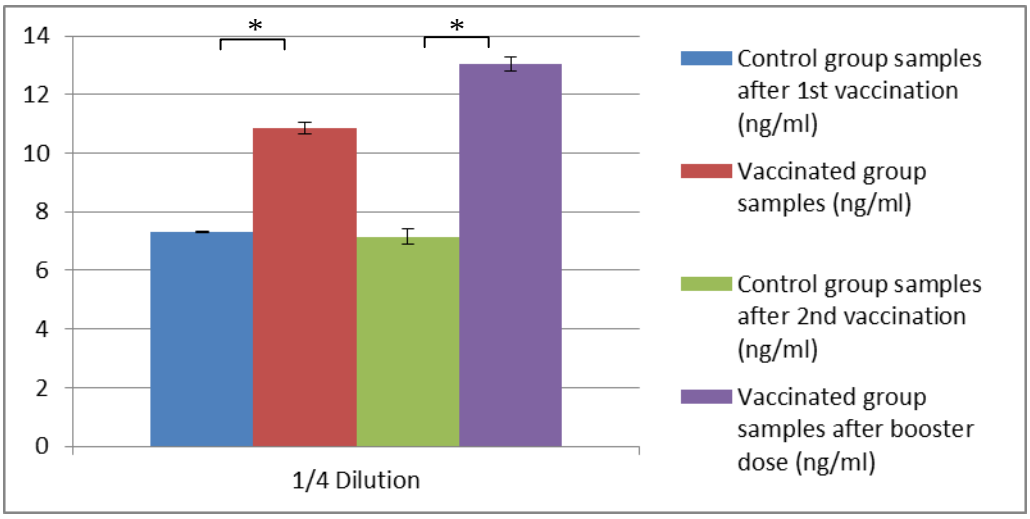


**Figure 24** IFN-γ levels in control and vaccinated mice (*p<0.05 between immunized and control groups).

When control and immunized mice with GlnH and BP0020 were compared in terms of the production of IFN-γ, it was found that there was a specific difference, showing that T lymphocytes of the immunized mice produced sufficient IFN-γ.

# CHAPTER 4

# CONCLUSION

- In this study immunoprotectivity of recombinant vaccine candidates glutamine-binding periplasmic protein GlnH and putative peptidoglycan binding-protein BP0020 was investigated in mouse model.

- In ELISA, serum-specific IgG levels were detected since they have importance in humoral immune response. There was a specific difference between the control mice and immunized mice with 10 μg recombinant GlnH and BP0020 proteins. In addition, it was observed that antigen-specific responses against GlnH were higher when compared to BP0020.

- The humoral immune response stimulated by an antigen does not necessarily entail its immunoprotective capability against *B. pertussis*. Cell-mediated immunity is a prerequisite for complete immunity against *B. pertussis*. Since IFN-γ production is a hallmark of cellular immune response, serum IFN-γ levels of vaccinated and control groups were measured. Serum IFN-γ levels of vaccinated mice were significantly higher in comparision to control group.

- Vaccinated mice group was compared with control group in terms of bacterial clearance of the lungs. Viable counts from lung homogenates on days 5 and 8 post-challenge showed that bacterial clearance was stimulated by combined vaccine formula including 10 μg recombinant GlnH and BP0020 proteins.

- In light of the owerall results it could be suggested that recombinant GlnH and BP0020 proteins are promisig candidates for new vaccine formulations. However further studies such as determination of antigen-specific IgG1/IgG2a levels against separate proteins with different concentrations and higher numbers of mice are required.

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# APPENDIX A

## 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 B

## COMPOSITIONS OF BUFFERS AND SOLUTIONS

**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

**TEN Buffer**

40 mM Tris

1 mM EDTA

150 mM NaCl

**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

**Transfer Buffer (1X) (1000ml)**

| | |
|---|---|
| 20% Methanol | 200 ml |
| 25 mM Tris | 3,63 g |
| 192 mM Glycine | 14,4 g |
| 0,037% SDS | 0,37 g |

**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

**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

**Coating Buffer (pH:7.4, 0.2 µm filtered)**

0.014 M NaCl

0.002 M $KH_2PO_4$

0.008 M $Na_2HPO_4$. $7H_2O$

0.01 M KCl

**Blocking Buffer**

4 % Bovine Serum Albumin, 5 % Sucrose in PBS

**Assay Buffer (pH:7.2-7.4)**

2 % Bovine Serum Albumin in PBS

**Wash Buffer (pH:7.0-7.5, prepared fresh)**

50 mM Tris, 0.2 % Tween-20 in PBS

**Stop Solution**

0.18 M $H_2SO_4$

**Coomassie Blue R-250 Staning**

| | |
|---|---|
| Coomassie blue R-250 | 0,25 g |
| Methanol | 125 g |
| Glacial acetic acid | 25 ml |
| $dH_2O$ | 100 ml |

**Blocking Solution**

2% BSA in washing solution

**Phenol-Chloroform Solution (water-saturated)**

Phenol 500 g

Chloroform 500 mL

Distilled water 400 mL

- The solution was stored at room temperature, protected from light.

## APPENDIX C

## CHEMICALS AND THEIR SUPPLIERS

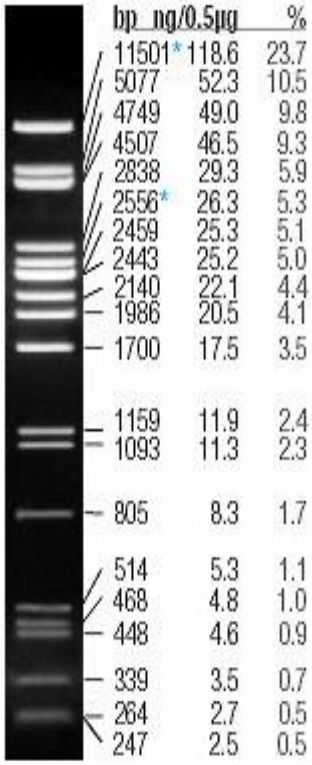
| **Chemicals** | **Supplier** |
|---|---|
| Agar Bacteriological | Merck |
| Agarose | Prona |
| Ampicillin | Sigma |
| Bovine Serum Albumin | Sigma |
| Bromophenol Blue | Sigma |
| $CaCl_2.2H_2O$ | Merck |
| Casamino Acid | Sigma |
| Coomassie Brilliant Blue G-250 | Merck |
| Coomassie Brilliant Blue R-250 | Sigma |
| dNTPs | MBI Fermentas |
| Dimethylformamide | Merck |
| EDTA | AppliChem |
| Ethanol | Botafarma |
| Ethidium Bromide | Sigma |
| Glacial Acetic Acid | Merck |
| Glycerol | Merck |
| Glycine | Merck |
| HCl | Merck |
| Imidazole | Merck |
| IPTG | Sigma |
| Kanamycin | Sigma |

| | |
|---|---|
| $KH_2PO_4$ | Merck |
| KCl | Merck |
| L-cystein | Sigma |
| Ligase Buffer (2X) | MBI Fermentas |
| Ligase Buffer (10X) | MBI 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 |
| SDS | Merck |
| Skim Milk | Fluka |
| Sucrose | Merck |
| Tris-base | Merck |
| Urea | Fluka |
| X-gal | MBI Fermentas |
| Yeast Extract | Difco |

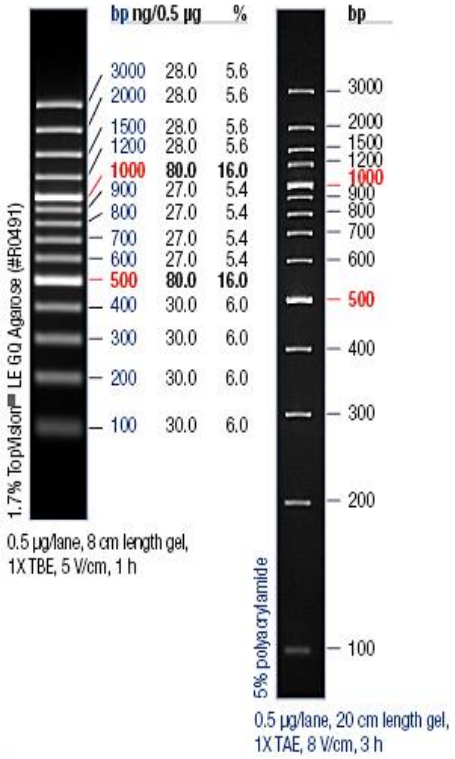
| **Enzymes** | **Supplier** |
|---|---|
| *BamH*I | MBI Fermentas |
| *Bgl*II | MBI Fermentas |
| *EcoR*I | MBI Fermentas |
| Lysozyme | AppliChem |
| *Nco*I | MBI Fermentas |
| T4 DNA Ligase | MBI Fermentas |
| *Tag* DNA Polymerase | MBI Fermentas |

## APPENDIX D

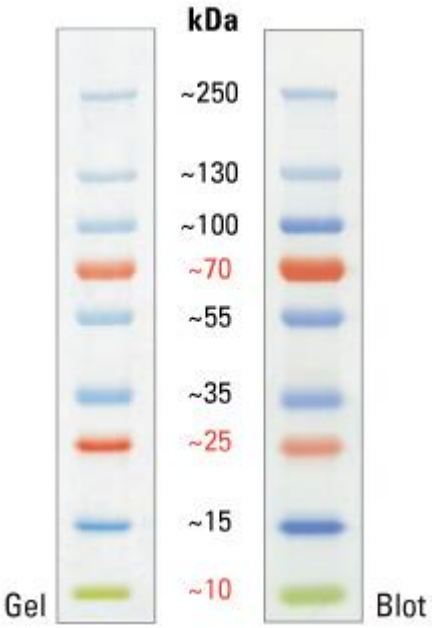
## MARKERS




Fermentas Lambda DNA/PstI, 24 Ladder




O'GeneRuler™ 100 bp Plus DNA ready-to-use, 100-3000bp




SDS-PAGE band profile of the Thermo Scientific PageRuler Plus Prestained Protein Ladder. Images are from a 4-20% Tris-glycine gel (SDS-PAGE)

Product number: 26619