ASSESSMENT OF IMMUNE PROTECTIVE CAPACITY OF THE RECOMBINANT IRON SUPEROXIDE DISMUTASE (FeSOD) FROM *BORDETELLA PERTUSSIS*

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ABSTRACT

ASSESSMENT OF IMMUNE PROTECTIVE CAPACITY OF THE RECOMBINANT IRON SUPEROXIDE DISMUTASE (FeSOD) FROM *BORDETELLA PERTUSSIS*

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Whooping cough (pertussis) is a highly contagious acute respiratory disease caused by the strict human pathogen *Bordetella pertussis*, a gram-negative coccobacillus. The worldwide mass-vaccination was started in 1940s and to date, a number of whole-cell (Pw) and acellular pertussis vaccine (Pa) formulations were developed. Yet the current vaccines are incapable of providing sustained, lifelong immunity and eliminating subclinical infections, which pose a threat especially for unimmunized infants as well as adolescents and adults. Thus, finding new protein candidates with high immune protective capacities is necessary to enhance the clinical efficacy of current acellular pertussis (Pa) vaccines.

In this study, iron-superoxide dismutase (FeSOD) protein was investigated for its capacity of conferring protectivity as well as stimulating humoral and cellular responses against *B. pertussis* infection in a mouse model. For this purpose, *sodB* gene, which encodes iron-superoxide dismutase FeSOD protein, was amplified from the genomic DNA of the universal *B. pertussis* strain 'Tohama I' and sequentially cloned to pGEM®-T subcloning and pET-28a(+) expression vectors. Afterwards

sodb/pET28a(+) construct was introduced to *E. coli* BL21(DE3) cells and the gene was overexpressed therein via IPTG induction. The expressed FeSOD protein was then purified by affinity chromatography and its previously reported immunogenicity was confirmed by Western blot. After filter-sterilization, the protein was adsorbed to alum [$Al(OH)_3$] adjuvant and introduced to BALB/c twice at three weeks intervals intraperitoneally at a concentration of 20 μg purified FeSOD protein/mouse. Another group of mice were immunized in tandem with heat-inactivated whole-cell suspension of *B. pertussis*. Ten days after the second immunization, mice were intranasally challenged with the local 'Saadet' strain of *B. pertussis*. Next the lungs of groups of mice were excised, homogenized and plated as serial dilutions on days 5, 8 and 14 post-challenge, and viable lung CFU counts were carried out. Whole cell immunization conferred complete bacterial clearance following *B. pertussis* intranasal infection while FeSOD immunization failed to attain such protection. In addition to the protectivity assay, ELISA was performed to assess the humoral (i.e. IgG) immune response triggered upon FeSOD- and whole-cell immunizations and a statistically significant increase in *anti*-FeSOD IgG production was observed in FeSOD-immunized group. Finally, cellular immune response was tested via cytokine (IFN-γ) assay, in which spleens of mice were excised, splenocytes were cultured and the level of IFN-γ production upon FeSOD addition to the cultures was measured via ELISA. This test showed that whole-cell immunization triggered IFN-γ production at significant levels while FeSOD-immunization did not; indicating the failure of alum-adsorbed FeSOD immunization in inducing cell-mediated immune response.

Keywords: Whooping cough, *Bordetella pertussis*, acellular vaccine, FeSOD protein, cellular immune response, humoral immune response, protectivity

ÖZ

BORDETELLA PERTUSSIS'E AİT REKOMBİNANT DEMİR SÜPEROKSİT DİSMUTAZ (FESOD) PROTEİNİNİN İMMÜN KORUYUCU KAPASİTESİNİN DEĞERLENDİRİLMESİ

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Boğmaca (pertussis), tek konağı insan olan *Bordetella pertussis* gram-negatif kokobasilinin sebep olduğu oldukça bulaşıcı bir solunum yolu hastalığıdır. 1940'larda dünya çapında geniş kapsamlı bir aşılama süreci başlamış ve bugüne kadar hastalığa karşı çeşitli tüm hücre ve asellüler aşı kompozisyonları geliştirilmiştir. Ancak mevcut aşılar, uzun soluklu immünite sağlama ve subklinik enfeksiyonları ortadan kaldırma konularında yetersiz kalmaktadır. Bu durum özellikle bağışıklık kazanmamış küçük çocuklarda olduğu kadar adölesanlar ve yetişkin bireyler için de tehlike oluşturmaktadır. Bu yüzden mevcut asellüler aşıların klinik etkinliğini artırmak için yüksek immün koruyucu özelliği taşıyan yeni proteinlerin bulunması önem arz etmektedir.

Bu çalışmada, demir süperoksit dismutaz (FeSOD) proteininin, *B. pertussis* enfeksiyonuna karşı sağladığı koruyuculuk ve aynı zamanda humoral ve hücresel bağışık yanıtlarını tetikleme kapasitesi, fare modeli kullanılarak incelenmiştir. Bunun için öncelikle FeSOD proteinini kodlayan *sodB* geni, tüm dünyada kullanılan *B. pertussis* suşu 'Tohama I' hücrelerinin genomik DNA'sından PCR

yardımıyla çoğaltılıp sırasıyla pGEM®-T Easy klonlama ve pET28a(+) ekspresyon vektörlerine klonlanmıştır. Rekombinant pET28a(+)/*sodB* vektörü, *E. coli* BL21(DE3) hücrelerine aktarılmış ve burada IPTG indüksiyonu yoluyla protein ifadesi gerçekleştirilmiştir. İfade edilen FeSOD proteini daha sonra afinite kromatografisi ile saflaştırılmış ve önceki çalışmalarda tespit edilen immunojenitesi Western blot yöntemiyle teyit edilmiştir. FeSOD proteini daha sonra $Al(OH)_3$ adjuvanıyla karıştırılmış ve bu karışım BALB/c farelerine, fare başına 20 µg saflaştırılmış protein düşecek şekilde, intraperitoneal enjeksiyonla üç hafta arayla iki defa verilmiştir. Aynı şekilde diğer bir grup fare de *B. pertussis* inaktive tam hücre süspansiyonu ile bağışıklanmıştır. İkinci aşılamadan 10 gün sonra, *B. pertussis* lokal 'Saadet' suşunun $2.5x10^9$ cfu/ml'lik süspansiyonundan bağışıklanan her fareye 100 µl'lik doz olacak şekilde intranazal yolla verilmiştir. Bu işlemi takip eden beşinci, sekizinci ve on dördüncü günlerde fare gruplarının akciğerleri alınmış ve farklı seyreltmelerle ekim yapılan ciğer homojenatlarından CFU sayımı yapılmıştır. Bunun sonucunda tam hücre aşılamasının *B. pertussis* enfeksiyonuna karşı tam koruma sağladığı, FeSOD immunizasyonunun ise bunu gerçekleştiremediği saptanmıştır. Koruyuculuk testinin yanı sıra, bağışıklanan farelerin serumu kullanılarak humoral bağışık yanıtı ELISA testi aracılığıyla tespit edilmiş, FeSOD aşılamalarının *anti*-FeSOD IgG üretiminde önemli ölçüde artışa sebep olduğu gözlenmiştir. Son olarak hücresel immun yanıtının tespiti, farelerin dalak örneklerinden kültür oluşturulup, kontrol ve aşılanmış farelerden elde edilen kültürlerin FeSOD proteininin eklenmesi üzerine ürettiği IFN-γ miktarları karşılaştırılarak yapılmış ve tam hücre aşılamasının hücresel immun yanıtı önemli ölçüde tetiklediği görülürken aluminyuma adsorbe edilmiş FeSOD aşılamasının bunu gerçekleştirmediği görülmüştür.

Anahtar Kelimeler: Boğmaca, *B. pertussis*, asellüler aşı, FeSOD proteini, humoral ve hücresel bağışık yanıtları, koruyuculuk

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bp(s)	: Base pair(s)
B. pertussis	: *Bordetella pertussis*
CFU	: Colony forming unit
CMI	: Cell-mediated immunity
DTPw	: Diphteria, Tetanus, whole cell Pertussis vaccine
DTPa	: Diphteria, Tetanus, acellular Pertussis vaccine
E. coli	: *Escherichia coli*
FHA	: Filamentous haemagglutinin
FIM	: Fimbriae
IFN-γ	: Interferon gamma
IgG	: Immunoglobulin G
IPTG	: Isopropyl-b D-thiogalactopyranoside
i.c.	: Intracerebral
i.p.	: Intraperitoneal
kDa	: Kilodalton
NCBI	: National Center for Biotechnology Information
OD	: Optical Density
PRN	: Pertactin
PT	: Pertussis Toxin
SDS-PAGE	: Sodium Dodecyl Sulphate-Polyacrylamide Gel Electrophoresis
s.c.	: Subcutaneous

CHAPTER 1

INTRODUCTION

1.1. The Disease "Whooping Cough"

Whooping cough (pertussis) is a highly contagious acute respiratory disease, main causative agent of which is the gram-negative coccobacillus *Bordetella pertussis*, a strict human pathogen (Belcher *et al*., 2000; Mattoo and Cherry, 2005). Despite significant reduction in incidence rates and death cases associated with pertussis from the 1940s, when whole cell vaccines were introduced, pertussis remains to be among the 10 infectious diseases with the highest morbidity and mortality worldwide (Mooi *et al*., 2007). In fact, a shift towards older age categories (i.e. from infants to adolescents and adults) in incidence of the disease has been observed for the last 25-30 years, which is a hallmark for waning vaccine-induced immunity (Mooi *et al*., 2007). Decreased immunity following vaccination in the absence of natural and vaccinal boosters, lower efficacy of current vaccines, vaccine-induced adaptation of the circulating strains (i.e. antigenic shift) as well as increased awareness and improvements in diagnostic tools could be numbered among the factors leading to the resurgence of pertussis (Cherry, 1997; Cherry and Olin, 1999; Mattoo and Cherry, 2005; Tondella *et al*., 2009).

1.1.1. History of Pertussis

The first recording of the disease dates back to 1540, in England (Lapin, 1943). In addition, the first outbreak of pertussis was reported in France, in 1578 (Cone, 1970). The disease was given its name "pertussis", which means 'violent

cough', in 1679 by Thomas Sydenham (Mattoo and Cherry, 2005). Furthermore, *B. pertussis* as the causative agent of whooping cough was isolated for the first time in 1906, by Bordet and Gengou (Bordet and Gengou, 1906). Preliminary attempts for whole-cell vaccine development against pertussis ensued the isolation of the organism in the laboratory. Yet whole-cell pertussis vaccines were combined with diphteria and tetanus toxoids, licenced and became widespread later in the 1940s and 1950s (section 1.3).

1.1.2. Pathogenesis and Clinical Manifestations

The route of infection by *B. pertussis* follows the four main steps specified as *i*) attachment to host cells (i.e. ciliated epithelial cells in the lower and upper respiratory tracts) (Fig 1.1), *ii*) evasion of host defense mechanisms (e.g. obstruction of phagocytosis mediated by polymorphonuclear neutrophils), *iii*) local tissue damage (i.e. extrusion of ciliated cells from the epithelial surface) and *iv*) systemic manifestations (e.g. bronchopneumonia, encephalopathy) (Mattoo and Cherry, 2005). Various virulence determinants, which will be discussed in subsequent sections, play role in each step.

Although several factors such as host age, previous immunity or infection, antibiotic treatment as well as host and pathogen genotypes may affect clinical manifestations, classic pertussis illness in general has three main stages lasting for 6-12 weeks or longer in total (Mattoo and Cherry, 2005). After a 7-10 day incubation period, the "catarrhal stage" shows itself by rhinorrhea, lacrimation and mild cough (fever and pharyngitis are generally not observed in classic illness). This 7-14 day long initial stage is followed by "paroxysmal stage" which lasts for 2-8 weeks and is characterized by severe and violent coughing fits referred to as 'paroxysms'. (The patient's inspiratory efforts during these forceful paroxysms causes the characteristic whooping sound). Vomiting, cyanosis, protrusion of tongue, salivation and distention of neck veins are also paroxysm-associated symptoms and frequently observed in

paroxysmal stage. The final "convalescent stage" is normally 1-2 weeks long and accompanied by a gradual decrease in frequency and severity of paroxysms. Last but not least; encephalopathy, cyanosis, otitis media, seizures and pneumonia are the complications commonly observed in classic illness (Mattoo and Cherry, 2005).

While classic pertussis illness is mainly observed in unimmunized children, asymptomatic infections as well as mild illness characterized by much less severe symptoms (e.g. coughing for less than a 2-week period, rhinorrhea, sore throat, tearing and sneezing) is common in adults and immunized infants (Mattoo and Cherry, 2005).

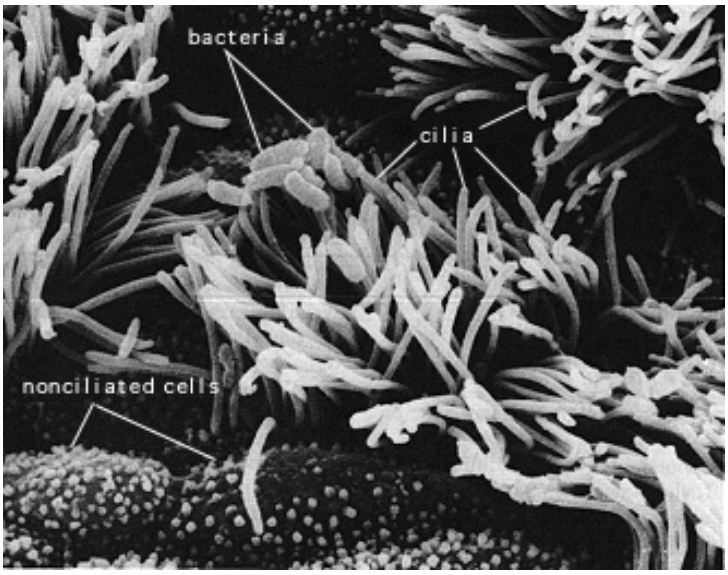


Fig. 1.1. Colonization of ciliated tracheal epithelial cells by *B. pertussis* (*www.textbookofbacteriology.net*).

1.1.3. Diagnosis and Treatment

Diagnosis of the classical illness could actually be made by observation of paroxysmal cough with post-tussive vomiting, whooping, absolute lymphocytosis,

and lack of fever. Yet a series of laboratory diagnostic techniques have been developed to distinguish the infections caused by *B. pertussis* or *B. parapertussis*$_{HU}$ from other microbial infections that bring on similar symptoms (Mattoo and Cherry, 2005). Culturing, fluorescein-conjugated antibody (DFA) staining, PCR, and serologic-based techniques are used for laboratory diagnosis (Müller *et al.*, 1997; Mattoo and Cherry, 2005). Culturing of fastidious *B. pertussis* cells from nasopharyngeal swab and aspiration specimens bears some difficulties in regard to specimen collection, specimen transport and culture media preparation (Müller *et al.*, 1997; Mattoo and Cherry, 2005). Unlike PCR or culturing techniques, DFA staining technique is not based on multiplication of *B. pertussis* cells or their genetic materials. Hence, it has low sensitivity (Müller *et al*., 1997). PCR amplification from specimens with primers designed for PT promoter region, *cyaA* gene or certain insertion sequences is another strategy (Müller *et al*., 1997). PCR technique is prominent among other techniques with its high sensitivity and specificity. Yet, care must be taken to prevent false positive results due to contamination (Mattoo and Cherry, 2005). Seriological techniques include either monitoring a couple of fold-increase in agglutinating antibody titers against PRN, FIM or LPS; or using ELISA method to detect IgA, IgM and IgG antibodies against certain antigens like PRN, FIM, FHA and PT (Müller *et al.*, 1997; Mattoo and Cherry, 2005; Tondella *et al*., 2009). The former method, which has high specificity but low sensitivity, has been replaced with the latter in recent years (Matto and Cherry, 2005).

Antibiotic treatment against pertussis is essentially effective when it is started at catarrhal stage (Bergquist *et al*., 1987; Güriş, 1999). Nevertheless, there are some studies suggesting that the treatment might also ameliorate disease progression when it is started at the onset of paroxysmal stage (Bergquist *et al*., 1987; Heininger *et al*., 1993). Erythromycin is the principal antibiotic used in a 7-day long effective treatment with determined doses for children and adults (Halperin *et al*., 1997; Mattoo and Cherry, 2005). Though not very common, some *B. pertussis* strains were found to be resistant to erythromycin (Lewis *et al*., 1995; Korgenski and Daly, 1997), so trimethoprim-sulfamethoxazole could be used in patients infected with an

erythromycin-resistant strain or those developing side effects to erythromycin (Hoppe *et al*., 1989). Lastly, azithromycin and clarithromycin are other two newly upcoming macrolides promising much less side effects (Wood and McIntyre, 2008).

1.1.4. Epidemiology of Pertussis

Whooping cough is caused essentially by *B. pertussis* and occasionally by *B. parapertussis*$_{HU}$. The frequency of the presence of *B. parapertussis*$_{HU}$ in pertussis patients varies from 1% to 40% in different countries (Watanabe and Nagai, 2003). It is an endemic disease with epidemic peaks of every 3-4 years (Mooi *et al*., 2007). It is also one of the most contagious infectious diseases with a basic reproductive number [i.e. average number of secondary cases of infection generated by one primary case in a susceptible population (Anderson and May, 1991)] of 12-17. The disease affects all races indiscriminately (Mooi *et al*., 2007).

Pertussis had the highest mortality rate among all infectious diseases affecting the children less than 14 years of age in the USA before the mass-vaccination era in the 1940s (Cherry, 1999). It had an incidence rate of 150 infections per 100 000 population in the same period. Although a dramatic decrease both in morbidity and mortality was observed following the mass-vaccination, resurgence of the disease has been reported since the 1980s even in countries with widespread and long-term vaccination stories (Bass and Wittler, 1994; De Serres *et al*., 1995; de Melker *et al*., 1997; Gzyl *et al*., 2004; Elomaa *et al*., 2005; Tiwari *et al*., 2005). Consequently, with 45 million cases and 409 000 deaths per year worldwide, pertussis remains one of the 10 infectious diseases with highest morbidity and mortality (Music, 2005). The resurgence of the disease was undoubtedly revealed by Tanaka *et al*. (2003), who compared the 1980s and the 1990s and showed a 49% increase in incidence of the disease among infants; especially among those too young to be fully or partially vaccinated (i.e. less than 5 months of age). Majority of the deaths due to pertussis occurs in the same age group of infants as well (Celentano *et al*., 2005).

Several studies propose a shift in incidence of the disease from infants to adolescents and adults (von Konig *et al*., 2002; Skowronski *et al*., 2002; Hewlett and Edwards, 2005) reflecting the insufficiency of the present pertussis vaccines and childhood pertussis to confer lifelong immunity. The incidence rate of the disease is underestimated in adults, though, due to much less severe symptoms or even subclinical cases (Cherry, 2005; de Melker *et al*., 2005; Ward *et al*., 2005). Yet the infected adults provide a reservoir for the disease and pose a threat for unvaccinated infants (von Konig *et al*., 2002; Schellekens *et al*., 2005).

1.1. The Organism *Bordetella pertussis*

1.2.1. Phylogeny and Evolution of Bordetellae

According to the recent studies, the genus *Achromobacter* belonging to the family *Alcaligenaceae* is the mostly related one to the *Bordetella* genus (Gerlach *et al*., 2001; Gerlach *et al*., 2004; Brenner *et al*., 2005). The nine species currently listed as the *Bordetella* species are *B. bronchiseptica, B. parapertussis, B. pertussis, B. holmesii, B. avium, B. trematum, B. hinzii, B. petrii* and *B. ansorpii* (Fig 1.2). Based on the comparative analyses of their 16S rDNA sequences, three *Bordetella* species found in human niche; *B. bronchiseptica, B. parapertussis,* and *B. pertussis* form a closely related lineage that can be easily seperated from the other species. Multilocus enzyme electrophoresis (MLEE) studies indicate that these three 'classical' *Bordetella* species are less genetically diverse compared to other bacterial pathogens (Musser *et al*., 1986; Musser *et al*., 1987). *B. bronchiseptica,* whose host range comprises many mammals, rarely causes respiratory infections in humans; mostly in immunocompromised individuals (Woolfrey and Moody, 1991). *B. parapertussis* has two distinct lineages denoted as *B. parapertussis*$_{HU}$ *and B. parapertussis*$_{OV}$, which have been isolated from human and sheep, respectively (Cullinane *et al*., 1987). Several insertion sequence (IS) element ploymorphism studies propose that

B. parapertussis is the closest one to the common ancestor among classical species and human-adapted *B. pertussis* and *B. parapertussis*$_{HU}$ arose distinctly from different *B. bronchiseptica* lineages (van der Zee *et al*., 1997; Reischl *et al*., 2001). Although closely related, these two human-restricted species are immunologically different (Khelef *et al*., 1993). In addition, multilocus enzyme electrophoresis (MLEE) (van der Zee *et al*., 1997), multilocus sequence typing (MLST) and CGH studies (Diavatopulos *et al*., 2005) indicate that *B. parapertussis* emerged more recently than *B. pertussis*. Comparing the genome size of these three species, *B. bronchiseptica* has much larger genome and greater number of functional genes than the other two. It is followed by *B. parapertussis,* and *B. pertussis* is the one with the smallest genome size and least number of functional genes (Parkhill *et al*., 2003). This indicates the role of genome reduction as well as large genome

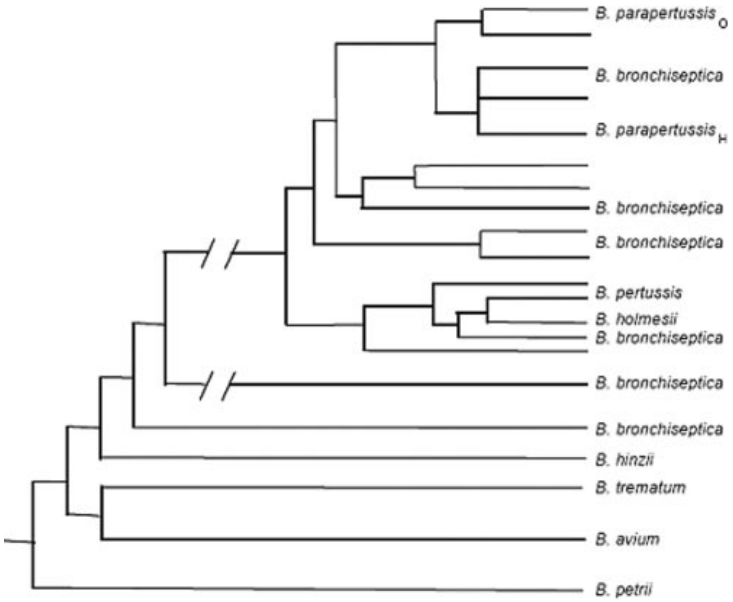


Fig.1.2. Phylogenetic relationship among the *Bordetella* species. (Mattoo and Cherry, 2005)

rearrangements mediated by IS elements in evolution of these two human-adapted pathogens. The role of genome rearrangements could be noticed by the fact that *B. parapertussis* genome has much greater co-linearity with that of *B. bronchiseptica* than *B. pertussis* genome (Cummings *et al*., 2004).

Selection for a change from chronic to acute infection and for avoidance of existing host immunity are considered to be the two selection pressures playing role in evolution of *B. pertussis* and *B. parapertussis* (Bjornstad and Harvill, 2005). For example, Pertussis toxin (PT) favors acute infection over chronic one and its expression is confined to *B. pertussis* (Kirimanjeswara *et al*., 2005). Likewise, a bacterial type three secretion system (TTSS) which promotes prolonged infection of the host cell (Cornelis, 2000) is functionally expressed in *B. bronchiseptica* whereas it is absent in *B. pertussis* and *B. parapertussis* (Matto and Cherry, 2005). Furthermore, lipid A structure of the LPS molecules of *B. pertussis* and *B. parapertussis* are modified to evade Toll-like receptor (TLR-4) mediated host cell immune response (Raetz and Whitfield, 2002; Mann *et al*., 2004; Mann *et al*., 2005).

As mentioned above, avoidance of existing immunity is also an important driving force for evolution of both *B. pertussis* and *B. parapertussis*. Aside from immunocompromised hosts and people in close contact with infected animals, *B. bronchiseptica* infection in human has diminished as it is replaced by infections of *B. pertussis* and *B. parapertussis*, evolution of which are presumably driven by the selection pressure for variants able to avoid existing anti-*B. bronchiseptica* immunity (Preston *et al*., 2007). For example, O antigen of *B. bronchiseptica*, which is a highly immunogenic protein, is absent in *B. pertussis* (Preston *et al*., 1999). As stated before, evolution of *B. parapertussis* is a more recent event and it could be suggested that existing anti-*B. pertussis* immunity has been the predominant selection pressure in evolution of *B. parapertussis* in human niche (Preston *et al*., 2007). In concordance with this, highly immunogenic Band A trisaccharide is not expressed in *B. parapertussis* while it is present in LPS of *B. pertussis* (Preston *et al*., 2006).

Interestingly, *B. parapertussis* does express O antigen (Zhang *et al*., 2009), which might suggest that during the evolution of *B. parapertussis*, anti-*B. bronchiseptica* immunity has lost its role of being the major selection pressure (Preston *et al*., 2007).

1.2.2. Microevolution of *B. pertussis*

The current low genetic diversity of *B. pertussis* is associated with a bottleneck possibly caused by the mass-vaccination implemented for the last 60 years or so (van Loo *et al*., 1999; Weber *et al*., 2001; van Loo and Mooi, 2002; Elomaa *et al*., 2005). Nevertheless, polymorphisms leading to amino acid substitution are currently observed in *prn, ptxA, tcfA and fim* genes which encode pertactin (Prn), pertussis toxin A subunit (PtxA), tracheal colonization factor A (TcfA) and fimbrial subunits, respectively. These proteins have also immunoprotective capacities and thus are involved in acellular vaccines (Cherry *et al*., 1998; Storsaeter *et al*., 1998; Taranger *et al*., 2000).

Polymorphism in repeat sequences is a mechanism which is developed in many pathogens to evade host immunity (Gravekamp *et al*., 1996; Delogu and Brennan, 2001; Franks *et al*., 2003). Eleven alleles for Prn have been identified so far (Mooi *et al*., 1998; Mooi *et al*., 2000) which differ in two repeat sequences denoted as R1 and R2. R1 consisting of GGxxP repeats is located very close to the RGD motif that is associated with adhesion to host cell (Leininger *et al*., 1992). Both R1 and R2 regions have been shown to involve protective epitopes which affect vaccine efficacy (Charles *et al*., 1991; King *et al*., 2001; Gzyl *et al*., 2004). Albeit its protective capacity, it has also been reported that Prn lacks cross-protectivity, as *B. pertussis* Prn did not confer protectivity against *B. parapertussis* in a mouse model (Khelef *et al*., 1993). Prn1 is found in most pertussis vaccines although Prn2 predominates in current *B. pertussis* populations (Mooi *et al*., 2007).

Pertussis toxin is composed of five subunits denoted as S1-S5 or PtxA-E (Hewlett and Donato, 2007). PtxA is the one with most immunogenicity and showing the highest varience (De Magistris *et al*., 1989; van Loo *et al*., 2002). Six *ptxA* alleles coding for four distinct PtxA subunits are present in *B. pertussis*. PtxA2 is found in most pertussis vaccines although PtxA1 is the predominant one in current *B. pertussis* populations (Mooi *et al*., 2007).

B. pertussis has three fimbrial genes, namely *fim2, fim3* and *fimX*. Fim2 and Fim3 are the ones which are predominantly expressed (Willems *et al*., 1992; Locht *et al*., 1992; Parkhill *et al*., 2003) and determine the serological types 2 and 3 of the pathogen (Mink *et al*., 1994). The reciprocal relationship between the predominant fimbrial serotype of the bacteria isolated from patients and that included in whole-cell vaccines reflects the immunoprotective capacity of the fimbrial antibodies (Preston, 1985). This protectivity has also been shown in other studies testing the efficacies of both acellular and cellular pertussis vaccines (Stanbridge and Preston, 1974; Hallander *et al*., 2005). Both *fim2* and *fim3* are polymorphic. Two alleles of *fim2* are formed through an SNP resulting in an amino acid substitution (van Loo *et al*., 2002) while three SNPs generate four alleles of *fim3* named as Fim3A, Fim3B and Fim3C (Tsang *et al*., 2004). The discrepancy between the frequency of the alleles found in clinical isolates and those involved in whole-cell vaccines is actually not confined to *fim* genes but is a general case which has been observed since the 1950s and reflects the phenomenon 'vaccine-driven evolution' (Mooi *et al*., 1998; Mooi *et al*., 2001; *Fry et al*., 2001; Packard *et al*., 2004; van Amersfoorth *et al*., 2005). This phenomenon is also considered to be one of the factors contributing to the resurgence of pertussis (Wendelboe *et al*., 2005).

1.2.3. Virulence Determinants and Molecular Pathogenesis

Expression of various virulence determinants (e.g. adhesins, toxins, autotransporters) of *B. pertussis* are regulated either through phenotypic modulation as a response to

changes in environmental conditions (Lacey, 1960) or through certain genetic modifications referred to as phase variation (Stibitz *et al*., 1989). Either mechanism is based on a two-component phosphorelay system called "BvgAS" (Fig 1.3) which is expressed from *bvgA/S* locus (Uhl and Miller, 1996). This system is overviewed in the following section while main virulence factors regulated by this system are discussed briefly in subsequent sections.

1.2.3.1. BvgAS System

BvgAS system is composed of BvgA and BvgS proteins (Fig 1.3). BvgS is a cytoplasmic membrane protein containing periplasmic (P), linker (L), transmitter (T), receiver (R) and histidine phosphotransfer (HPD) domains (Cotter and Dirita, 2000). P domain is linked to cytoplasmic T, R and HPD domains through L domain, and is capable of sensing environmental changes. At 37°C and very low $MgSO_4$ and nicotinic acid concentrations, this sensing results in autophosphorylation of T domain at its H729 residue (Uhl and Miller, 1994; Uhl and Miller, 1996). This phosphoryl group in turn is transferred to R and HPD domains, respectively, and finally to the receiver (R) domain of BvgA protein. When phosphorylated, BvgA protein acts as a transcriptional activator via its helix-turn-helix (HTH) domain, binds to promoter regions and activates transcription of several genes referred to as Bvg^+-phase-specific, or virulence-activated genes (*vag*) (Mattoo and Cherry, 2005). BvgA also activates a regulator protein named as BvgR, which represses another group of genes called Bvg^--phase-specific or virulence-repressed genes (*vrg*) by binding to their operator sequences (Matto and Cherry, 2005). At temperatures lower than 37°C and high $MgSO_4$ and nicotinic acid concentrations, all of the signaling cascade described above is reversed: BvgAS system as well as BvgR regulation is inactivated, whereby *vag* genes are down-regulated and *vrg* genes are derepressed (Locht *et al*., 2001). In fact, the shutdown of BvgAS system is not an all-or-none phenomenon. In addition to Bvg^+ and Bvg^-, there has also been established an intermediary phase Bvg^i (Fig1.3) which arises at certain environmental conditions (e.g. a defined range of nicotinic acid concentration) (Mattoo *et al*., 2005). Bvg^i is characterized by

expression of a subset of *vag* genes, repression of *vrg* genes and expression of some genes confined to Bvg^i (Mattoo *et al*., 2005). The regulation of BvgAS system could be triggered either by environmental changes described above (i.e. phenotypic modulation) or by frameshift mutations in *bvgAS* locus (i.e. phase variation) (Stibitz *et al*., 1989).

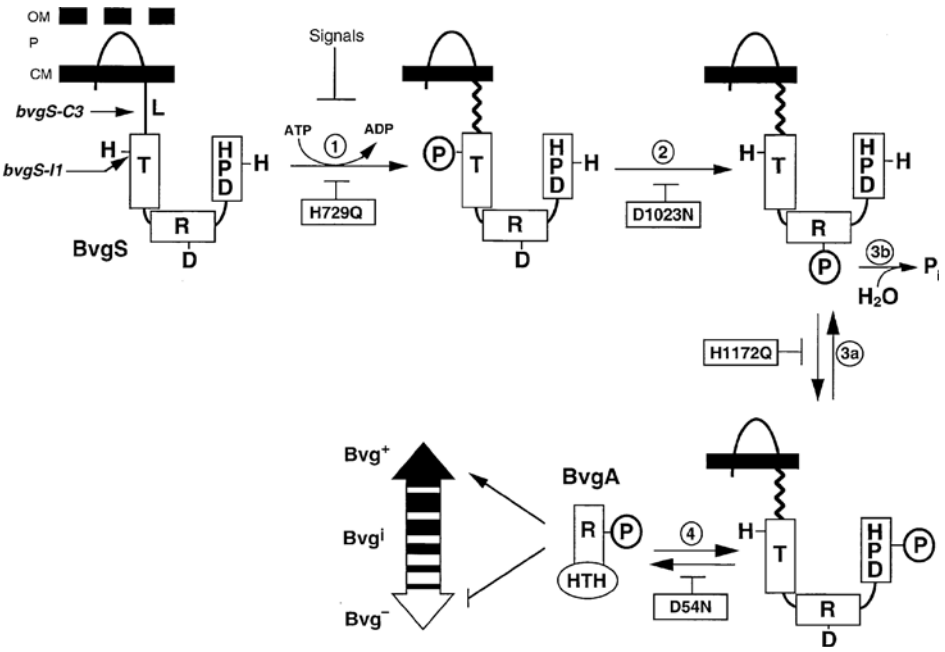


Fig. 1.3. BvgAS phosphorelay system(Cotter and Dirita, 2000).

1.2.3.2. Filamentous Haemagglutinin (FHA)

Filamentous haemagglutinin (FHA) is the major adhesin of *B. pertussis* (Locht *et al*., 2001). It is encoded by *fhaB* virulence-activated gene as a 367-kDa precursor. This molecule is subsequently cleaved to its 220-kDa hairpin-like mature form (Mattoo and Cherry, 2005). Secretion of FHA across the outer membrane is mediated by the help of an accessory protein encoded by *fhaC* gene. This protein forms an FHA-specific transmembrane pore in outer membrane (Fig 1.4) (Jacob-Dubuisson *et al*., 1999). FHA is capable of binding to VLA-5 integrin through its RGD (Arg-Gly-Asp) domain. This binding confers attachment of FHA to monocytes, macrophages and

bronchial epithelial cells (Ishibashi *et al*., 2001; Mattoo *et al*., 2005). FHA also has a carbohydrate binding domain (CRD) and a heparin-binding site, which enable the molecule to bind ciliated respiratory epithelial cells and to trigger FHA-mediated hemagglutination, respectively (Menozzi *et al*., 1991; Prasad *et al*., 1993). FHA has also been known for its various immunomodulatory actions. It was shown that following *B. pertussis* infection in a mouse model, FHA-specific T regulatory cells secreted IL-10 and restrained protective Th1 response thereupon (McGuirk *et al*., 2002). Furthermore, neutrophile-dependent phagocytosis of *B. pertussis* is attenuated by anti-FHA antibodies in convalescent human serum (Mobberley-Schuman *et al*., 2003). FHA is also known to play role in precluding *B. pertussis*-infected monocytes from inducing antigen-specific $CD4^+$ T-cell proliferation (Boschwitz *et al*., 1997).

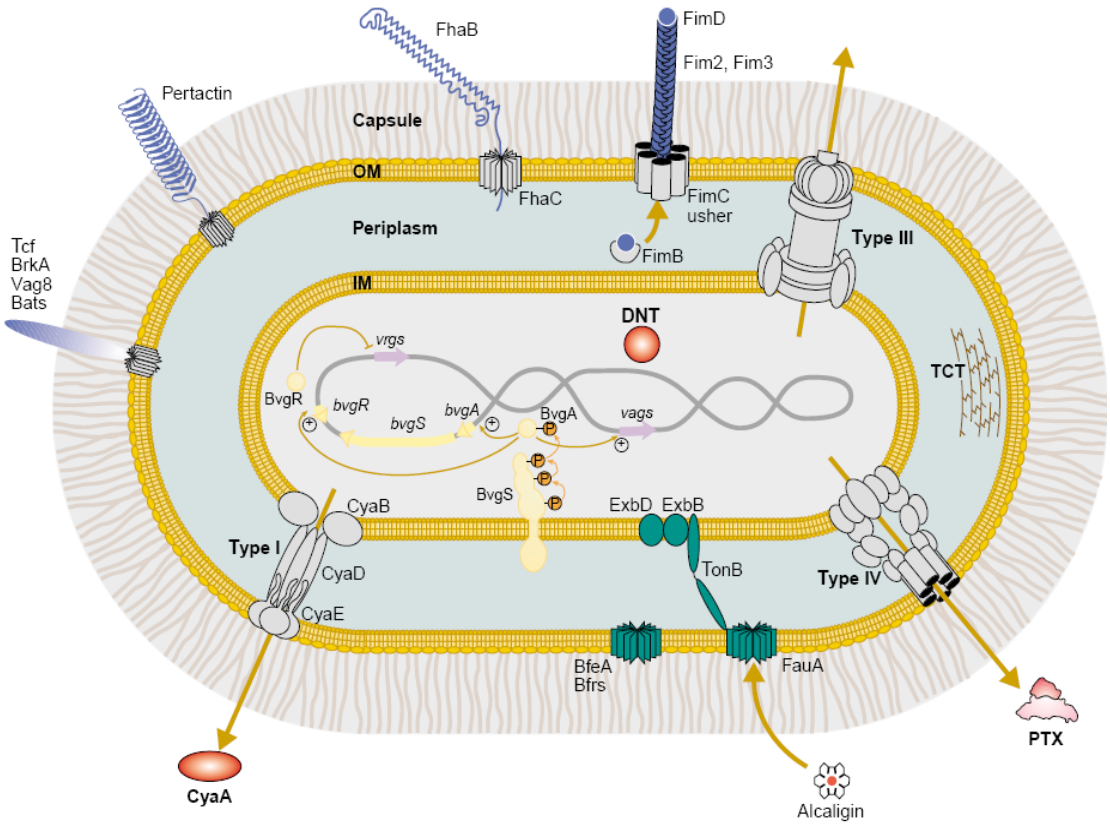


Fig. 1.4. The virulence factors of *B. pertussis* (Locht *et al*., 2001).

1.2.3.3. Fimbriae (FIM)

Fimbriae (FIM) are filamentous multimeric adhesins expressed by all *Bordetella* species including *B. pertussis* (Fig 1.4) (Mattoo *et al*., 2005). Fim2 and Fim3 are encoded by *fim2* and *fim3* genes, respectively. They are the major fimbrial subunits and determine the fimbrial serotypes 2 and 3 of *B. pertussis* (Mooi *et al*., 1987). Both Fim2 and Fim3 are accompanied at their tips by another subunit FimD which is encoded by the fimbrial biogenesis locus *fimBCD* (Willems *et al*., 1992). This locus also encode FimB and FimC accessory proteins which act as a chaperon and an usher protein, respectively (Willems *et al*., 1992). Interestingly, *fimBCD* operon lies between *fhaB* and *fhaC* loci (Willems *et al*., 1992) and binding of FimD to VL-5 integrin on macrophages induces the expression CR3 which is a receptor for FHA (Hazenbos *et al*., 1995). Taken together, Fimbriae and FHA could be speculated to cooperate in attachment and colonization of upper respiratory tract by *B. pertussis* (Mattoo *et al*., 2005). Fimbriae are also known as immunomodulatory molecules for their stimulating early T-independent IgM and Th2-mediated immune responses (Mattoo *et al*., 2000).

1.2.3.4. Pertussis Toxin (PT)

Pertussis toxin is a hexameric protein composed of five different subunits named as S1 to S5 which are encoded by the genes *ptxA* to *ptxE*, respectively (Locht *et al*., 1986). The protein is secreted across the outer membrane via a nonamer complex of its specific accessory protein Ptl, constituting a type IV secretion system (Weiss *et al*., 1993). PT is composed of two moieties A and B. The pentameric B component comprises S2, S3, two S4 and S5 subunits while A component corresponds to S1 (Mattoo *et al*., 2005). B component takes on the attachment of toxin to surface of the target cell and entry of A subunit therein (Tamura *et al*., 1982). Once inside the cell, A component transfers ADP-ribosyl group from NAD to trimeric G proteins and impede their activities (Katada *et al*., 1983). G proteins take part in certain signal transduction pathways and their inactivation brings about several systemic

symptoms like histamine sensitization, elevated insulin secretion accompanied by hypoglycemia, and lymphocytosis (Mattoo and Cherry, 2005; Carbonetti *et al*., 2007). In contrast to immunostimulatory functions like acting as an adjuvant (Ryan *et al*., 1998), PT also exhibits several immunosuppressive features such as delaying chemotaxis and migration of and lysosomal enzyme release from neutrophils and macrophages, reducing serum anti-*B. pertussis* antibody levels upon *B. pertussis* infection, and decreasing the expression of major histocompatibility complex class II (MHC-II) molecules on the surface of monocytes (Meade *et al*., 1984; Brito *et al*., 1997; Carbonetti *et al*., 2004; Shumilla *et al*., 2004; Mattoo and Cherry, 2005).

1.2.3.5. Pertactin (PRN) and Other Autotransporters

As their name implies, autotransporters are proteins that do not require any accessory protein for their transport across the outer membrane (Henderson and Nataro, 2001). Instead, their conserved C-terminal domain facilitates the transport of effector N-terminal domain. Pertactin (PRN) is the firstly discovered autotransporter expressed in all Bordetellae (Mattoo *et al.*, 2005). Owing to its leucine-rich, proline-rich and RGD motifs, PRN is considered to play a substantial role in cell attachment (Emsley *et al*., 1994). Immunostimulatory effect of anti-PRN antibodies could be evidenced by the fact that PRN-containing dTPa vaccines are much more efficient than those lacking this antigen (Gustafsson *et al*., 1996; Cherry, 1997; Cherry and Heininger, 2004). BrkA is another auto-transporter adhesin that provides *B. pertussis* cells with resistance to complement-mediated killing (Fernandez and Weiss, 1996). Still another autotransporter SphB1 was reported to take part in C-terminal maturation of FHA (Coutte *et al*., 2001) while TcfA is exclusively expressed in *B. pertussis* and is the only autotransporter protein secreted to extracellular milieu rather than being kept on cell surface (Finn and Stevens, 1995).

1.2.3.6. Adenylate Cyclase (CyaA)

Adenylate cyclase is a bifunctional toxin that exhibits both adenylate cyclase and haemolytic activities (Ladant and Ullmann, 1999). The transport of the toxin through cell and outer membranes is facilitated by accessory proteins CyaB, CyaD and CyaE, which together constitute a Type-1 secretion system (Fig 1.4) (Locht *et al.*, 2001). C-terminal domain of the toxin which is activated by another protein CyaC through palmitoylation shows haemolytic activity. This domain is also responsible for recruitment of N-terminal domain to the target cell surface and internalization of it by pore formation (Hewlett *et al*., 1988). Once inside the cell, N-terminal domain is activated by calmodulin and catalyzes the production of cAMP from ATP which brings about a dramatic increase in intracellular cAMP concentration (Wolff *et al*., 1980; Confer *et al*., 1984). Immunomodulatory effect of this sharp increase comes out as inhibition of phagocytosis of *B. pertussis* cells by neutrophils and induction of macrophages' apoptosis at the onset of infection (Khelef *et al*., 1993; Weingart *et al*., 2000).

1.2.3.7. Dermonecrotic Toxin (DNT)

When injected intradermally, dermonecrotic toxin causes localized necrotic lesions in mice. It is also lethal if given intravenously (Locht *et al*., 2001; Mattoo *et al*., 2005). Similar to PT, DNT is an A-B toxin with its N-terminal receptor binding domain and C-terminal effector domain (Matto *et al*., 2005). Yet, DNT is not secreted as PT is; but localized in the cytoplasm (Fig. 1.4) (Locht *et al*., 2001). Upon receptor binding, the toxin is internalized by a dynamin-dependent endocytosis and activated via mammalian proteases (Matsuzawa *et al*., 2004). When activated, N-terminal domain deaminates or polyaminates Rho GTPase, causing constitutive activation of the protein and alterations in certain signaling cascades (Schmidt *et al*., 1999; Masuda *et al*., 2000). The actual role of DNT –if any- in *Bordetella* pathogenesis is yet to be established (Mattoo and Cherry, 2005).

1.2.3.8. Type III Secretion System (TTSS)

Type III secretion system is a very complex protein apparatus found in a variety of gram-negative bacteria and it confers direct transportation of certain effector proteins into the cytoplasm of eukaryotic cells (Galan and Collmer, 1999). These effector proteins in turn alter signaling cascades within the cells and contribute to pathogenicity (Lee, 1997). Unlike *B. bronchiseptica* and *B. parapertussis*$_{OV}$, *B. pertussis* fails to elicit TTSS-mediated cytotoxicity on mammalian cells *in vitro* although it actively produces proteins incorporated into TTTS (Mattoo *et al*., 2004). Post-transcriptional modifications are suspected to be responsible for differential regulation of TTSS in *B. pertussis* (Mattoo and Cherry, 2005). Eventually, the exact role of TTSS in virulence of *B. pertussis* remains to be elucidated (Locht *et al*., 2001).

1.2.3.9. Bvg-independent Virulence Determinants

Tracheal cytotoxin (TCT) is in fact the disaccharide-tetrapeptide monomer of peptidoglycan which forms the cell wall of gram-negative bacteria (Mattoo and Cherry, 2005). Thus it is constitutively produced owing to cell wall metabolism in a BvgAS-independent manner. Unlike other gram-negative species, *B. pertussis* lacks the AmpG-dependent mechanism keeping TCT inside the cell and therefore releases it to extracellular milieu (Mattoo and Cherry, 2005). TCT exerts its cytotoxic effects predominantly on ciliated cells in an indirect manner: it induces non-ciliated cells to produce excessive nitric oxide (NO·) which in turn diffuses into ciliated cells (Flak and Goldman, 1996). Diffusion of NO· into ciliated cells brings severe consequences like mitochondrial damage as well as extrusion and loss of ciliated cells from the epithelial surface, thus local tissue damage (Wilson *et al*., 1991).

Like lipopolysaccharide (LPS) molecules of other gram-negative bacteria, the LPS of *B.pertussis* is also an endotoxin which is pyrogenic, toxic and mitogenic (Watanabe *et al*., 1990). Unlike those of *B. bronchiseptica* and *B. parapertussis*$_{HU}$, LPS

molecule of *B. pertussis* lacks O-antigen, which is a highly immunogenic protein (Peppler, 1984; Zhang *et al*., 2009). *B.pertussis* LPS is composed of 'band A' and 'band B'. Band B includes 'lipid A' molecule linked to a oligosaccharide core structure while band A comprises band B as well as an additional trisaccharide component (Caroff *et al*., 1990). Some proteins playing role in modification of LPS molecule (e.g. palmitoylation of LPS in *B. bronchiseptica* by PagA protein) are BvgAS-regulated (van den Akker, 1998), which leads to a speculation about possible roles of LPS molecules in *Bordetella* pathogenesis. These modifications may not only determine the host specificity, but also contribute to resistance against antibody-dependendent serum killing as well as to persistence of colonization in respiratory tract (Harvill *et al*., 2000; Mattoo and Cherry, 2005).

Systems by which iron metal is recruited to *B. pertussis* cell surface and subsequently internalized is crucial for survival of the pathogen in iron-depleted host environment (Locht *et al*., 2001). The mechanism composed of alcaligin siderophore which act synergistically with an energy-producing system (i.e.TonB/ExbBD) and alcaligin-specific receptor (i.e. FauA) (Brickman and Armstrong, 1999) is such a system which is not regulated by BvgAS (Fig 1.4) (Locht *et al*., 2001). Impaired ability of TonB-deficient mutants in colonizing mouse respiratory tract emphasizes the importance of this system as well as other iron uptake systems (e.g. BfeA, Bfrs) in *B. pertussis* virulence (Pradel *et al*., 2000).

1.3. Pertussis Vaccines

Although the production of first whole-cell pertussis vaccine (Pw) started soon after 1906 when the causative pathogen *Bordetella pertussis* was identified for the first time (Bordet and Gengou, 1906), it is in 1940s and 1950s when Pw was combined with Tetanus and Diphteria vaccines (DTPw), and started to become more and more available worlwide, especially in developed countries (Storasaeter *et al*., 2007). From 1970s to mid-2000s, the vaccination coverage of children with DTP throughout the world had been increased from 5% to 78% via the efforts made essentially by World

Health Organization (WHO) along with other organizations (WHO, 2005). According to the estimates of WHO, this mass-vaccination in 2003 limited morbidity and mortality rates to 17.6 million and 280 000 respectively which would exceed 50 million and 800 000 otherwise (WHO, 2005). This worldwide vaccination also resulted in changes in epidemiology profile of pertussis: it was children of pre-school age which showed the highest incidence rate of pertussis in pre-vaccination era (Cherry and Heininger, 2004) whereas infants and adolescents surpassed other age groups following mass-vaccination (Andrews *et al*., 1997; Edwards, 2005).

1.3.1. Whole Cell Vaccines

Development of Pw is based essentialy on inactivation of whole *B. pertussis* cells by certain physical (i.e heat) or chemical (e.g. formalin treatment) means and this strategy remained nearly unchanged since the very beginning of Pw history. Yet current Pw vaccines vary considerably regarding reactogenicity and efficacy (Galazka, 1993). Differences in composition such as amount of impurities (e.g. lipopolisaccharides) as well as different inactivation methods may account for these variances (Relyveld *et al*., 1991; Galazka, 1993). Dramatic discrepancies regarding efficacy was also emphasized in a study in which the average absolute efficacy of DTPw vaccines was reported as 78% (Jefferson *et al*., 2003). Some local (i.e. pain, redness, swelling) and systemic (i.e. fever, drowsiness, anorexia, irritability) adverse reactions could be observed in more than 50% of DTPw-vaccinated children (Cody *et al*., 1981). Though less common, other adverse effects such as prolonged crying, febrile convulsion as well as hypotonic-hyporesponsive episodes (HHE) characterized by reduced muscle tone, hyporesponsiveness, skin pallor and cyanosis are also associated with Pw vaccinations (Howson *et al*., 1992; Barlow *et al*., 2001; Gold *et al*., 2002). Owing to its inexpensiveness and availability, Pw still maintains its popularity throughout the world although its successor Pa provided less reactogenicity as well as more specific and predictable immune responses (Storsaeter *et al*., 2007).

1.3.2. Acellular Vaccines

First trials for development of acellular vaccine (Pa) date back to 1940s (Pillemer *et al*. 1947). Initial formulations were mainly dependent on preparation of cell extracts treated with chemicals (e.g. trisodium phosphate) and adsorbed to human erythrocytes or aluminum compounds (Pillemer *et al*., 1947; Weihl *et al*., 1963). The idea of separating toxins and protective antigens found together in cell extracts, thereby reducing the reactogenicity brought about several studies in which Pa was formulated to contain purified antigens. Filamentous hemagglutinin (FHA) and pertussis toxin (PT) were the first two proteins whose protectivities were confirmed by mouse potency assays (Sato *et al*., 1974) and consequently used in formulation of the first licensed Pa in the late 1970s in Japan (Storsaeter *et al*., 2007). Successive studies showed that this Japanese formulation of Pa was both less reactogenic and more efficacious as compared to Pw (Gustafsson *et al*., 1996; Greco *et al*., 1996). Fimbriae (Fim) (Zhang *et al*., 1985) and pertactin (Prn) (Brennan *et al*., 1988) were the proteins which came after FHA and PT, and were included in protective antigen-based Pa vaccines.

1.3.3. Composition, Efficacy and Safety of Acellular Vaccines

Many different combinations of two- to five-component Pa were generated by different manufacturers in 1980s and 1990s in the precedence order of PT, FHA, Prn and Fim. That is to say, all Pa formulations contained PT toxoid while Fim2 and Fim3 were only found in four- or five-component vaccines (Table 1.1). Gustafsson *et al*. (1996) showed that five-component Pa including FHA, PT, Prn, Fim2 and Fim3 was more efficacious than the two-component Pa composed of FHA and PT only. Likewise, another study comparing one- or two-component vaccines to those with at least three components reported the overall efficacy as 67-70% and 80-84%, respectively (Jefferson *et al*., 2003). In addition to antigen composition, variable adsorption capacities of vaccine components to different aluminum salts also affects

the efficacy of a particular Pa (Denoel *et al*., 2002). Furthermore, the short-term protectivity of a Pa does not necessarily mean that it also shows a sustained, long-term effectiveness (Gustafsson *et al*., 2005).

Immune responses triggered by Pa and Pw exhibit more or less differences. Dirix *et al.* (2009) compared antigen-specific Th1 and Th2-based cytokine secretions in Pa- or Pw-vaccinated infants and concluded that Pw elicited a Th1-predominated cytokine profile which corresponds to the cellular immune response, while Pa stimulated a mixed Th1/Th2-based cytokine secretion, the latter of which stands for the humoral immune response. The ability of Pa to induce both cellular and humoral responses was also shown in adolescents and adults (Meyer *et al*., 2007). It was stressed in the same study that the persistence of the cellular immune response one year after vaccination was much more appreciable than that of the humoral immune response, reflecting the presumable role of cellular immune response in sustainable protection (Meyer *et al*., 2007).

In view of several studies, HHE and febrile convulsions as well as other local and systemic adverse events listed in section 1.3.1 are much less frequently observed upon Pa administration compared to Pw (Bernstein *et al*., 1993; Schmitt-Grohe *et al*., 1997; Le Saux *et al.*, 2003; Geier and Geier, 2004). This said, whole-limb swelling might be observed as an adverse reaction after administration of –if not initial-repetitive, booster doses of Pa (Schmitt *et al*., 1997; Rennels *et al*., 2000). Recent studies propose that the use of reduced antigen content vaccine (dTpa) as a booster may reduce the risk of facing adverse events brought on by administration of pediatric, conventional DTPa, especially in adolescents and adults (Zepp *et al*., 2006; Mertsola *et al*., 2010; Zepp *et al*., 2011). dTpa poses no difference from DTPa with respect to antigen composition, yet it contains lower quantities of each component (Storsaeter et al., 2007).

1.3.4. Combined Vaccines

As discussed above, combination of Pw or Pa with diphteria and tetanus toxoids has been applied for at least 60 years. More recently, this combination has been expanded by adding several other components such as hepatitis B vaccine (HBV), conjugated *Haemophilus influenzae* type b vaccine (Hib) and inactivated poliovirus vaccine (IPV) (Storsaeter *et al*., 2007). These combinations provide parents and physicians with a more timely and complete vaccination schedule, which permits less injection and is somewhat money-saving (Dodd, 2003; Marshall *et al*., 2007). Combination vaccines included Pw first in several versions like DTPw-HBV, DTPw-HBV-Hib and DTPw-IPV-Hib (Table 1.1) (Storsaeter *et al*., 2007). Pa was involved in these combinations through subsequent studies (Table 1.1). The immunogenicity and safety of Pa combinations have been extensively investigated and verified by several recent studies (Halperin *et al*., 2009; Kilpi *et al*., 2009; Johns and Hutter, 2010; Li *et al*., 2010).

1.3.5. Current Issues with Pertussis Vaccines

First generation (whole-cell) and second generation (acellular) pertussis vaccines have undoubtedly contributed to curb morbidity and mortality rates of pertussis so far. Yet these current vaccines still show inadequacies in several points such as providing sustainable immunity, preventing adverse actions in higher doses (i.e. reactogenicity) and avoiding subclinical disease thereby reducing the risk of transmission to unprotected infants (Storasaeter *et al*., 2007).

Limited duration of vaccine-induced immunity against pertussis has been concerned for years (Edwards, 2005). For instance, Pw-driven immunity was revealed to reduce by half after 4 years of vaccination (Jenkinson, 1988). Another study also estimated Pw-induced protective immunity lasting for 6-9 years (Torvaldsen and McIntyre, 2003). Duration of immunity conferred by Pa was reported to be more or less the

Table 1.1. Composition of acellular vaccines and combinations from major manufacturers of acellular vaccines (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

GSK — GlaxoSmithKline Biologicals, SP — Sanofi Pasteur, HBV — hepatitis B vaccine, IPV — inactivated poliovirus vaccine, Hib —conjugated *Haemophilus influenzae* type b vaccine

same with that by Pw (Wendelboe *et al*., 2005). Insufficiency of current Pa and Pw vaccines in providing a life-long immunity could also be inferred from ascending incidence rate of pertussis among adolescents vaccinated in their childhood. It is also stated that this age group plays a crucial role in transmitting the disease to vulnerable, unvaccinated infants; mostly by household contact (Edwards, 2005).

The role of adolescents as potential reservoirs for *B. pertussis* does not only reflect the issue of sustainable immunity, but it also brings the fact that the effective prevention of subclinical pertussis remains yet to be fulfilled. Adolescents mostly experience an asymptomatic disease which current vaccines seemingly fail to handle (Edwards, 2005). Some, but not all, antibodies are able to inactivate BrkA-mediated resistance mechanism of *B. pertussis* which enables the pathogen to circumvent bactericidal activity of the complement system (Weiss *et al*., 1999). Given that pertussis vaccines could struggle with the disease either by neutralizing toxin activities of *B. pertussis* and interfering with bacterial adherence or by conferring direct bacterial clearence, most of the current Pa vaccines are likely to follow the first route (Weiss *et al*., 1999; Kubler-Kielb *et al*., 2011). In accordance with this, among the antigens predominantly found in current Pa vaccines (i.e. FHA, PT, Prn and Fim), only pertactin (Prn) was found to stimulate the production of antibodies mediating complement-dependent bacterial clearence (Hellwig *et al*., 2003). Several studies also indicate that in addition to protein antigens, core oligosaccharide (OS) component of *B. pertussis* lipopolysaccharide (LPS) also elicits an effective bactericidal activity (Archambault *et al*., 1991; Mountzouros *et al*., 1992; Kubler-Kielb *et al*., 2011). Thus exploring novel protein candidates to enhance bactericidal capacity would be a plausible strategy in development of new generation Pa vaccines (Weiss *et al*., 1999; Weingart *et al*., 2000; Weiss *et al*., 2004).

1.4. Iron-Superoxide Dismutase (FeSOD)

Superoxide dismutases (SODs) constitute a metalloenzyme family in prokaryotes and eukaryotes that struggles against oxidative damage to DNA, RNA, proteins and lipids caused by reactive oxygen intermediates (ROIs) (Hassan, 1984). SODs convert toxic superoxide (O_2^-) into O_2 and H_2O_2, the latter being subsequently detoxified by other enzymes such as catalases (Hassan, 1989). SODs are classified with respect to their metal cofactors. *sodB* encodes FeSOD, cofactor of which is iron metal (Khelef *et al*., 1996). FeSOD is exclusively found in prokaryotes, mostly in cytoplasm (Wu *et al*., 1998; Battistoni *et al*., 2000). The immunoprotemics group in our laboratory recently showed that FeSOD is found in the surfaceome of *B. pertussis* 'Tohama I' and 'Saadet' strains (Tefon *et al*., 2011). Along with other SODs, the action of FeSOD as a virulence factor has been reported (Bakshi *et al*., 2006). Mutant live vaccine strain of *Francisella tularensis* ($sodB_{Ft}$) with decreased FeSOD expression showed much less virulence capacity in mice as compared to its wild-type form. It has also been shown that *sodB* deletion in *Legionella pneumophila* results in loss of cell viability (Sadosky *et al*., 1994). Moreover, absence of FeSOD also affects cell growth rate of *Escherichia coli* and *B. pertussis* (Carlioz *et al*., 1986; Khelef *et al*., 1996). In addition, Δ*sodB* mutant of *B. pertussis* Tohama I strain had no detectable expression of two of the main virulence factors adenylate cyclase-hemolysin (AC-Hly) and pertactin (PRN) (Khelef *et al*., 1996). Unlike parental strain, Δ*sodB* mutant also failed to induce apoptosis in J774A.1 macrophages and to effectively colonize and persists in lungs of mice.

1.5. Aim of the Study

The immunogenicity of FeSOD protein in mice immunized with both local and universal strains of *B. pertussis* was shown recently by the immunoproteome group in our laboratory (Tefon *et al*., 2011). In the present study, the protective efficacy and immunostimulatory capacity of the recombinant iron-superoxide dismutase

(FeSOD) from *Bordetella pertussis* was investigated with the aim of development of a new generation acellular pertussis vaccine. Following cloning, expression and purification steps, mouse immunization and lung colonization assays were performed to check the protectivity while ELISA and IFNγ assays were implemented to monitor humoral and cellular immune responses triggered by the protein in a mouse model.

CHAPTER 2

MATERIALS & METHODS

2.1. Bacterial Strains and Plasmids

The *B. pertussis* strains used in this study were 'Tohama I' and 'Saadet'. Tohama I is a standard strain of *B. pertussis* used worldwide for research. Saadet is a local *B. pertussis* strain which was isolated in 1948 in Turkey and given the name of the patient from whom it was isolated. Both strains were kindly provided by Dr. Erkan Özcengiz (VBR Vaccine Res. Co., Ankara). *Escherichia coli* DH5α and *E. coli* BL21 strains were used for cloning and expression studies, respectively. pGEM®-T cloning vector (Promega) (Fig. 2.1) was used for cloning of s*odB* amplicon, which was subsequently cloned into pET-28a(+) expression vector (Novagen) for protein expression (Fig. 2.2).

2.2. Culture Media

Contents of culture media are listed in the Appendix A.

2.3. Buffers and Solutions

Contents and instructions for preparation of buffers and solutions are listed in the Appendix B.

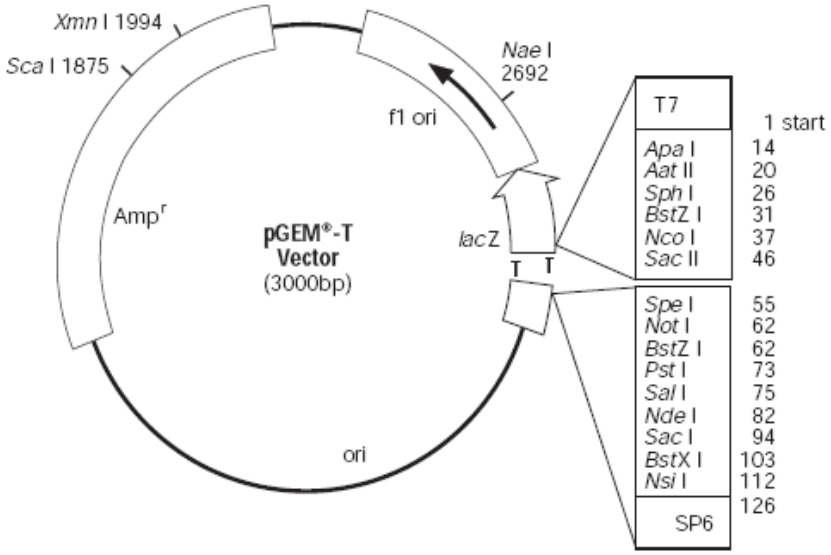


Fig. 2.1. Genetic map of the pGEM®-T vector.

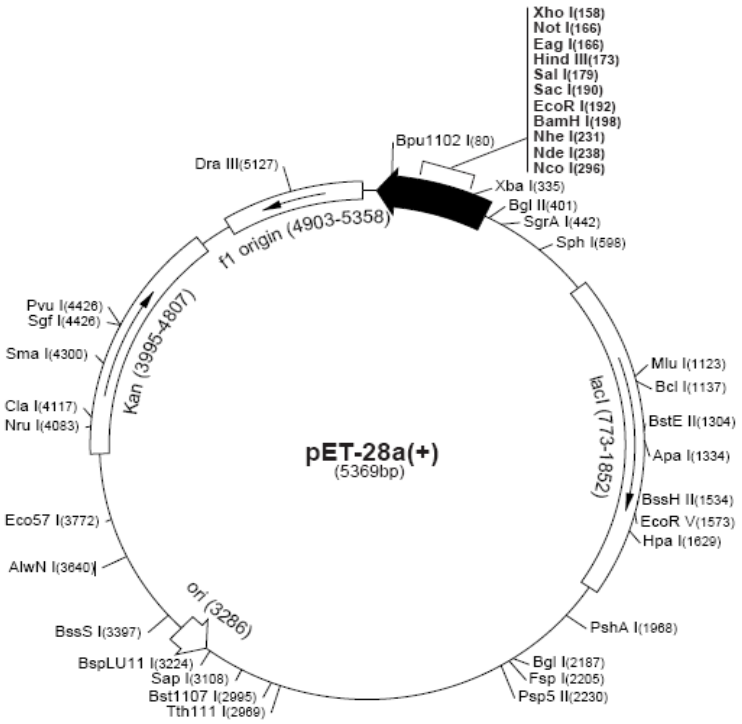


Fig. 2.2. Genetic map of the pET-28a(+) vector.

2.4. Chemicals and Enzymes

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

2.5. Maintenance of Bacterial Strains

B. pertussis Tohama I and Saadet strains were grown on Cohen-Wheeler agar medium (Appendix A) for 72 h at 37°C, stored at 4°C and subcultured at 2 week intervals. *E. coli* DH5α and BL21 cells were grown at 37°C at 180-200 rpm in Luria-Bertani (LB) liquid medium containing selective antibiotic (100 µg/mL ampicillin for cells carrying pGEM®-T and 30 µg/mL kanamycin for those carrying pET-28a(+)). LB agar plates (Appendix A) containing these two antibiotics at the same concentrations were used for the maintenance of transformants/recombinants at 4°C.

2.5. Genomic DNA Isolation from *B.pertussis* Strain Tohama I

A modified version of a procedure previously described by Woods *et al*. (1993) was used for genomic DNA isolation from Tohama I strain of *B. pertussis*. Briefly, the cells were grown on charcoal agar for 60 hours at 37°C, then the scrapings of colonies from the agar plate were suspended in 1 mL TEN Buffer (40 mM Tris, 1 mM EDTA, 150 mM NaCl). Next the suspension was incubated at 100°C for 10 min followed by a centrifugation for 2 min at 13,000 rpm. The supernatant was stored at 4°C and used as the genomic DNA template in PCR reactions.

2.6. Primer Design

The reference gene sequence was extracted from NCBI database (*www.ncbi.nlm.nih.gov*) and the primers given below were designed and ordered to the IONTEK Research Company (İstanbul, Turkey) accordingly:

sodB (Gene ID: 2666341):

Forward → 5' GGATCCATGGCACACACTCTT 3' Tm: 61.2°C

Reverse → 5' AGATCTTTAGGCGAAATTCTTCG 3' Tm: 59.2°C

Expected size: 591 bp

2.7. PCR Reactions

PCR reactions were performed in 50 µL reaction mixtures. Final concentrations of the dNTP mix, $MgCl_2$, primers and *Taq* Polymerase Buffer (Fermentas) were 0.2 mM, 2.5 mM, 0.4 µM and 1X, respectively. 1 µL of *Taq* Polymerase enzyme (Fermentas, 5U/µL) was added to each reaction mixture. Optimized PCR program used for *sodB* gene amplification was as follows: initial denaturation at 94°C for 5 min, then 35 cycles composed of three steps as 30 s denaturation at 94°C, 30 s annealing at 58°C, and 30 s extension at 72°C. A final extension at 72°C for 10 min was performed at the end and the samples were stored at 4°C.

2.8. Agarose Gel Electrophoresis

Electrophoresis was implemented on a horizontal submarine electrophoresis apparatus. Agarose gel concentration differed between 1% to 2% (w/v) in accordance with the specific use. 1% gels were preferred to run PCR reactions while more dense gels were prepared to seperate enzyme digestion reactions. 1X TAE Buffer (Appendix B) was used in agarose gel preparation and as running buffer during electrophoresis. Each gel contained 0.5 µg/ml ethidium bromide and electrophoresis was carried out at 100V for 40-45 min. The gels were visualized under shortwave UV transilluminator (UVP) and their photographs were taken by Vilber Lourmat Gel

Imaging System. Lambda DNA/PstI marker (Fermentas) was used as reference to determine the molecular weights of DNA bands.

2.9. Ligation Reactions

For ligation of PCR products to pGEM®-T cloning vector, the DNA bands were purified from agarose gel via the Gel Elution Kit (GeneMark) by following instructions in manufacturer's manual. The concentration of the eluted PCR product was measured by Thermo Scientific NanoDrop 2000 spectrophotometer. 10 µl ligation reaction was prepared as follows: 0.5 µl pGEM®-T vector (50 ng/µl) was mixed with PCR product at a molar ratio of 1:1 (the formula ''insert(ng) = insert(bp)/vector(bp) × vector(ng) × insert : vector ratio'' was used to determine the amount of PCR product to be added). 5 µl of 2X rapid ligation buffer and 1 µl of 3 U/µl T4 DNA ligase (Promega) were also added and the total volume of the ligation reaction was completed to 10 µl with molecular biology grade water (AppliChem). Finally, this mixture was incubated at 4°C overnight.

In pET28a(+) ligations, both pET28a(+) vector and insert-ligated pGEM®-T plasmids were digested with selected restriction enzymes (plasmid vector isolation isolation was made previously from 100-ml overnight culture via the Qiagen MidiPrep Kit by following manufacturer's protocol). Then the digested molecules were run on agarose gel electrophoresis, and the bands corresponding to pET28a(+) vector and the insert were cut from the gel and purified. After their concentrations were measured, pET28a(+) was dephosphorylated by rAPid Alkaline Phosphatase (Roche) by following manufacturer's intructions. Then the insert and dephosphorylated vector were mixed in a 10 µl-ligation reaction mixture with a molar vector:insert ratio of at least 1:10 (30 ng vector was included in ligation reaction and the amount of insert to be added was calculated accordingly). 1 µl each from 5U/µl T4 DNA ligase (Fermentas) and 10X ligation buffer was added to the mixture and the total volume was completed to 10 µl with molecular grade water. Lastly the mixture was incubated at 4°C overnight.

2.10. Restriction Enzyme Digestion

*Bgl*II and *Bam*HI (10U/µl) restriction enzymes (Fermentas) were used in double digestion reactions for cloning procedures while *Xho*I (Fermentas) was used for confirmation of directional cloning. 40-50 µl of reaction mixtures for these enzymes were prepared. 1 µl from each enzyme was added to the reaction mixture and the mixture was incubated at 37°C for 3-3.5 hours.

2.11. Competent Cell Preparation

100 µl of *E.coli* DH5α glycerol stock was thawn on ice and inoculated into 10 ml of LB medium (Appendix A) which was incubated for 12-16 h at 190-200 rpm at 37°C. 3 ml of overnight culture was inoculated into 200 ml of sterile LB medium. The culture was incubated at 190-200 rpm at 37°C until OD_{600} of the culture reaches between 0.4-0.7 (preferentially around 0.6). Then the culture was incubated on ice for 15 min. Afterwards it was centrifuged at 3,500 rpm for 5 min at 4°C. The supernatant was discarded while the pellet was resuspended on ice with 20 ml of Buffer I (Appendix B). The suspension was centrifuged at 3,500 rpm for 5 min at 4°C and the supernatant was discarded. The pellet was resuspended in 8 ml of Buffer II (Appendix B) this time and the suspension was divided into autoclaved 1.5 ml-Eppendorf tubes as 100µl-aliquots. These aliquots were incubated on ice for 15-30 min, freezed in liquid nitrogen and stored at -80°C.

2.12. Transformation

100 µl of *E.coli* DH5α competent cells stored in -80°C was thawn on ice. Ligation reaction mixture (10µl) was added and the tube was further incubated on ice for 30 min. Next, the tube was soaked for 40-50 seconds to the water bath previously heated to 42°C and immediately put back to ice for an additional 5 min incubation. Then 900 µl of sterile LB medium was added to the cells and the tube was incubated for 70-80 min at 37°C at 180-200 rpm shaking. Afterwards the tube was centrifuged at 3,000 rpm for 10 min and the supernatant was discarded. The pellet was resuspended in residual supernatant gently and finally spread-plated onto the selective LB agar medium plate. The plate was incubated at 37°C overnight.

In transformation of ligation products in which pGEM®-T vector was used, LB agar plates were prepared so to contain 100 µg/ml ampicillin, while those prepared for pET28a(+) ligations contained 30 µg/ml kanamycin.

In pGEM®-T transformations, 4 µl of 100mg/ml IPTG (isopropyl-beta-D-thio-galactopyranoside) and 40µl 20 mg/ml X-Gal (5-bromo-4-chloro-3-indolyl-beta-d-galactopyranoside) were added and spreaded onto the plates prior to spreading of transformant cells, so that white colonies carrying insert-ligated pGEM®-T vector and blue colonies carrying the empty vector could be distinguished through blue/white screening.

2.13. DNA Sequencing Reactions

DNA sequencing was carried out in McLab Molecular Cloning Laboratories (San Fransisco, USA). Universal sequencing primers designed for pGEM®-T Easy vector was supplied by the company. The chromatograms of the sequencing reactions were checked by FinchTV DNA sequencing chromatogram trace viewer program

(Geospiza) and screened for mutational analysis by NCBI BLAST program (*www.ncbi.nlm.nih.gov/BLAST/*).

2.14. Manual Plasmid Isolation

A modified version of the procedure presented by Ehrt and Schnappinger (2003) was performed for manual plasmid isolation. Streak-plated, overnight grown colonies were harvested in 100 µl STE solution (Appendix B). The tubes were vortexed and incubated on ice for 20 min. 60 µl lysis solution (Appendix B) was added to the tubes, vortexed and icubated at room temperature for 10 min. Then 160 µl basic phenol:chloroform:isoamyl alcohol (25:24:1) was added and the tubes were again vortexed. After 5 min centrifugation at 13,000 rpm, the upper phase containing DNA was saved and run on 1% agarose gel.

2.15. Glycerol Stock Preparation of Bacterial Colonies

The colony to be stocked was inoculated into sterile LB medium containing selective antibiotic and incubated overnight at 37°C at 180-200 rpm shaking. The next day 750 µl of the overnight culture was mixed with 250 µl of 60% sterile glycerol. The mixture was frozen by liquid nitrogen and stored at -80°C.

2.16. Protein Expression

A modified version of the procedure previously described by Confer *et al*. (2003) was followed for protein expression. First, -80°C glycerol stock of a transformant *E. coli* BL21 carrying the pET28a(+) plasmid with directionally cloned gene inside was thawn on ice and 100 µl of it was inoculated into 5 ml sterile LB medium. Following 6-7 h incubation at 37°C at 180-200 rpm shaking, 200 µl

from the culture was inoculated into 10 ml sterile LB medium and incubated at 37°C overnight at 180-200 rpm. 2 ml from overnight culture was inoculated into two 100 ml sterile LB broth in 500 ml-Erlenmeyer flasks (one of the 100 ml-cultures would be used for protein expression and the other as negative control). These two cultures were incubated at 37°C at 180 rpm shaking about 3-3.5 hours until their OD_{600} absorbances reached 0.6. At this point, 100 µl of 1M IPTG was added for induction of protein expression to one of the cultures so that the final concentration of IPTG in culture was 1 mM. Then the flasks were kept incubated. 3 hours after addition of IPTG, half of the both induced and uninduced cultures were kept incubated while the other halves were transferred sterically into centrifuge tubes and centrifuged at 6000 rpm for 15 min at 4°C. The supernatant in each tube was discarded while the pellet was resuspended in 1 ml DSB Buffer (Appendix B) and vortexed. Then the tubes were kept at -80°C for 15 min, thawn and vortexed thoroughly. This freeze-thaw step was repeated once more. Afterwards the suspensions were homogenized by the sonicating apparatus (Ultrasonic Processor, Cole Parmer) for 6x10 seconds at 80% amplitude for enhancement of cell lysis. Next the tubes were centrifuged at 15000 rpm for 15 min at 4°C and the supernatants were saved. After their OD_{280} absorbance values were measured by spectrophotometer (a 1/100 diluted sample was prepared for OD_{280} measurements; DSB buffer was used both for dilution and as blank solution), the supernatants were stored at -20°C. 5 hours after addition of IPTG, the procedure described above was repeated for the rest of the cultures.

2.17. Protein Purification

Protein purification was carried out by immobilized metal ion affinity chromatography (IMAC). Specifically manufactured columns (Protino®Ni-TED 2000 Packed Columns, MACHEREY NAGEL) containing dry silica-based resin precharged with N^{2+} ions were used for purification of the recombinant polyhistidine-tagged protein (it is the interaction between N^{2+} ions and polyhistidine

tag which provides binding of the recombinant protein to the column). The procedure in manufacturer's protocol was followed with slight modifications: First, the column was equilibrated with 4 ml DSB Buffer and allowed to drain by gravity. Then the cleared protein lysate obtained from 5 h-IPTG induced culture was added to the pre-equilibrated column and the column was again drained (flowthrough obtained after this step was saved and stored in -20°C). Afterwards the column was washed with 4 ml DSB Buffer. This washing step was repeated several times and OD_{280} of flowthrough was recorded after each washing. When OD_{280} value of the last flowthrough reached almost 0, washing was ceased. Finally the polyhistidine-tagged protein bound to the column was eluted with 3 ml DSB Buffer (pH 8.0) containing 250 mM imidazole. OD_{280} of the elute was recorded and then stored at -80°C.

Lastly the purified protein eluate was dialysed through cellulose membrane (Sigma) against 1 L dialysis buffer (Appendix B). Dialysis was carried out at 4°C overnight with gentle stirring. The dialysed protein solution was filtered through 0.2 μm filter (Millipore) and stored at –80°C.

2.18. SDS-PAGE

Proteins were seperated on 4% stacking and 12% separating polyacrylamide slab gels (Table 2.1) under denatured conditions. Mini-PROTEAN Tetra Electrophoresis System (Bio-Rad) was used for electrophoresis. Unstained Protein Molecular Weight Marker (Fermentas #SM0431) was used to determine the molecular weight of proteins. Electrophoresis was carried out at 16 mA for 2-2.5 hours.

2.19. Coomassie Blue Staining

Following SDS-PAGE, the gel was incubated in fixation buffer (40% ethanol + 10% acetic acid) for 1 h with gentle shaking. Then it was rinsed with dH_2O twice. After 1 h incubation in Coomassie Blue staining solution (Appendix B) with gentle

shaking, the gel was rinsed with (20% ethanol + 5% acetic acid) buffer to reduce the background. Finally, the gel was put back to dH_2O.

Table 2.1. The contents of SDS-PAGE gels.

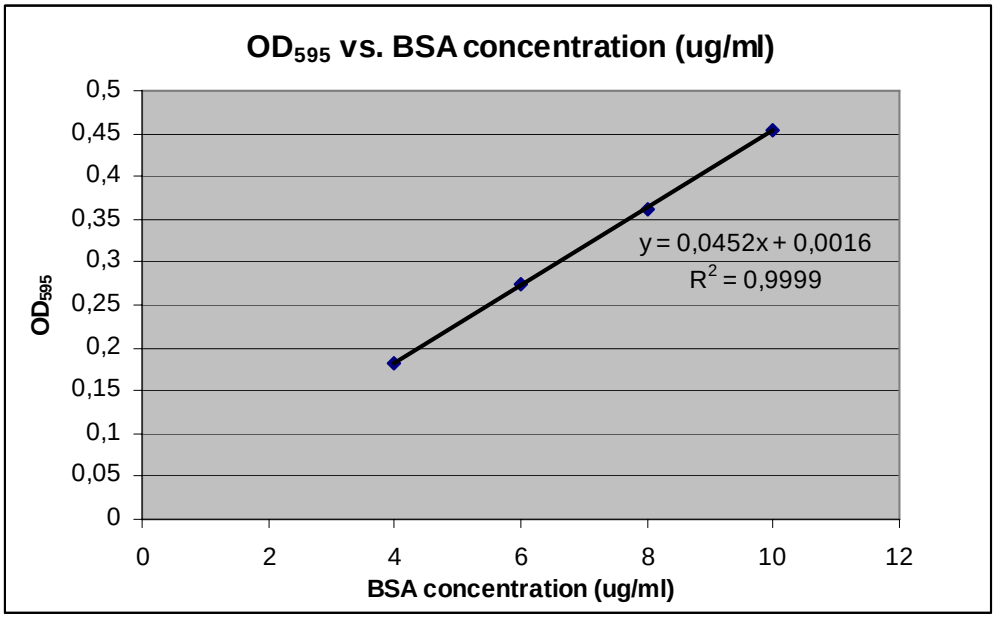
	Stacking Gel (0.125 M Tris, pH 6.8)	**Separating Gel** (0.375 M Tris, pH 8.8)
Monomer Concentration	4%	12%
Acrylamide/bis	650 µl	4 ml
Distilled water	3.05 ml	3.35 ml
1.5 M Tris-HCl, pH 8.8	-	2.5 ml
1.5 M Tris-HCl, pH 6.8	1.25 ml	-
10% (w/v) SDS	50 µl	100 µl
10% Ammonium persulphate	25 µl	50 µl
TEMED	5 µl	5 µl
TOTAL MONOMER	**5 ml**	**10 ml**

2.20. Measurement of Protein Concentration

Bradford quantification method was used in measurement of protein concentration (Bradford, 1976). The method is based on binding of Coomassie Brilliant Blue G-250 to proteins and the concomitant shift in the absorption maximum of the dye from 465 to 595 nm. 5X Bradford assay reagent was prepared as follows: 100 mg

Coomassie Blue G-250 was dissolved in 50 ml 95% ethanol. Then 100 ml 85% phosphoric acid and 50 ml distilled water was added to this solution. This stock solution was stored at 4°C. When needed, the stock solution was diluted to 1X with distilled water and filtered through Watman No. 1 filter paper. Then the standard solutions [1 mg/ml Bovine Serum Albumin (BSA) was used as standard protein] and sample solutions were prepared as described (Table 2.2). Three sample solutions were prepared to take their average OD_{595} value. A standard curve of absorbance (OD_{595}) versus concentration (µg/ml) was plotted based on readings and the concentration of the sample was calculated accordingly (Fig 2.3).

Figure 2.3. Standard Curve for Bradford assay.



2.21. Semi-Dry Western Blotting

SDS-PAGE gel, 12 pieces of Watman No 1. filter paper and a nitrocellulose membrane cut in same dimensions with that of the gel (6x8 cm) were soaked into transfer buffer (Appendix B). Six Watman papers washed with transfer buffer were put onto semi-dry blotting apparatus (Cleaver Scientific) one on the top of the other. Then the membrane, gel and 6 additional Watman papers were put, respectively, in the same way. The proteins were transferred at 1.5 mA/cm^2 (75 mA for 6x8 cm gel) current for 1 h. After transfer, the membrane was put into 10% (w/v) skim milk in 1X TBS Buffer (Appendix B) and incubated at 37°C for 2 h with gentle shaking. Then it was washed with 0.5% (v/v) Twin-20 in 1X TBS solution for 10 min with gentle shaking at room temperature. Afterwards the membrane was incubated with 5% skim milk/1X TBS including serum at 1/300 dilution, with gentle shaking at 37°C. The membrane was again washed with 0.5% (v/v) Twin-20/1X TBS for 10 min and then incubated for 1 h at room temperature in 5% skim milk/TBS solution containing rabbit anti-mouse IgG (Sigma) at a concentration of 10 µl/100 ml. Then the membrane was washed with 1X TBS (without Twin) for 10 min. AP Conjugate Substrate Kit (Bio-Rad) was used for colorimetric detection. A mixture composed of reagent A, reagent B, development buffer and dH_2O was prepared by following manufacturer's instructions. The membrane was incubated with this mixture at dark conditions until the bands appear. Finally, the reaction was stopped by replacing the reaction mixture with dH_2O.

2.22. Mouse Immunization Experiments

Male 8-10 week-old BALB/c mice weighing between 15-20 g were supplied from Faculty of Medicine, Ankara University.

2.22.1. Immunization

The preparation of FeSOD/adjuvant mixture was kindly provided by Dr. Erkan Özcengiz (VBR Inc., Ankara, Turkey). The concentrations of the purified recombinant FeSOD protein and alum [$Al(OH)_3$] adjuvant were 40 μg/ml and 0.9 mg/ml, respectively. 27 mice for the three groups (9 PBS control + 9 whole-cell immunized + 9 FeSOD-immunized) were used in total. Each FeSOD-immunized mouse was given 0.5 ml protein/adjuvant mixture intraperitoneally while 0.5 ml 1X PBS was injected to each control mice. For whole-cell immunization, *B. pertussis* Saadet cells grown on Bordet-Gengou agar were suspended in 0.85% NaCl solution at a concentration of ~1.25 x 10^9 CFU/ml. The suspension was heat-inactivated at 56°C for 40 min and each mouse was injected with 0.5 ml of this suspension intraperitoneally. Immunizations were repeated three weeks later in the same way. Blood was collected from the tail veins of mice (a few drops from each mouse) on the preceding day and 10 days after the second immunization. Blood samples were incubated at room temperature for 1 h, centrifuged at 3000 rpm for 10 min and the upper phase (i.e. serum) was saved and stored at -20°C. Blood uptake was repeated 10 days after the second immunization.

2.22.2. Intranasal Challenge

The following day of second blood uptake, mice were partially anaesthetized with ketamine (at a concentration of 100 mg / kg body weight) and xylasine (5 mg / kg body weight) mixture at first. (These two agents were diluted in sterile 0.85 % NaCl solution to provide a final dose of 0.1 ml per 10 g of body weight, and injected inraperitoneally). Then each mouse was given 100 μl ~2.5×10^9 CFU/ml *B. pertussis* Saadet cells suspended in 0.85% (w/v) NaCl solution with 1% casamino acid by administration of the bacterial suspension drop by drop into the two nostrils equally using a 1cc insulin syringe.

2.22.3. Lung Viable CFU Countings

On the days 5, 8 and 14 post-challenge, three mice each from FeSOD-immunized, PBS-injected control and whole-cell immunized groups were sacrificed and their lungs were transferred sterically into 5 ml 0.85% NaCl with 1% casamino acid. The lungs were cut into small pieces by razor blade and vortexed. 1:10 and 1:100 dilutions were prepared from the original suspensions. 100 µl from each suspension (i.e. undiluted, 1:10 and 1:100 diluted) was plated onto the Cohen-Wheeler agar medium containing cephalexin at a concentration of 40 mg/l. (Three replicates were prepared for each plate). The plates were incubated at 37°C for 5 days and colony numbers were recorded. The average $\log_{10}$CFU/ml was calculated for each lung sample and a $\log_{10}$CFU vs time plot was obtained accordingly.

2.23. Enzyme-Linked Immunosorbent Assay (ELISA)

Firstly, Thermo Immulon 1B ELISA microtiter plate (Thermo Scientific) was coated with 1 µg of purified FeSOD protein per well in 50 µl 1X PBS (Appendix B). Then the plate was covered with parafilm and incubated overnight at 4°C. Following day the wells were blocked with 200 µl blocking solution (Appendix B) and the plate was incubated at 37°C for 2 h. After washing five times with wash buffer (Appendix B), 50 µl of test serum mixed with 1X PBS was added to each well with serial dilutions as 1:50, 1:100, 1:200…1:6400, and the plate was incubated at 37°C for 2 h. After repeated washing step, 50 µl of alkaline phosphatase-conjugated rabit anti-mouse IgG (Sigma), 1:2000 diluted in T-cell buffer (Appendix B), was added to each well. Following 1 h incubation at 37°C, the plate was again washed five times with washing solution. PNPP substrate (*p*-Nitrophenyl Phosphate Disodium Salt, Therno Scientific) was used for colorimetric detection. A mixture composed of a PNPP tablet, 5X diethanolamine substrate buffer and dH_2O was prepared by following manufacturer's instruction and 50 µl from this mixture was added to each well. After 30 min incubation at RT, OD_{405} of the wells were measured via the ELISA

Microplate Reader (μQuant, BioTek) and OD_{405} vs dilution factor was plotted for each sample serum accordingly.

2.24.1. Culturing of Mouse Splenocytes

One week after the second immunization, the spleens of two mice each from PBS control, FeSOD-vaccinated and whole-cell-vaccinated groups were dissected and sterically transferred to 5 ml of RPMI 1640 medium containing 10% Fetal Bovine Serum (FBS) and 1% penicillin/streptomycin (Biochrom). Then they were homogenized through 70 μm nylon cell strainer (BD Falcon) and cell counting was carried out by using hemocytometer. Next the cells from six samples were distributed on a 6-well plate with a concentration of $1x10^7$ cells/well in 2.5 ml RPMI 1640 medium. The plate was incubated for 24h at 37°C in a CO_2 incubator with 5% CO_2. The next day, 30 μg of purified FeSOD protein was added to each well except for the one allocated to one of the two PBS-injected mice. Culture media in six wells were harvested on 3rd day (72 h) of FeSOD addition and stored at -20°C till to be used in subsequent IFN-γ assay.

2.24.2. Cytokine (IFN-γ) Assay

Mouse IFNγ ELISA MiniKit (Thermo Scientific) was used for the assay. The manufacturer's protocol was applied with slight modifications. Firstly, 35 μl of coating antibody was mixed with 11 ml coating buffer and 100 μl from this mixture was added to the wells of an ELISA microtiter plate. The plate was covered and incubated at room temperature overnight. The next day coating antibody solution was discarded, the plate was aspirated and 300 μl of blocking buffer (Appendix B) was added to each well. Then the plate was covered and incubated at room temperature for 1 h. Next, blocking buffer was discarded, the plate was aspirated and 100 μl culture medium in duplicates of serial dilutions (i.e. 1/16, 1/32 and 1/64

diluted in assay buffer; Appendix B) was added to each well. The plate was covered and incubated at room temperature overnight. The next day wells were washed three times with 300 µl of wash buffer (Appendix B) and aspirated. Then 35 µl of detection antibody was mixed with 11 ml of assay buffer and 100 µl from this mixture was added to each well. The plate was covered and incubated at room temperature for 1 h. Following aspiration and washing three times with wash buffer, 100 µl of streptavidin-horseradish peroxidase conjugate (SA-HRP), 1/10 000 diluted in assay buffer, was added to each well and the plate was incubated at room temperature for 30 min. After repeated aspiration and washing steps, 100 µl of TMB (3,3'5,5'-Tetramethyl benzidine dihydrochloride) substrate solution at room temperature was added to each well. Next the plate was covered and incubated at dark place at room temperature for 30 min. Finally, the reaction was stopped by adding 100 µl of stop solution (Appendix B) per well, and OD_{450} absorbances of the wells were measured via the ELISA Microplate Reader (Bio-Rad). A standard "A_{450} vs IFN-γ (pg/ml)" curve was plotted with the help of recombinant mouse IFN-γ standards supplied by the kit and then the IFN-γ concentrations in culture supernatants were calculated accordingly.

2.25. Statistical Analyses

Results obtained from the two ELISA assays were evaluated by one-way ANOVA Tukey's test. The unpaired *t*-test with one-tailed *p* value was used in comparison of lung viable CFU counts of FeSOD-immunized and PBS-injected mice. Values with $p < 0.05$ were considered as statistically significant. "GraphPad Prism 5" (GraphPad Software, Inc.) program was used for statistical analyses.

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Cloning and Subcloning of *sodB* Gene

3.1.1. PCR for Amplification of *sodB* Gene

The primers for amplification of *B. pertussis* Tohama I *sodB* gene (GenBank accession number: 2666341) were designed according to the sequence data obtained from NCBI database (*www.ncbi.nlm.nih.gov*). Cloning of *sodB* to pET28a(+) expression vector would be performed via double restriction enzyme digest, so *Bam*HI restriction site (5'-GGATCC-3') was added to 5' end of the forward primer while BglII site (5'-AGATCT-3') was added to that of the reverse primer. Whole *sodB* gene sequence (579 bp) was checked whether it contained any of these two restriction sites to prevent cutting from internal gene sequence. The "WebCutter" program (*http://rna.lundberg.gu.se/cutter2/*) was used for this purpose. Melting temperatures (Tm) of the primers designed were established using the "Oligo Calc: Oligonucleotide Properties Calculator" program (*http://www.basic.northwestern.edu/biotools/oligocalc.html*). Among the three Tm's calculated by the program, salt (Na^+) adjusted Tm was chosen and the PCR reaction was set up accordingly: 61.2°C and 59.2°C were the Tm's of the forward and reverse primers, respectively. So a temperature-gradient PCR with an annealing temperature range between 56-60°C was implemented first to determine the annealing temperature at which the primer couple works best. The primers performed slightly better at 58°C than the other temperatures (data not shown), so PCR reaction was repeated with this annealing temperature. Genomic DNA of *B.pertussis* Tohama I was used as DNA template (Fig 3.1).

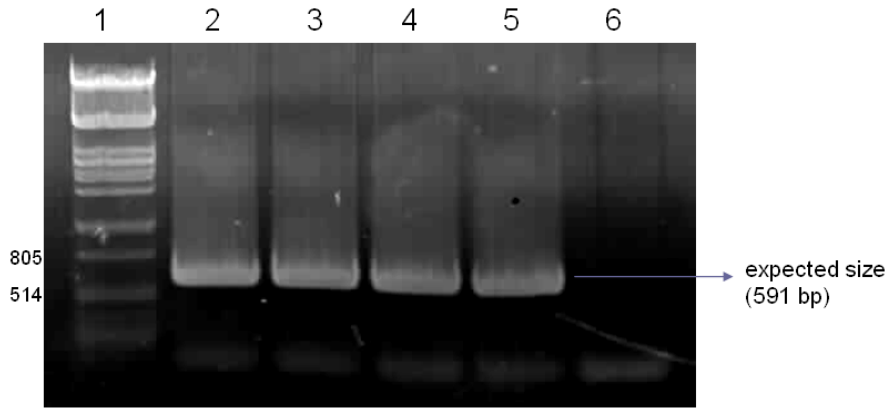


Fig. 3.1. PCR amplification of *sodB* gene at 58°C annealing temperature. Lane 1 — Lambda DNA/PstI Marker (Fermentas). Lane 2-5 — PCR products with DNA template. Lane 6 — PCR product with no DNA template (negative control).

3.1.2. Cloning of *sodB* Gene to pGEM®-T Easy Vector

In order to clone *sodB* PCR product to pGEM®-T Easy TA subcloning vector, PCR product was eluted from the gel. After concentration of the elute was measured, a 10µl-ligation reaction mixture was prepared with an insert:vector molar ratio of 1:1, which was the optimized ratio recommended by the vector's manufacturer (Invitrogen). Transformation of the competent *E.coli* DH5α cells with ligation mixture gave 4 seemingly white colonies. These 4 colonies along with a blue colony as a negative control were streak plated and the next day manual plasmid isolation was carried out. Two colonies (Lanes 3 and 6 in Fig 3.2) out of 4 were found out to carry insert as was deduced from size difference between the plasmids obtained from those two colonies and that from the negative control carrying empty pGEM®-T (Fig 3.2).

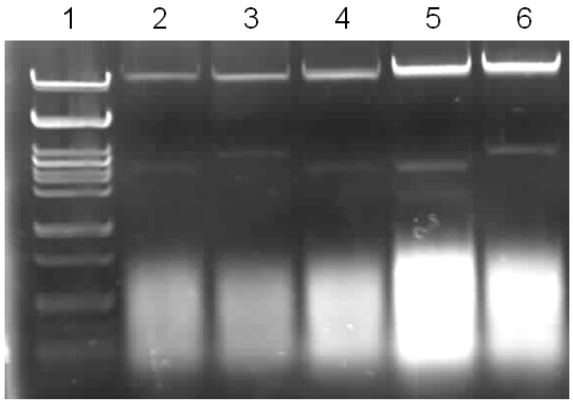


Fig. 3.2. Manual plasmid isolation for *sodB*/pGEM®-T Easy constructs. Lane 1 — Lambda DNA/PstI Marker (Fermentas). Lanes 2-3, 5-6 — plasmids from putative recombinant colonies. Lane 4 — negative control (empty pGEM®-T Easy vector).

Presence of *sodB* gene within the vector was further verified by double enzyme digestion (Fig 3.3). The plasmids were isolated via Plasmid Miniprep Purification Kit (Genemark) this time to obtain purified plasmids free from genomic DNA. This digestion also showed that both restriction enzyme sites (i.e. BglII and BamHI sites) were intact and not mutated so that the insert could be easily extracted from pGEM®-T vector for further subcloning.

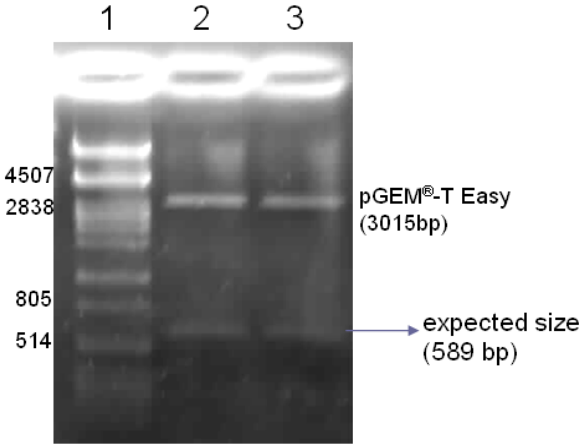


Fig. 3.3. Verification of cloning of *sodB* gene to pGEM®-T Easy vector. Lane 1 — Lambda DNA/PstI Marker (Fermentas). Lanes 2 and 3 — two colonies carrying insert/vector product.

3.1.3. Subcloning of *sodB* to pET28a(+) Expression Vector

Once cloning of *sodB* to pGEM®-T Easy vector was confirmed, the two insert/vector constructs from two different colonies were sequenced before proceeding to subcloning steps. Sequencing results were compared with *B.pertussis* Tohama I *sodB* sequence (GeneID: 2666341) through NCBI BLAST program (*http://blast.ncbi.nlm.nih.gov/Blast.cgi*) and no mutation was detected for both of the colonies. Next, empty circular pET28a(+) expression vector was single digested with *Bam*HI while *sodB*/pGEM®-T construct was double digested with *Bam*HI and *Bgl*II (These two enzymes create the same "GATC" sticky end although their recognition sites differ by two bases). Another ligation reaction without *sodB* elute was also prepared as a negative control. At the end of ligation and transformation, 6 colonies were obtained in positive ligation reaction while no colonies were detected in negative control. These 6 colonies along with a colony carrying pET28a(+) vector only were streak plated and manual plasmid isolation was performed the next day. Size diffference between those *sodB*-ligated and empty pETa(+) vectors confirmed successful subcloning (Fig 3.4).

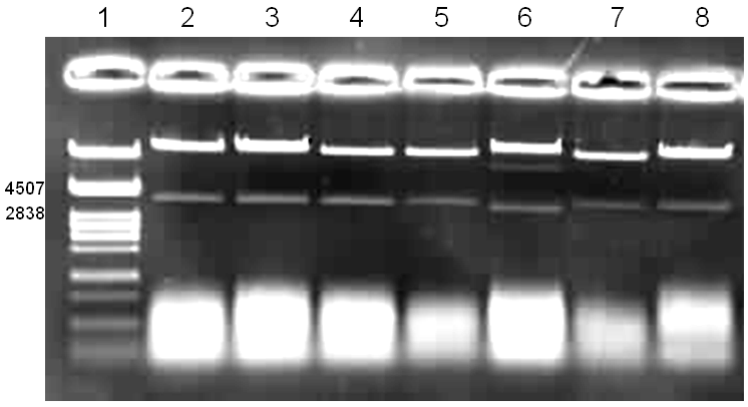


Fig. 3.4. Manual plasmid isolation from putative *sodB*/pET28a(+) colonies. Lane 1 — Lambda DNA/PstI Marker (Fermentas). Lanes 2-5; 7,8 — screened colonies. Lane 6 — negative control [pET28a(+) only].

3.1.4. Verification of Directional Cloning of *sodB* to pET28a(+)

Although insertion of *sodB* to pET28a(+) was verified by manual plasmid isolation (Fig 3.4), the direction of the insertion was yet to be determined since cloning in correct direction was critical for the gene being in frame and ready to be over-expressed later on. *Xho*I restriction enzyme had one restriction site (CTCGAG) at around 70th position of 579 bp-long gene (a list of restriction enzymes cutting internal sequence of the gene is available via the WebCutter program), and another one in multiple cloning site (MCS) of pET28a(+), at a position 40 bp further downstream of that of BamHI (Fig 2.2). Thus, if the gene was directionally cloned, a 550 bp-long; if inversely ligated, a 110bp-long fragment would be expected upon cutting the constructs with *Xho*I. Therefore, 6 constructs obtained from previous ligation were digested with *Xho*I and all but one construct gave the expected result confirming the correct orientation (Fig 3.5).

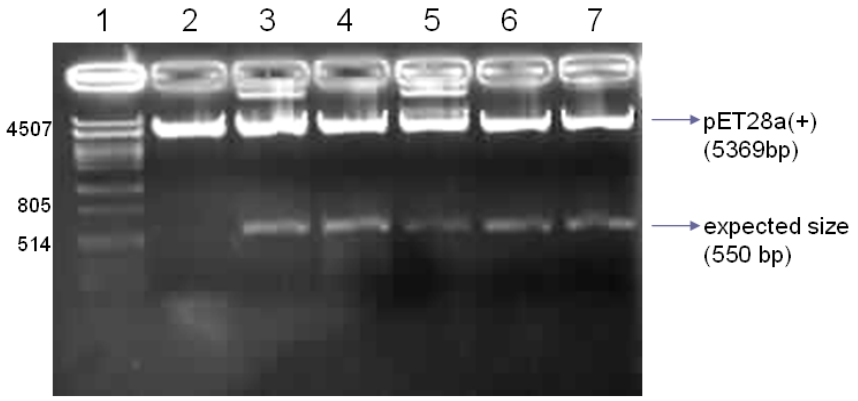


Fig. 3.5. Verification of directional cloning of *sodB* to pET28a(+) expression vector. Lane 1 — Lambda DNA/PstI Marker (Fermentas). Lane 2-7 — digestion profile of putative recombinant plasmids.

3.2. Over-expression and Purification of FeSOD

For *sodB* gene to be expressed in high levels, the recombinant plasmid had to be transferred to another *E. coli* strain, which is suitable for recombinant protein expression. BL21 is the most frequently used strain in recombinant protein expression studies (Sørensen *et al*., 2005). Following confirmation of cloning in correct orientation, *sodB*/pET28a(+) construct was isolated from DH5α cells and introduced to competent *E. coli* BL21 cells. As lysogenized by a DE3 phage fragment, BL21 cells are able to synthesize T7 RNA polymerase, which binds to IPTG-inducible T7/*lac* promoter of pET vectors and catalyzes transcription of the recombinant gene (Dubendorf and Studier, 1991). The absence of certain proteases like 'omT' and 'lon' also facilitates the expression of intact, recombinant proteins in BL21 cells. (Sørensen *et al.*, 2005).

The optimal duration of IPTG-induction was determined by comparing the protein yield of 3h- and 5h-induced cultures (data not shown), and 5h-induction was chosen. Recombinant protein recovery procedure was based on the strategy to recover native, biologically active proteins from insoluble "incluson bodies". This dynamic form of protein aggregation arises as a result of the cells' metabolic responses -mimicking those in stress conditions- to intense protein expression, and is accompanied by expression of molecular chaperones (Villaverde and Carrió, 2003). In this context, the purification procedure must feature vigorous cell lysis, high-speed centrifugation, solubilization in high denaturant concentration (i.e. 8M urea) and dialysis steps following purification to eventually obtain a properly folded, immunologically active protein (Villaverde and Carrió, 2003).

As discussed in the previous Chapter, protein purification involved the use of immobilized metal ion affinity chromatography based on the interaction between Ni^{2+} ions and hexa-His (6xHis) tags, which were located on both termini of the recombinant protein. Samples were saved after each treatment (e.g. washing) throughout the purification procedure. The samples of crude 5h-IPTG-induced and

uninduced cultures were loaded in SDS-PAGE gel to document the validity of each step in reaching from no-expression lysate to purified protein eluate (uninduced culture was used as negative control for protein expression). Visualization of the bands by Coomassie Blue staining revealed that a clear FeSOD protein band sucessfully purged from non-specific proteins was obtained at the end of purification procedure (Fig 3.6).

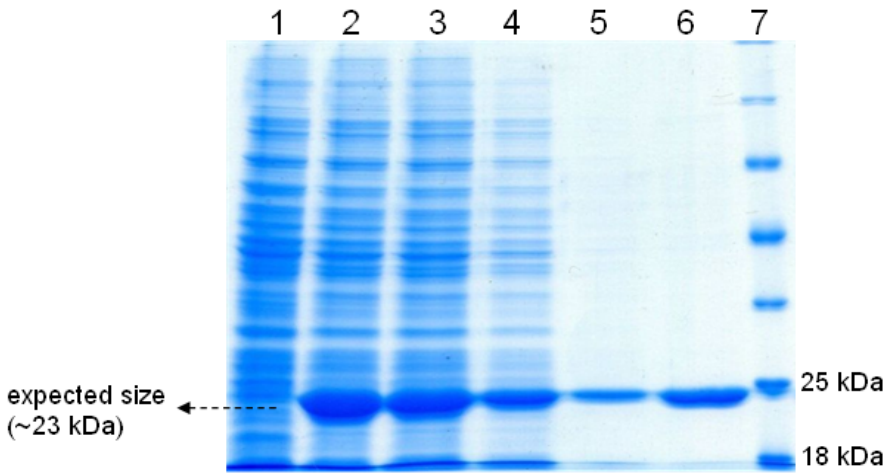


Fig. 3.6. Expression and purification of FeSOD protein (~23 kDa). Lane 1 — uninduced culture. Lane 2 — IPTG-induced culture. Lane 3 — flowthrough. Lane 4 — first wash. Lane 5 — second wash. Lane 6 — final eluate. Lane 7 — unstained protein marker (Fermentas #SM0361).

Having confirmed that FeSOD protein was purified successfully, dialysis against 4M urea and 150 mM NaCl was performed to reduce high urea and salt concentrations of the eluate that might interfere with correct refolding of the protein (Villaverde and Carrió, 2003). Afterwards, protein concentration of the eluate was measured via the Bradford assay. Precise measurement of protein concentration was essential for preparation of a protein/adjuvant mixture of known quantity, which would be introduced to mice in vaccination studies. A series of standard solutions were prepared and a standard curve was plotted accordingly (Fig 3.7). A nearly perfect

curve was obtained with the four standard solutions and the concentration of the FeSOD eluate was calculated as ~780 µg/ml. This value was further confirmed by OD_{280} absorbance measurement of 1:100 diluted elute as 0.008, which corresponded to 800 µg/ml.

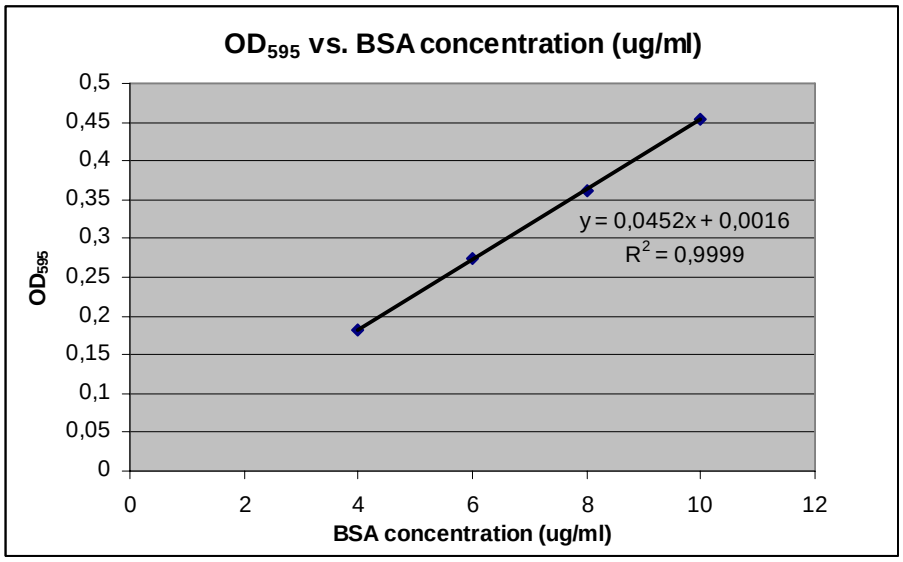


Fig. 3.7. Standard curve for Bradford assay.

3.3. Western Blotting

The immunogenicity of FeSOD protein was reported in different pathogens including the plant trypanosomatid genus *Phytomonas* (Marin *et al*., 2004; Villagran *et al*., 2005) and *Mycobacterium immunogenum* Gupta *et al*., 2009). A recent immunoproteome study conducted in our laboratory revealed that FeSOD was also found in surface proteome of *B. pertussis* Tohama I and Saadet cells, and was immunogenic (Tefon *et al*., 2011). Yet a further confirmation specifically for our current study was required whether the purified protein maintained its immunogenic activity after expression, purification and dialysis steps. Thus two Western blottings were performed with sera obtained from mice subcutaneously immunized with heat-

inactivated *B. pertussis* Tohama I cells and from those immunized with Saadet cells, respectively. Uninduced and IPTG-induced whole culture lysates were included as controls. Results of these two Western blots confirmed the immunogenicity of purified FeSOD protein (Fig 3.8 A and B), protective and immunostimulatory capacities of which remained to be investigated in successive colony counting, ELISA and cytokine assays.

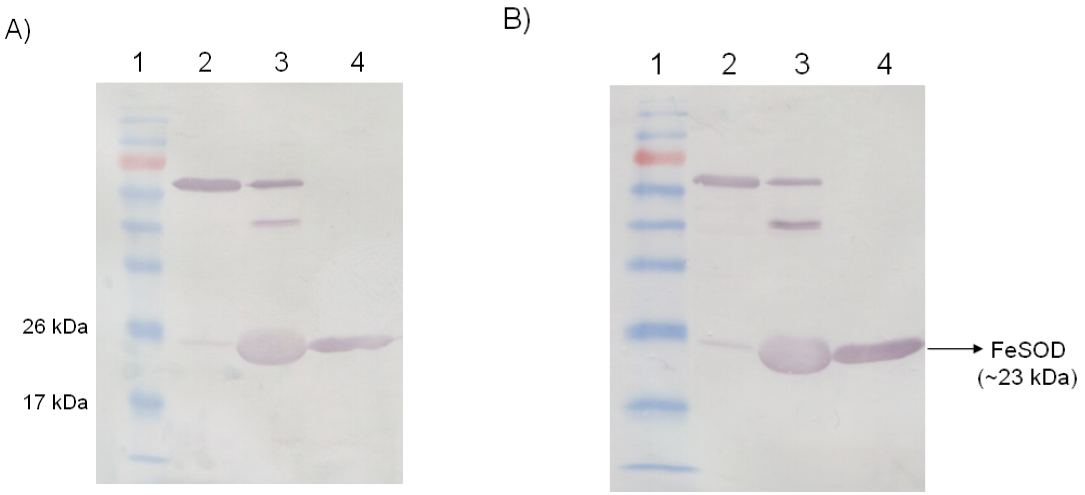


Fig. 3.8. Western blot analyses with A) *anti*-Saadet and B) *anti*-Tohama sera. Lane 1 — pre-stained protein marker (Fermentas). Lane 2 — uninduced protein extract. Lane 3 — IPTG-induced protein extract. Lane 4 — purified FeSOD protein.

3.4. Enzyme-linked Immunosorbent Assay (ELISA)

The potency test for a particular antigen to induce production of antibodies specific to that antigen at significant levels has been applied extensively in acellular pertussis vaccine studies. In this manner, proteins involved in current commercial Pa formulation (i.e. FHA, PT, PRN, FIM) had been tested by ELISA and confirmed to induce humoral immune response (Sato and Sato, 1984; Robinson *et al*., 1989; Kimura *et al*., 1990; Shahin *et al*., 1990). Likewise, *anti*-FeSOD IgG antibody response in mice raised against immunization by heat-inactivated whole-cell

suspension of *B. pertussis* cells and that by recombinant FeSOD protein was monitored in our study by using the ELISA method. Sera collected from tail veins of PBS-injected control, whole-cell immunized and FeSOD-immunized mice after the first and second immunization steps were used in assessment of *anti*-FeSOD IgG response. The results of the ELISA test are presented as OD_{405} readings plotted against serial serum dilutions (Figures 3.9.a and 3.9.b). Although the general trend in absorbances was unambiguously monitored in Figures 3.9.a and b, one particular point (i.e. 1/3200 dilution) was chosen to compare OD_{405} values of the tree groups (Fig 3.10.a and 3.10.b). 1/3200 dilution point was chosen because the absorbance value of FeSOD-immunized group responded to serum dilution barely at this point and started to decrease notably.

The results indicate that after the first and second immunizations, a statistically significant ($p < 0.01$) increase was observed only in FeSOD-immunized mice compared to the PBS-injected control group while whole-cell-immunizations generated no statistically significant rise either after the first or second immunization (Fig. 3.10.a and 3.10.b). There is also a remarkable difference between the first and second immunizations with respect to the absorbance values of FeSOD-immunized group. This might be considered as an expected result since IgG dominates the antibody pool on a second exposure to an antigen while IgM is the prevalent one when the antigen is encountered for the first time (i.e. primary response) (Ahmed *et al*., 2007). Another outcome that is of great importance is that immunization by recombinant FeSOD protein induced much stronger anti-FeSOD IgG response than the whole-cell immunization did (Figs 3.9.a, b; and 3.10.a, b). The level of IgG raised against a particular antigen in response to Pw and Pa immunizations may differ greatly. This divergence in favor of Pa has been reported for some other antigens such as PT (Mills *et al*., 1998) and FHA (Roduit *et al*., 2002).

It is noteworthy that immune response to all IgG subclasses as a whole was monitored in the current study. Yet it was previously reported that IgG1, which is associated with Th2 response, dominates the IgG pool in mice immunized with

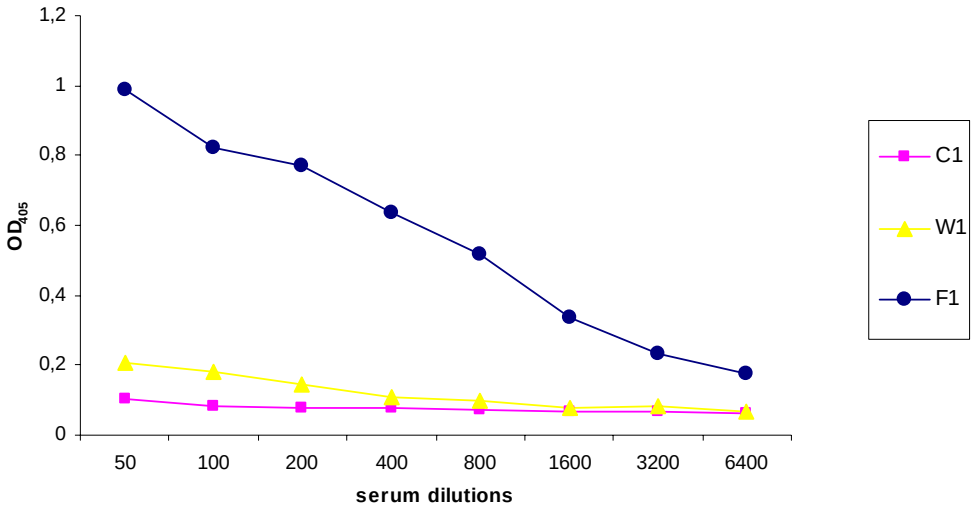


Fig. 3.9.(a). Absorbance (OD_{405}) vs. serum dilution factor curve for the sera obtained from PBS-injected control (C1), whole-cell-immunized (W1) and FeSOD-immunized (F1) mice after the 1st immunization.

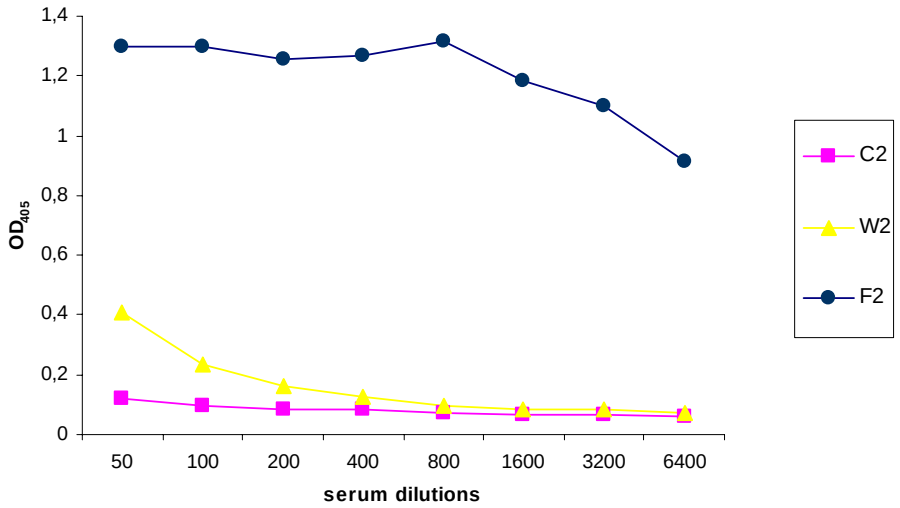


Fig. 3.9.(b). Absorbance (OD_{405}) vs. serum dilution factor curve for the sera obtained from PBS-injected control (C2), whole-cell-immunized (W2) and FeSOD-immunized (F2) mice after the 2nd immunization.

alum-adjuvanted acellular vaccines while IgG2a response is the predominant one in those immunized with Pw (Mills *et al*., 1998, van den Berg *et al*., 2000, Sugai *et al*., 2005).

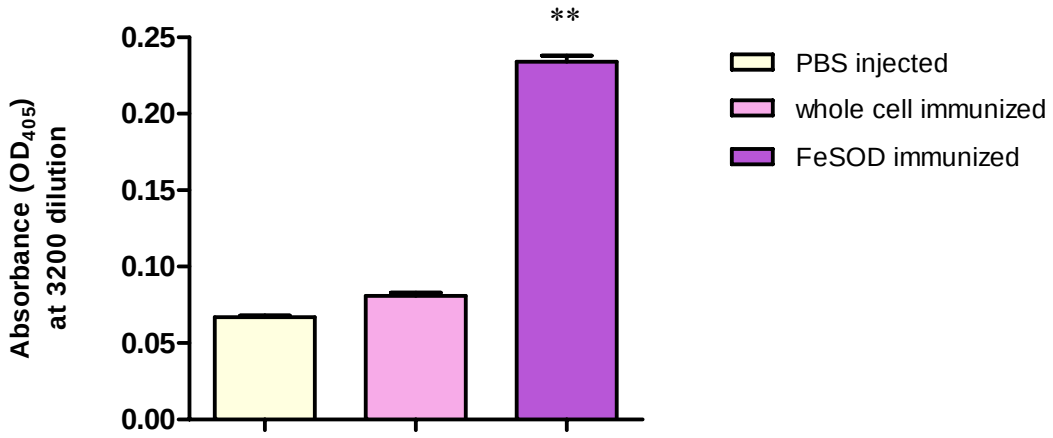


Fig. 3.10.(a). Comparison of OD_{405} absorbances of 1/3200 diluted sera obtained from PBS-injected control, whole-cell-immunized and FeSOD-immunized mice after the 1st immunization.

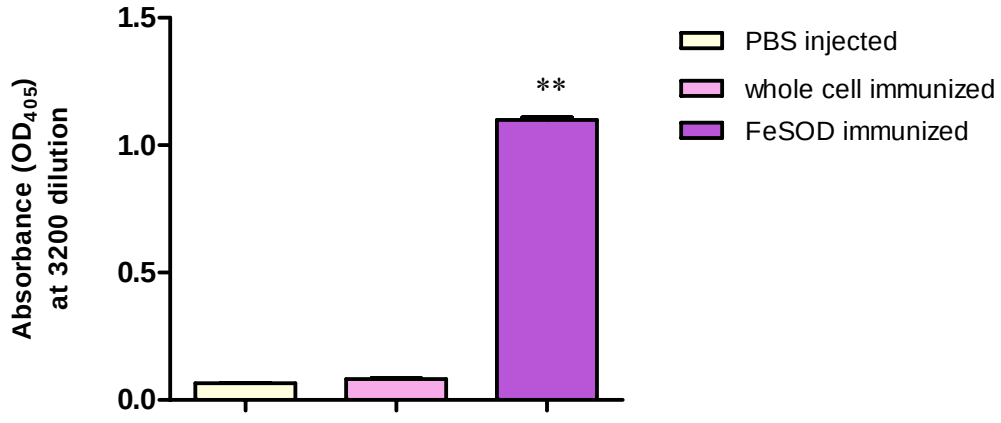


Fig. 3.10.(b). Comparison of OD_{405} absorbances of 1/3200 diluted sera obtained from PBS-injected control, whole-cell-immunized and FeSOD-immunized mice after the 2nd immunization.

Albeit a promising clue, IgG immune response test alone is far from being a direct evidence for any protein to its protectivity (Mills *et al*., 1998, Ausiello *et al*., 2003). Mills *et al*. (1998) and Tondella *et al*. (2009) pointed out the dual mechanism of immunity against *B. pertussis* involving both humoral and cellular immune responses which are directed against a number of protective antigens. According to some studies, assessment of antibody responses in the clinical trials is not sufficient to establish a clear correlation between serum antibody responses against a single *B. pertussis* antigen and the protection it confers (Edwards *et al*., 1995; Gustafsson *et al*., 1996; Miller *et al*., 1997; Mills *et al*., 1998). In some other studies, there was found a significant correlation between IgG1 levels induced by a particular acellular vaccine and the ability of the vaccine to eliminate *B. pertussis* cells from trachea and lungs of mice (van den Berg *et al*., 2000). This correlation might actually be attributed to the complement-fixing activity of IgG1 along with IgG3, which collaborate in stimulating complement-mediated bactericidal killing (Weiss *et al*., 2004). Last but not least, the potency of FeSOD to induce IgG1 and IgG2a responses could not be resolved seperately by the ELISA test performed. This distinction would be crucial, however, as IgG1 response is linked to Th2 type humoral immune response, while IgG2a is associated with Th1 and cellular immune responses (Sugai *et al*., 2005; Gracia *et al*., 2011). As discussed in detail in following sections, conversion of the Th2-predominated immune response driven by the current Pa into a more balanced Th1/Th2 response is one of the major objectives in current acellular pertussis vaccine studies (Gracia *et al*., 2011). Taken all these together, the ELISA results for FeSOD protein had to be reinforced by cell-mediated immunity (CMI) test (i.e. IFNγ assay) and more importantly, by mouse protection test (i.e. lung colonization assay) before coming to a judgement about the protective capacity of this protein.

3.5. Cytokine (IFN-γ) Assay

It was previously accepted that *B. pertussis* is an exclusively extracellular pathogen and Th2-dependent humoral immune response is necessary and sufficient for effective elimination of *B. pertussis* cells colonized in host lung (Bromberg, 1989). However, it was discovered soon after that infection of *B. pertussis* is not only confined to epithelial surfaces but also comprises intracellular association with pulmonary alveolar macrophages of immunocompromised patients (Bromberg *et al*., 1991). The ability of *B. pertussis* cells to invade human and mouse macrophages was further supported by some other studies thereafter (Saukkonen *et al*., 1991; Mills *et al*., 1993). This new finding attracted the common interest to the putative role of Th1-dependent cell-mediated immunity (CMI) in ultimate bacterial clearence and protection (Mills *et al*., 1993; Corbel *et al*., 1999), since Th1 cells are known to mediate immune responses against intracellular pathogens by activating macrophages and neutrophils as well as by facilitating the formation of opsonizing antibodies (Mahon *et al*., 1996; Mills *et al*., 2001; Sugai *et al*., 2005). The concomitant roles of humoral and cellular immune responses in full protection against *B. pertussis* have been reported to date by different groups (Barnard *et al*., 1996; Mills *et al*., 1998; Meyer *et al*., 2007). In line with this, the analysis of CMI as a key parameter in efficacy assessment of different Pa formulations has also been emphasized so far by several studies using murine respiratory infection models. (Mills *et al*., 1998; van den Berg *et al*., 2000; Ausiello *et al*., 2003). In this respect, IFN-γ production as representative of CMI was investigated in our current study as well. Firstly spleen tissues were excised from whole-cell immunized, PBS-injected and FeSOD-immunized mice groups following the binary immunization period. Then these specimens were cultured seperately in previously defined conditions (section 2.24.1) and induced for IFN-γ production by the addition of purified recombinant FeSOD protein to all but one culture. This exceptional ‘blank’ culture (Fig 3.11) was a member of the PBS-injected group and served as an internal control. The blank culture ensured that any significant rise in IFN-γ level would not occur due to protein addition alone but as a consequence of immunization. Lastly the

supernatants of the cultures were harvested and IFN-γ levels were calculated and analyzed as described previously (sections 2.24.2 and 2.25).

The results indicate that the splenocyte cultures generated from whole-cell-immunized mice produced a considerable amount of IFN-γ as a response to stimulation by FeSOD protein (Fig 3.11). FeSOD-immunized mice on the other hand showed no difference compared to the PBS control group indicating that FeSOD immunization did not elicit a significant level of Th1-dependent cell-mediated immune response (Fig 3.11). The ability of whole-cell vaccines (WCV) to stimulate CMI was reported previously; hence the splenocyte cultures derived from whole-cell immunized mice functioned as a positive control group and generated an expected result in this IFN-γ assay especially considering the findings of previous studies (Redhead *et al*., 1993; Barnard *et al*., 1996; van den Berg *et al*., 2000). Contrary to the whole-cell vaccines, acellular pertussis vaccine compositions have been known to trigger a Th2-biased, humoral immune response in mice (Redhead *et al*., 1993; Barnard *et al*., 1996). Yet it was reported that Pa compositions could also stimulate IFN-γ production in humans at remarkable levels depending on the identity of antigens they involve (Meyer *et al*., 2007). PT, FHA and PRN, for instance, are three of the antigens able to stimulate antigen-specific IFN-γ production (Mills *et al*., 1998; van den Berg *et al*., 2000; Ausiello *et al*., 2003; Meyer *et al*., 2007). Hence the current IFN-assay has presented an invaluable data for the assessment of the recombinant FeSOD protein whether it could be numbered among the rare antigens capable of activating CMI or not. As mentioned in section 3.5, a definite activation of CMI remains to be crucial to enhance the potency of current Pa compositions (Gracia *et al*., 2011).

In addition to the antigen composition, the type of the adjuvant used is also proposed to affect the efficacy of a certain Pa. Mansour *et al*. (2007) reformulated the licensed, alum-adsorbed DTPa-IPV vaccine in a liposome/oil emulsion adjuvant and determined the efficacy of a single dose of this new formulation as equivalent to that

conferred by the three repetitive doses of the licensed DTPa-IPV. Sugai *et al*. (2005) found out that addition of CpG adjuvant in an oligodeoxynucleotide form to the conventional alum-adsorbed DTPa greatly decreased *anti*-PT IgG1/IgG2a ratio -without decreasing the original amount of IgG1- and disrupted the huge imbalance

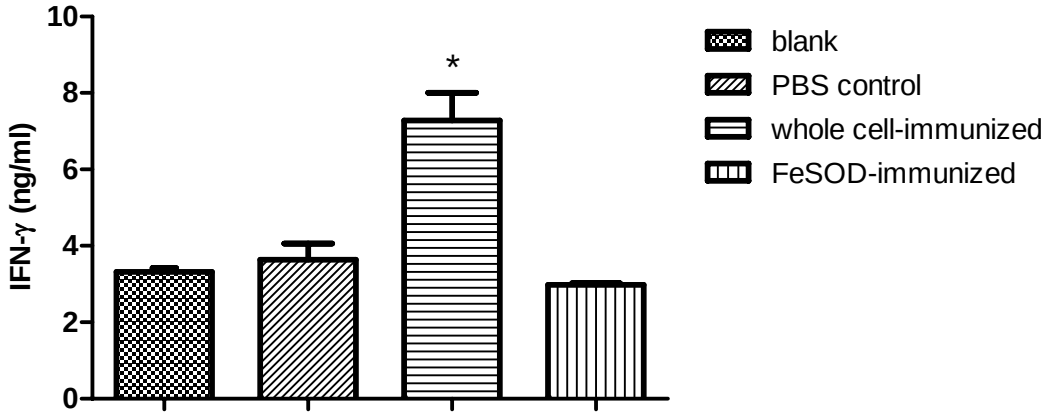


Fig 3.12. IFN-γ levels in FeSOD-stimulated splenocyte culture supernatants collected from differentially treated mice groups. Among the four groups, the mean value of only whole-cell immunized group was significantly different from the others (*$p < 0.05$).

which was initially in favor of IgG1. Gracia *et al*. (2011) recently expanded this bicomponent (CpG & alum) adjuvant platform by adding new components namely polyphosphazenes (PPs) and cationic innate defence regulator peptides (IDRs). Addition of these components to the genetically detoxified pertussis toxoid (PTd) resulted in an enhanced IgG2 response. Since IgG2a is related to the Th1-type immune response (Sugai *et al*., 2005; Gracia *et al*., 2011), it could be speculated that such adjuvant cocktails instead of alum alone could boost CMI activation capacity of any Pa. The recombinant FeSOD protein was adsorbed to alum adjuvant alone in the current study (section 2.22.1). Alum satisfactorily induces humoral immunity; whereas its insufficiency in triggering cell-mediated immunity is also known (Gupta *et al*., 1995; Gupta *et al*., 1998). Therefore, it could be plausible to try different adjuvant platforms in further studies to balance the cellular and humoral immune

responses and to enhance the overall protectivity conferred by the novel Pa composition.

3.6. Intranasal Challenge and Viable Lung CFU Counts

In addition to the analyses on stimulation of humoral and cellular immune responses, intranasal challenge followed by viable lung CFU counts was carried out to assess the protective capacity of recombinant FeSOD protein immunization in mice against *B. pertussis* infection. The two types of respiratory infection models; namely intranasal and aerosol challenges have been considered as useful tools for efficacy assessment of recently developed Pw or Pa compositions (Mills *et al*., 1993; Mahon *et al*., 1996; Hormozi *et al*., 1998; Xing *et al*., 1999; Ausiello *et al*., 2003). One alternative to these respiratory infection models is the Kendrick's intracerebral (ic) challenge model (Kendrick *et al*., 1947), which is accepted as inconvenient for being assayed in potency tests of Pa but useful for those of Pw (Mills *et al*., 1998; Watanabe *et al*., 2002). Some studies manifested a strong correlation between the potency of a recently developed Pa composition in a mouse respiratory challenge model and the degree of protection conferred by the same Pa composition in human-orientated clinical trials (Mills *et al*., 1998; Guiso *et al*., 1999). This strong correlation renders the practices on murine respiratory challenge models much more crucial than humoral and cellular immune response assays in such efficacy assessment studies (Mills *et al*., 2001).

Intranasal challenge followed by lung viable colony counting has particularly been considered as a useful efficacy test owing to its several appealing features. First, the route of infection in intranasal challenge successfully mimicks that in natural infection. Second, it is a non-lethal model for mice and allows recovery from challenge, which helps one to trace the mechanisms of protection in detail. Third, intranasal challenge does not generate a clinical disease and causes no or very little distress in mice, which makes this model favorable in terms of ethical concerns

(Corbel *et al*., 1999). Despite all these advantages listed, this model also has several drawbacks: it is a tedious procedure and requires expertise. Furthermore, highly variable outcomes could be obtained due to a number of factors such as the mouse and bacterial strains, which may substantially affect the results. Last but not least, this technique lacks a standardized methodology. Days post-challenge on which mice groups are sacrificed, bacterial and mouse strains used, the dose of challenge and the statistical analysis of lung CFU data are among the parameters which greatly differ from one study group to another (Corbel *et al*., 1999; Corbel *et al*., 2004; Tondella *et al*., 2009). Nevertheless, the model enables one to assess the potency of the Pa in question at least through comparison of immunized and negative control groups provided there is a firmly established procedure within a particular study.

In the present study, 8-10 week old BALB/c mice were used for the intranasal challenge. This specified pathogen free (SPF) inbred mouse strain has been extensively used in the analogous studies (Guiso *et al*., 1999; van den Berg *et al*., 2000; Denoël *et al*., 2002; Ausiello *et al*., 2003). The days post-challenge were chosen based on the previous trial experiments. Those experiments had revealed that the lung colonization of unimmunized mice by *B. pertussis* cells peaks at day 5 post-intranasal challenge (data not shown). Hence it would be wise to trace and compare the rates of bacterial clearance from the 5^{th} day on to establish the differences –if any- between immunized and negative control groups. Log_{10}CFU values reaching the top at 4-5 day post-challenge and the pattern of decrease from day 5 to day 14 was previously reported by some other studies in which the efficacies of different Pa compositions were compared (Guiso *et al*., 1999; Boursaux-Eude *et al*., 1999). The dose of intranasal challenge was established as 2.5×10^8 CFU per mouse in the current study while the doses used in other studies ranges from 1×10^6 (Hormozi *et al*., 1999) to 2×10^7 (Cheung *et al*., 2006). Yet it should be noted that the international reference strain 18323 of *B. pertussis* was employed in most of the studies (Hormozi *et al*., 1999; Guiso *et al*., 1999; Ausiello *et al*., 2003; Cheung *et al*., 2006) while the local isolate 'Saadet' was the challenge *B. pertussis* strain in the current assay. Cheung *et al*. (2006) stressed that the bacterial strain used is one of the

parameters affecting the outcome of an intranasal challenge assay. Antigenic differences among the ubiquitous vaccine strains (e.g. 18323) and the clinical isolates may account for this diversity (Khattak and Matthews, 1993). Consequently, the challenge dose determined for the Saadet strain did not necessarily have to be within the range constituted for 18323. Log_{10}CFU values obtained in the current study (Fig 3.12) are quite close to those in other studies, so the applied challenge dose appears to be reasonable for the Saadet strain.

The results of the challenge assay revealed that the whole-cell-immunized mice group again featured as a perfect positive control, which was similar to the case in IFN-γ assay: a full protection was inferred from the observation that lung homogenates of the whole-cell-immunized mice generated no viable CFU at any of the days post-challenge (Fig 3.12). For *B. pertussis*, complete bacterial clearance by day 5 post-challenge as the result of Pw immunization was also detected by previous studies (Redhead *et al*., 1993; Mills *et al*., 1998; Roduit *et al*., 2002). FeSOD-immunized and PBS-injected control groups, on the other hand, did not attain a complete clearance even day 14 post challenge; yet they showed not much significantly different pattern of decrease. Nonetheless, the rate of decrease in viable CFU numbers in FeSOD-immunized mice was slightly higher as compared to that in PBS-control group (Fig 3.12). To make sure whether this slight difference is statistically significant or not, area under the curve (AUC) was calculated for both of the groups by applying the formula [AUC = mean log_{10}cfu/ml x days ± standard error (SE)] as previously described (van den Berg *et al*., 2000, Roduit *et al*., 2002). Then the results were compared via unpaired *t*-test with one-tailed *p*-value. This test ensured that the slight difference observed in Fig 3.12 was not significant ($p > 0.05$) (Fig 3.13), meaning that FeSOD-immunization does not confer an additional protection in terms of bacterial clearance. The results obtained in this test showed a kind of parallelism with those in the previous IFN-γ assay. The strong link between CMI and protection had been discussed in detail previously (section 3.6). Hence the results obtained in the intranasal challenge assay could also be interpreted as a

validation of the indispensable role of CMI in protection against *B. pertussis* infection (Mills *et al*., 2001).

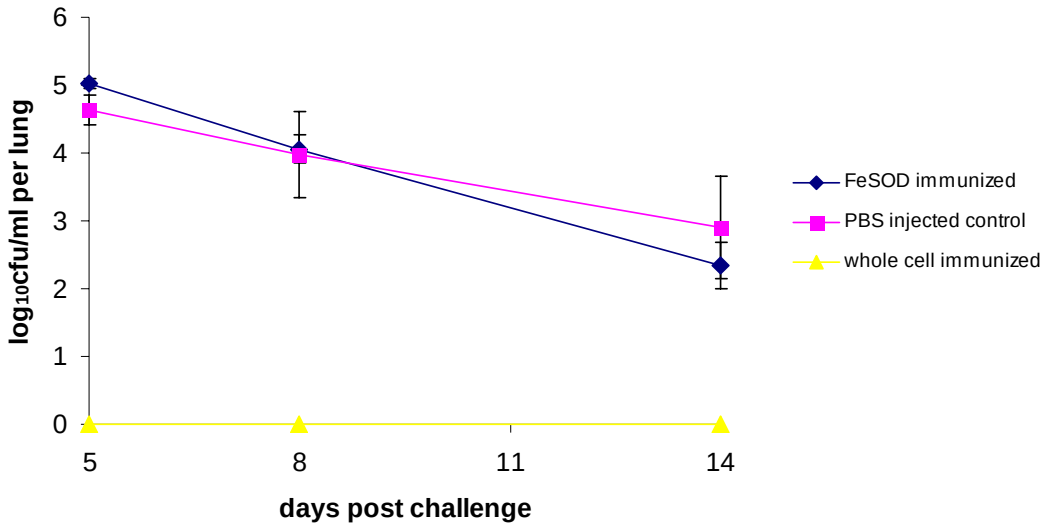


Fig 3.13. Colony numbers of viable *B. pertussis* cells recovered from lung homogenates of FeSOD-immunized, whole-cell immunized and PBS control mice at 5, 8 and 14 days post-intranasal challenge.

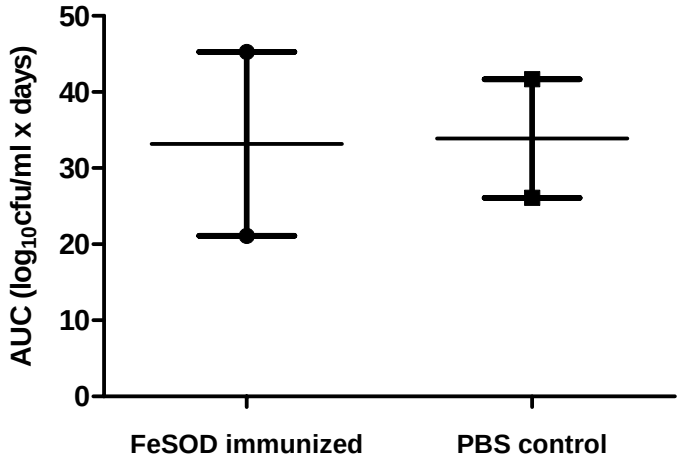


Fig 3.14. Statistical comparison of FeSOD-immunized and PBS control mice with respect to lung colonization patterns. Neither the means nor the variances of the two groups were significantly different ($p > 0.05$).

CHAPTER 4

CONCLUSION

- Iron superoxide dismutase (FeSOD) from *B. pertussis* was recently reported as a highly immunogenic protein. The capacity of the protein to induce humoral immune response (i.e. production of *anti*-FeSOD IgG) was shown in this study by injecting BALB/c mice the purified recombinant FeSOD protein. Heat-inactivated whole cell immunization on the other hand resulted in no significant level of *anti*-FeSOD IgG production. In addition, secondary exposure to the antigen triggered much more *anti*-FeSOD IgG production in mice than the primary exposure.

- Splenocyte cultures obtained from whole-cell immunized mice produced a significant amount of IFN-γ as a response to stimulation by FeSOD protein *in vitro*. In contrast, splenocytes obtained from FeSOD-immunized mice did not develop a IFN-γ response higher than the PBS control group. IFN-γ production is a hallmark of the cell-mediated immune response. Therefore, immunization by recombinant FeSOD protein adsorbed to alum adjuvant does not appear to induce cell-mediated immunity (CMI) in mice.

- While whole-cell immunization conferred a complete protection in mice against intranasal infection by *B. pertussis*, and lung homogenates of such mice were totally free from *B. pertussis* cells on 5, 8 and 14 days post-challenge, lung homogenates obtained from FeSOD-immunized and PBS control groups on the other hand exhibited a similar, almost linear rate of decrease in terms of mean log10CFU from the day 5 to 14, and neither of the groups achieved full bacterial clearance even day 14 post-challenge. The

small difference between the rates of decrease was not significant ($p > 0.05$) among these two groups. It follows that recombinant FeSOD immunization did not confer protectivity in mice against intranasal *B. pertussis* infection.

- The humoral immune response triggered by an antigen does not necessarily entail its protectivity against *B. pertussis* infection. Cell-mediated immune response is an indispensable part of the complete immunity against *B. pertussis*. Hence, CMI response tests supported by murine respiratory infection models provide much more reliable data to the assessment of protectivity of any antigen.

- The identity of the adjuvant used is of a great importance in controlling the type and intensity of the immune response triggered by any Pa composition. The impaired cellular immune response monitored upon FeSOD/alum immunization in the present study could be enhanced by using other adjuvant platforms such as CpG or liposome/oil emulsion in future studies.

- In light of the overall results, the recombinant FeSOD protein itself is not a promising candidate to constitute a new-generation acellular pertussis vaccine. Yet, as suggested by high levels of IgG response it induced, its potential as a component of new-generation multi-component pertussis vaccine cannot be ruled out.

REFERENCES

Ahmed N., Dawson M., Smith C., Wood E. (2007). The immune system (Owen E., Ed.). Biology of disease (pp. 73-97). New York, U.S.: Taylor & Francis Group.

Anderson R. M., May, R. M. (1991). Infectious Diseases of Humans, Dynamics and Control. Oxford University Press, Oxford.

Andrews R., Herceg A., Roberts C. (1997). Pertussis notifications in Australia, 1991 to 1997. Commun Dis Intell, 21(11): 145-148.

Archambault D., Rondeau P., Martin D., Brodeur B.R. (1991). Characterization and comparative bactericidal activity of monoclonal antibodies to *Bordetella pertussis* lipo-oligosaccharide A. J Gen Microbiol, 137(4):905-11.

Ausiello C., Lande R., Stefanelli P., Fazio C., Fedele G., Palazzo R., Urbani F., Mastrantonio P. (2003). T-cell immune response assessment as a complement to serology and intranasal protection assays in determining the protective immunity induced by acellular pertussis vaccines in mice. Clinical and Vaccine Immunology, 10(4): 637-642.

Bakshi CS, Malik M, Regan K, Melendez JA, Metzger DW, Pavlov VM, Sellati TJ. (2006). Superoxide dismutase B gene (sodB)-deficient mutants of *Francisella tularensis* demonstrate hypersensitivity to oxidative stress and attenuated virulence. J Bacteriol, 188(17):6443-8.

Barlow W. E., Davis R. L., Glasser J. W., Rhodes, P. H., Thompson R. S., Mullooly J. P., Black S. B., Shinefield H. R., Ward J. I., Marcy S. M., DeStefano F., Chen R. T., Immanuel V., Pearson J. A., Vadheim C. M., Rebolledo V., Christakis D., Benson P. J., Lewis N. (2001). The risk of seizures after receipt of whole-cell pertussis or measles, mumps, and rubella vaccine. N Engl J Med, 345(9): 656-661.

Barnard A., Mahon B. P., Watkins J., Redhead K., Mills K. H. (1996). Th1/Th2 cell dichotomy in acquired immunity to *Bordetella pertussis*: variables in the in vivo priming and in vitro cytokine detection techniques affect the classification of T-cell subsets as Th1, Th2 or Th0. Immunology, 87(3): 372-80.

Bass J. W. and Wittler R. R. (1994). Return of epidemic pertussis in the United States. Pediatr Infect Dis J, 13: 343-345

Battistoni A., Pacello F., Folcarelli S., Ajello M., Donnarumma G., Greco R., Grazia M., Ammendolia D., Touati G., Valenti P. (2000). Increased expression of periplasmic Cu,Zn superoxide dismutase enhances survival of Escherichia coli invasive strains within nonphagocytic cells. Infect Immun, 68:30–37.

Belcher C. E., Drenkow J., Kehoe B., Gingeras T. R., McNamara N., Lemjabbar H, Basbaum C, Relman D A. (2000). The transcriptional responses of respiratory epithelial cells to Bordetella pertussis reveal host defensive and pathogen counter-defensive strategies. PNAS, 97(25): 13847-13852.

Bergquist S. O., Bernander S., Dahnsjo H., Sundelof B.O. (1987). Erythromycin in the treatment of pertussis: a study of bacteriologic and clinical effects. Pediatric Infectious Disease Journal, 6(5): 461-465.

Bernstein D.I., Smith V.E., Schiff G.M., Rathfon H.M., Boscia J.A. (1993). Comparison of acellular pertussis vaccine with whole cell vaccine as a booster in children 15 to 18 months and 4 to 6 years of age. Pediatr Infect Dis J, 12(2):131-5.

Bjornstad O.N., Harvill, E.T. (2005). Evolution and emergence of Bordetella in humans. Trends Microbiol, 13: 355-359.

Bordet J., and Gengou O. (1906). Le microbe de la coqueluche. Ann. Inst. Pasteur 20:48–68.

Boschwitz J. S., Batanghari J. W., Kedem H., and Relman D. A. (1997). Bordetella pertussis infection of human monocytes inhibits antigen-dependent CD4 T cell proliferation. J. Infect. Dis, 176: 678–686.

Boursaux-Eude C., Thiberge S., Carletti G., Guiso N. (1999). Intranasal murine model of Bordetella pertussis infection: II. Sequence variation and protection induced by a tricomponent acellular vaccine1. Vaccine, 17(20-21): 2651–2660.

Bradford M. M. (1976*).* A rapid and sensitive method for the quantitation of microgram quantities of protein utilizing the principle of protein-dye binding. Analytical Biochemistry, 72(1-2): 248-254.

Brennan M. J., Li, Z. M., Cowell J. L., Bisher M. E., Steven A. C., Novotny P., Manclark C. R. (1988). Identification of a 69-kilodalton nonfimbrial protein as an agglutinogen of Bordetella pertussis. Infect Immun, 56(12): 3189-95

Brenner D.J., Krieg N.R., Staley J.T. (eds) (2005). Family III Alcaligenaceae. In: Bergey's Manual of Systematic Bacteriology, Part C. Springer, pp.647-671.

Brickman, T. J., Armstrong S. K. (1999). Essential role of the iron-regulated outer membrane receptor FauA in alcaligin siderophore-mediated iron uptake in Bordetella species. J Bacteriol, 181(19): 5958-5966.

Brito G. A., Souza M. H., Melo-Filho A. A., Hewlett E. L., Lima A. A., Flores C. A., Ribeiro R. A. (1997). Role of pertussis toxin A subunit in neutrophil migration and vascular permeability. Infect. Immun, 65:1114–1118.

Bromberg K., Tannis G., Steiner P. (1991). Detection of *Bordetella pertussis* associated with the alveolar macrophages of children with human immunodeficiency virus infection. Infection and immunity, 59(12): 4715–4719.

Carbonetti N. H., Artamonova G. V., Andreasen C., Dudley E., Mays R. M., and Worthington Z. E. (2004). Suppression of serum antibody responses by pertussis toxin after respiratory tract colonization by Bordetella pertusis and identification of an immunodominant lipoprotein. Infect. Immun, 72:3350– 3358.

Carbonetti, N. H. (2007). Immunomodulation in the pathogenesis of Bordetella pertussis infection and disease. Curr Opin Pharmacol, **7**(3): 272-278.

Carlioz, A., Touati D. (1986). Isolation of superoxide dismutase mutants in Escherichia coli: is superoxide dismutase necessary for aerobic life? EMBO J, 5:623–630.

Caroff M., Chaby R., Karibian D., Perry J., Deprun C., and Szabo L. (1990). Variations in the carbohydrate regions of Bordetella pertussis lipopolysaccharides: electrophoretic, serological and structural features. J. Bacteriol, 172:1121–1128.

Celentano L.P., Massari M., Paramatti D., Salmaso S., Tozzi A.E. (2005). Resurgence of pertussis in europe. Pediatr Infect Dis J, 24: 761-765

Charles I. G., Li J., Roberts M., Beesley K., Romanos M., Pickard D. J., Francis M., Campbell D., Dougan G., Brennan M.J., Manclark C.R., Jensen M.A., Heron I., Chubb A., Novotny P., Fairweather N.F. (1991). Identification and characterization of a protective immunodominant B cell epitope of pertactin (P.69) from *Bordetella pertussis.* European Journal of Immunology, 21(5): 1147-1153.

Cherry J. D. (1997). Comparative efficacy of acellular pertussis vaccines: an analysis of recent trials. Pediatr Infect Dis J 16:S90-96.

Cherry J. D., Gornbein J, Heininger U., and Stehr K. (1998). A search for serologic correlates of immunity to *Bordetella pertussis* cough illnesses. Vaccine, 16:1901–1906.

Cherry J. D. (1999). Epidemiological, clinical and laboratory aspects of pertussis in adults. Clin Infect Dis, 28 Suppl 2, S112-S117.

Cherry J.D. (2005). The epidemiology of pertussis, a comparison of the epidemiology of the disease pertussis with the epidemiology of *Bordetella pertussis* infection. Pediatrics, 115: 1422-1427.

Cherry J. D., Heininger U. (2004). Pertussis and other *Bordetella* infections, p. 1588–1608. In R. D. Feigin, J. D. Cherry, G. J. Demmler, and S. Kaplan (ed.), Textbook of pediatric infectious diseases, 5th ed. The W. B. Saunders Co, Philadelphia, Pa

Cherry J. D. and Olin P. (1999). The science and fiction of pertussis vaccines. Pediatrics 104:1381-1383.

Cheung G. Y. C., Xing D., Prior S., Corbel M. J., Parton R., Coote J. G. (2006). Effect of different forms of adenylate cyclase toxin of *Bordetella pertussis* on

protection afforded by an acellular pertussis vaccine in a murine model. Infection and immunity, 74(12): 6797-805.

Cody C. L., Baraff L. J., Cherry J. D., Marcy S. M., Manclark C. R. (1981). Nature and rates of adverse reactions associated with DTP and DT immunizations in infants and children. Pediatrics, 68(5): 650-660.

Cone T. E. J. (1970). Whooping cough is first described as a disease sui generis by Baillou in 1640. Pediatrics 46:522.

Confer D. L., Slungaard A. S., Graf E., Panter S. S., Eaton J. W. (1984). Bordetella adenylate cyclase toxin: entry of bacterial adenylate cyclase into mammalian cells. Adv. Cyclic Nucleotide Protein Phosphorylation Res, 17: 183–187.

Confer A.W., Ayalew S., Panciera R. J., Montelongo M., Whitworth L. C. Hammer J. D. (2003). Immunogenicity of recombinant Mannheimia haemolytica serotype 1 outer membrane protein PlpE and augmentation of a commercial vaccine. Vaccine, 21(21-22): 2821-2829

Corbel M. J., Xing D. K., Kreeftenberg J. G. (1999). Informal consultation with manufacturers and WHO Ad hoc working group on mouse protection models for acellular pertussis vaccines national institute for biological standards, 12 november 1998. Biologicals: journal of the International Association of Biological Standardization, 27(2): 189-93.

Corbel M., Kreeftenberg J., Knezevic I. (2004). WHO Working Group on the standardisation and control of pertussis vaccines—report of a meeting held on 6–7 May 2003, Ferney Voltaire, France. Vaccine, 22(3-4): 293-300.

Cornelis G.R. (2000). Type III secretion: a bacterial device for close combat with cells of their eukaryotic host. Philos Trans R Soc Lond B Biol Sci, 355: 681-693.

Cotter P.A., Dirita V.J. (2000). Bacterial virulence gene regulation: an evolutionary perspective. Annual Review of Microbiology, 54: 519-565.

Coutte L., Antoine R., Drobecq H., Locht C., Jacob-Dubuisson F. (2001). Subtilisin-like autotransporter serves as maturation protease in a bacterial secretion pathway. EMBO J. 20:5040–5048.

Cullinane L.C., Alley M.R., Marshall R.B., Manktelow B.W. (1987). *Bordetella parapertussis* from Lambs. N Z Vet J, 35:175.

Cummings C.A., Brinig M.M., Lepp P.W., van de Plas S., Relman D.A. (2004). Bordetella species are distinguished by patterns of substantial gene loss and host adaptation. J Bacteriol, 186: 1484-1492.

De Serres G., Boulianne N., Douville F. M., Duval B. (1995). Pertussis in Quebec, ongoing epidemic since the late 1980s. Can Commun Dis Rep, 21: 45-48.

Delogu G., Brennan M.J. (2001). Comparative immune response to PE and PE_PGRS antigens of *Mycobacterium tuberculosis*. Infect Immun, 69(9): 5606-11.

De Magistris M.T., Romano M., Bartoloni A., Rappuoli R., Tagliabue A. (1989). Human T cell clones define S1 subunit as the most immunogenic moiety of pertussis toxin and determine its epitope map. JEM, 169(5): 1519-1532.

Denoel P., Poolman J., Carletti G., Veitch K. (2002). Effects of adsorption of acellular pertussis antigens onto different aluminium salts on the protective activity in an intranasal murine model of *Bordetella pertussis* infection. Vaccine, 20(19-20): 2551-2555.

deMelker H., Versteegh F. G., Schellekens J., Teunis P., Kretzsschmar, M. (2005). The incidence of *Bordetella pertussis* infections estimated in the population from a combination of serological surveys. J Infect, 53: 106-113.

Diavatopoulos D.A., Cummings C.A., Schouls L.M., Brinig M.M., Relman D.A., Mooi F.R. (2005). *Bordetalla pertussis*, the causative agent of whooping cough, evolved from a distinct, human-associated lineage of *B. bronchiseptica*. PloS Pathog 1, e45

Dirix V., Verscheure V., Goetghebuer T., Hainaut M., Debrie A.S., Locht C., Mascart F. (2009). Vaccine, 27(43): 6042-7.

Dodd D. (2003). Benefits of combination vaccines: effective vaccination on a simplified schedule. Am J Manag Care, 9(1 Suppl):S6-12.

Dubendorf J. W., Studier F. W. (1991). Controlling basal expression in an inducible T7 system by blocking the target T7 promoter with *lac* repressor. Journal of Molecular Biology, 219(1): 45-59

Edwards K. M. (2005). Overview of pertussis: focus on epidemiology, sources of infection, and long term protection after infant vaccination. Pediatr Infect Dis J, 24(6 Suppl): S104-108.

Ehrt S., Schnappinger D. (2003). Methods in Molecular Biology, 235: (75-78).

Elomaa A., Advani A., Donnelly D., Antila M., Mertsola J., Hallander H., He Q. (2005). Strain variation among *Bordetella pertussis* isolates in Finland, where the whole-cell pertussis vaccine has been used for 50 years. J Clin Microbiol, 43: 3681-3687.

Emsley P., McDermott G., Charles I. G., Fairweather N. F., Isaacs N. W. (1994). Crystallographic characterization of pertactin, a membrane-associated protein from *Bordetella pertussis*. J. Mol. Biol, 235:772–773.

Fernandez R. C., Weiss A. A. (1996). Susceptibilities of *Bordetella pertussis* strains to antimicrobial peptides. Antimicrob. Agents Chemother, 40: 1041–1043.

Finn T. M., Stevens L. A. (1995). Tracheal colonization factor: a *Bordetella pertussis* secreted virulence determinant. Mol. Microbiol, 16:625–634

Flak T. A., and Goldman W. E. (1996). Autotoxicity of nitric oxide in airway disease. Am. J. Respir. Crit. Care Med, 154:S202–S206.

Franks S., Baton L., Tetteh K., Tongren E., Dewin D., Akanmori B.D., Koram K.A., Ranford-Cartwright L., Riley E.M. (2003). Genetic diversity and antigenic polymorphism in Plasmodium falciparum: extensive serological cross-reactivity between allelic variants of merozoite surface protein 2. Infect Immun, 71(6): 3485-95

Frey A., Di Canzio J., Zurakowski D. (1998). A statistically defined endpoint titer determination method for immunoassays. Journal of immunological methods, 221(1-2): 35-41.

Fry, N. K., Neal S., Harrison T. G., Miller E., Matthews R., George R. C. (2001). "Genotypic variation in the Bordetella pertussis virulence factors pertactin and pertussis toxin in historical and recent clinical isolates in the United Kingdom." Infect Immun 69(9): 5520-5528.

Galan, J. E., Collmer A. (1999). Type III secretion machines: bacterial devices for protein delivery into host cells. Science 284(5418): 1322-1328.

Galazka A. (1993). Control of pertussis in the world. World Health Stat Q, 45 (2-3): 238-47.

Geier D.A, Geier M.R. (2004). An evaluation of serious neurological disorders following immunization: a comparison of whole-cell pertussis and acellular pertussis vaccines. Brain Dev, (5):296-300

Gerlach G., Von Wintzingerode F., Middendorf B., Gross R. (2001). Evolutionary trends in the genus *Bordetella*. Microbes Infect, 3:61-72.

Gerlach G., Jansen S., Beier D., Gross R. (2004). Functional characterization of the BvgAS two-component system of *Bordetella holmesii*. Microbiology, 150: 3715-3729.

Gold M.S. (2002). Hypotonic-hyporesponsive episodes following pertussis vaccination: a cause for concern?. Drug Saf, 25(2): 85-90.

Grabowska K., Wang X., Jacobsson A., Dillner J. (2002). Evaluation of cost-precision rations of different strategies for ELISA measurement of serum antibody levels. Journal of immunological methods, 271(1-2): 1-15.

Gracia A., Polewicz M., Halperin S. A., Hancock R. E. W., Potter, A. A., Babiuk L. A., Gerdts V. (2011). Antibody responses in adult and neonatal BALB/c mice to immunization with novel *Bordetella pertussis* vaccine formulations. Vaccine, 29(8): 1595-604.

Gravekamp C., Horensky D.S., Michel J.L., Madoff L.C. (1996). Variation in repeat number within the alpha C protein of group B streptococci alters antigenicity and protective epitopes. Infect Immun, 64(9): 3576-83.

Greco D., Salmaso S., Mastrantonio P., Giuliano M., Tozzi A. E., Anemona A., Ciofi degli Atti M. L., Giammanco A., Panei, P., Blackwelder W. C., Klein D. L., Wassilak S. G. (1996). A controlled trial of two acellular vaccines and one whole-cell vaccine against pertussis. Progetto Pertosse Working Group. N Engl J Med, 334(6): 341-8.

Guiso N., Capiau C., Carletti G., Poolman J., Hauser P. (1999). Intranasal murine model of *Bordetella pertussis* infection. I. Prediction of protection in human infants by acellular vaccines. Vaccine, 17(19): 2366-76.

Gupta R. K., Rost B. E., Relyveld E., Siber G. R. (1995). Adjuvant properties of aluminum and calcium compounds. Pharmaceutical Biotechnology, 1995(6): 229-48.

Gupta R. K. (1998). Aluminum compounds as vaccine adjuvants. Advanced Drug Delivery Reviews, 32: 155-172.

Gupta M. K., Subramanian V., Yadav J. S. (2009). Immunoproteomic identification of secretory and subcellular protein antigens and functional evaluation of the secretome fraction of *Mycobacterium immunogenum*, a newly recognized species of the *Mycobacterium chelonae-Mycobacterium abscessus* group. J Proteome Res, 8(5): 2319-2330.

Gustafsson L., Hallander H. O., Olin P., Reizenstein E., and Storsaeter J. (1996). A controlled trial of a two-component acellular, a five-component acellular, and a whole-cell pertussis vaccine. N. Engl. J. Med, 334:349–355.

Gustafsson L., Hallander H.O., Olin P. (2005). Seven year report. Pertussis surveillance in Sweden. Progress report October 1997-September 2004. Avdelningen För Immunologi Och Vaccinologi Smittskyddsinstitutet.

Güriş D., Strebel P. M., Bardenheier B., Brennan M., Tachdjian R., Finch E., Wharton M., Livengood J. R. (1999). Clin Infect Dis. 28(6): 1230-1237.

Gyzl A., Augustynowicz E., Rabczenko D., Gniadek G., Slusarczyk J. (2004). Pertussis in Poland. Int J Epidemiol, 33: 358-365

Gyzl A., Augustynowicz E., Rabczenko D., Gniadek G., **Dulnyc G., lusarczyk J. (2004)**. Sequence variation in pertussis S1 subunit toxin and pertussis genes in *Bordetella pertussis* strains used for the whole-cell pertussis vaccine produced in Poland since 1960: Efficiency of the DTwP vaccine-induced immunity against currently circulating *B. pertussis* isolates. Vaccine, 22(17-18): 2122-2128

Hallander, H. O., A. Advani, Donnelly D., Gustafsson L., Carlsson, R. M. (2005). Shifts of Bordetella pertussis variants in Sweden from 1970 to 2003, during three periods marked by different vaccination programs. J Clin Microbiol 43(6): 2856-2865.

Halperin S. A., Bortolussi R., Langley J. M., Miller B., Eastwood B. J. (1997). Seven days of erythromycin estolate is as effective as fourteen days for the treatment of *Bordetella pertussis* infections. Pediatrics, 100(1): 65-71.

Halperin S.A., Tapiero B., Diaz-Mitoma F., Law B.J., Hoffenbach A., Zappacosta P.S., Radley D., McCarson B.J., Martin J.C., Brackett L.E., Boslego J.W., Hesley T.M., Bhuyan P.K., Silber J.L. (2009). Safety and immunogenicity of a hexavalent diphtheria-tetanus-acellular pertussis-inactivated poliovirus-Haemophilus influenzae b conjugate-hepatitis B vaccine at 2, 3, 4, and 12-14 months of age. Vaccine, 27(19):2540-7.

Hanahan D., Jessee J., Bloom F. (1995). Techniques in transformation of *E. coli*. (Rickwood D., Hames B. D., Eds.). *DNA Cloning 1: Core Techniques* (pp.1-36). New York: Oxford University Press.

Harvill E. T., Preston A., Cotter P. A., Allen A. G., Maskell D. J., Miller J. F. (2000). Multiple roles for Bordetella lipopolysaccharide molecules during respiratory tract infection. Infect. Immun, 68:6720–6728.

Hassan H. M. (1984). Determination of microbial damage caused by oxygen free radicals, and the protective role of superoxide dismutases. Methods Enzymol, 105:404–412.

Hassan H. M. (1989). Microbial superoxide dismutases. Adv. Genet, 26: 65–97

Hazenbos W. L., Geuijen C. A., van den Berg B. M., Mooi F. R., van Furth R. (1995). Bordetella pertussis fimbriae bind to human monocytes via the minor fimbrial subunit FimD. J Infect Dis, 171: 924–929.

Heininger U., Cherry J.D., Eckhardt T., Lorenz C., Christenson P., Stehr K. (1993). Clinical and laboratory diagnosis of pertussis in the regions of a large vaccine efficacy trial in Germany. Pediatr Infect Dis J, 12(6):504-9.

Hellwig S.M., Rodriguez M.E., Berbers G.A., van de Winkel J.G., Mooi F.R. (2003). Crucial role of antibodies to pertactin in Bordetella pertussis immunity. J Infect Dis, 188(5):738-42

Henderson, I. R., Nataro J. P. (2001). Virulence functions of autotransporter proteins. Infect. Immun, 69:1231–1243.

Hewlett E. L., Gordon V.M. (1988). Adenylate cyclase toxin of Bordetella pertussis, p. 193–209. In A. C. Wardlaw and R. Parton (ed.), Pathogenesis and immunity in pertussis. John Wiley & Sons, Inc., New York, N.Y

Hewlett E.L. and Edwards K.M. (2005). Clinical Practice. Pertussis-not Just for Kids. New Eng J Med, 352: 1215-1222.

Hoppe J. E., and Eichhorn A. (1989). Activity of new macrolides against Bordetella pertussis and Bordetella parapertussis. Eur. J. Clin. Microbiol. Infect. Dis, 8:653–654.

Hormozi K., Parton R., Coote J. (1999). Adjuvant and protective properties of native and recombinant *Bordetella pertussis* adenylate cyclase toxin preparations in mice. FEMS immunology and medical microbiology, 23(4): 273-82.

Howson C. P., Fineberg H. V. (1992). Adverse events following pertussis and rubella vaccines. Summary of a report of the Institute of Medicine. JAMA, 267(3): 392-396.

Ishibashi Y., Relman D. A., Nishikawa A. (2001). Invasion of human respiratory epithelial cells by Bordetella pertussis: possible role for a filamentous hemagglutinin Arg-Gly-Asp sequence and alpha5beta1 integrin. Microb Pathog, 30: 279–288.

Jacob-Dubuisson F., El-Hamel C. , Saint N., Guedin S., Willery E., Molle G., Locht C. (1999). Channel formation by FhaC, the outer membrane protein involved in the secretion of the Bordetella pertussis filamentous hemagglutinin. J Biol Chem, 274: 37731–37735

Jefferson T., Rudin M., DiPietrantonj, C. (2003). Systematic review of the effects of pertussis vaccines in children. Vaccine, 21(17-18): 2003-2014.

Jenkinson D. (1988). Duration of effectiveness of pertussis vaccine: evidence from a 10 year community study. Br Med J (Clin Res Ed), 296(6622):612-4.

Johns T.L., Hutter G.E. (2010). New combination vaccines: DTaP-IPV (Kinrix) and DTaP-IPV/Hib (Pentacel). Ann Pharmacother, 44(3):515-23.

Katada T., Tamura M., Ui M. (1983). The A protomer of islet-activating protein, pertussis toxin, as an active peptide catalyzing ADP-ribosylation of a membrane protein. Arch. Biochem. Biophys, 224:290–298.

Kendrick P. L., Eldering G. (1947). Mouse protection tests in the study of pertussis vaccine; a comparative series using the intracerebral route for challenge. Am J Public Health Nations Health, 37(7): 803-810.

Khattak M. N., Matthews R. C. (1993). A comparison of the DNA fragment patterns of the mouse-virulent challenge strains and clinical isolates of *Bordetella pertussis*. J Infect, 27(2): 119-124.

Khelef N., Danve B., Quentin-Millet M.J., Guiso N. (1993). *Bordetella pertussis* and *Bordetella parapertussis*, two immunologically distinct species. Infect Immun, 61: 486-490.

Khelef N., DeShazer D., Friedman R. L., Guiso N. (1996). In vivo and in vitro analysis of *Bordetella pertussis* catalase and Fe-superoxide dismutase mutants. FEMS Microbiol. Lett, 142:231–235.

Kilpi T.M., Silfverdal S.A., Nilsson L., Syrjänen R., Belloni C., Desole M., Triban C., Storsaeter J., Soila M., Jacquet J.M. (2009). Immunogenicity and reactogenicity of two diphtheria-tetanus-acellular pertussis-hepatitis B-inactivated polio virus-Haemophilus influenzae type b vaccines administered at 3, 5 and 11-12 months of age. Hum Vaccin, 5(1):18-25.

Kimura A., Mountzouros K. T., Relman D. A., Falkow S., Cowell J. L. (1990). Bordetella pertussis filamentous hemagglutinin: evaluation as a protective antigen and colonization factor in a mouse respiratory infection model. Infect Immun, 58(1): 7-16.

King A.J., Berbers G., van Oirschot H.F., Hoogerhout P., Knipping K., Mooi F.R. (2001). Role of the polymorphic region 1 of the Bordetella pertussis protein pertactin in immunity. Microbiology, 147(11): 2885-95.

Kirimanjeswara G.S., Agosto L.M., Kennett M.J., Bjornstad O.N., Harvill E.T. (2005). Pertussis toxin inhibits neutrophil recruitment to delay antibody-mediated clearance of *Bordetella pertussis*. J Clin Invest, 115: 3594-3601.

Korgenski E. K. and Daly J. A. (1997). Surveillance and detection of erythromycin resistance in Bordetella pertussis isolates recovered from a pediatric population in the intermountain west region of the United States. Journal of Clinical Microbiology, 35(11): 2989-2991.

Kubler-Kielb J., Vinogradov E., Lagergård T., Ginzberg A., King J.D., Preston A., Maskell D.J., Pozsgay V., Keith J.M., Robbins J.B., Schneerson R. (2011). Oligosaccharide conjugates of Bordetella pertussis and bronchiseptica induce bactericidal antibodies, an addition to pertussis vaccine. Proc Natl Acad Sci U S A, 108(10):4087-92.

Lacey B. W. (1960). Antigenic modulation of *Bordetella pertussis*. J Hyg, 58: 57.

Ladant D., Ullmann A. (1999). Bordetella pertussis adenylate cyclase: a toxin with multiple talents.Trends Microbiol 7(4): 172-176.

Lapin J. H. (1943). Whooping cough. Charles C Thomas, Springfield, Ill.

Lee C. A. (1997). Type III secretion systems: machines to deliver bacterial proteins into eukaryotic cells? Trends Microbiol, 5:148–156.

Leininger E., Ewanowich C.A., Bhargava A., Peppler M.S., Kenimer J.G., Brennan M.J. (1992). Comparative roles of the Arg-Gly-Asp sequence present in the Bordetella pertussis adhesins pertactin and filamentous hemagglutinin. Infect Immun, 60(6): 2380-5.

Le Saux N., Barrowman N.J., Moore D.L., Whiting S., Scheifele D., Halperin S. (2003). Decrease in hospital admissions for febrile seizures and reports of hypotonic-hyporesponsive episodes presenting to hospital emergency departments since switching to acellular pertussis vaccine in Canada: a report from IMPACT. Pediatrics, 112(5):e348.

Lewis K., Saubolle M. A., Tenover F. C., Rudinsky M. F., Barbour S. D., Cherry J. D. (1995). Pertussis caused by an erythromycin-resistant strain of *Bordetella pertussis*. Pediatr Infect Dis J, 14(5): 388-91.

Li Y.P., Zhang S.M., Ye Q. (2010). Safety and immunogenicity of the diphtheria, tetanus, accellular pertussis plus hemophilus influenza type b combined vaccine (DTaP-Hib) to Chinese infants. Zhongguo Yi Miao He Mian Yi, 16(2):97-104.

Locht C., Keith J. M. (1986). Pertussis toxin gene: nucleotide sequence and genetic organization. Science, 232: 1258–1264.

Locht C., Geoffroy, M. C., Renauld G. (1992). Common accessory genes for the Bordetella pertussis filamentous hemagglutinin and fimbriae share sequence similarities with the papC and papD gene families. EMBO J, 11(9): 3175-83.

Locht, C., Antoine R., Jacob-Dubuisson, F. (2001). *Bordetella pertussis*, molecular pathogenesis under multiple aspects. Curr Opin Microbiol, 4(1): 82-89.

Mahon B., Ryan M., Griffin F., Mills K. (1996). Interleukin-12 is produced by macrophages in response to live or killed *Bordetella pertussis* and enhances the efficacy of an acellular pertussis vaccine by promoting induction of Th1 cells. Infect. Immun., 64(12): 5295-5301.

Mann P.B., Kennett M.J., Harvill E.T. (2004). Toll-like receptor 4 is critical to innate host defense in a murine model of bordetellosis. J Infect Dis, 189: 833-836.

Mann P.B., Wolfe D., Latz E., Golenbock D., Preston A., Harvill E.T. (2005). Comparative toll-like 4-mediated innate host defense to *Bordetella* infection. Infect Immun, 73: 8144-8152.

Mansour M., Brown R. G., Morris A., Smith B., Halperin S. A. (2007). Improved efficacy of a licensed acellular pertussis vaccine, reformulated in an adjuvant emulsion of liposomes in oil, in a murine model. Clinical and vaccine immunology: CVI, 14(10): 1381-3.

Marin C., Hitos A. B., Rodriguez-Gonzalez I., Dollet M., Sanchez-Moreno M. (2004). *Phytomonas* iron superoxide dismutase: a possible molecular marker. [Research Support, Non-U.S. Gov't]. FEMS Microbiol Lett, 234(1): 69-74.

Marshall G.S., Happe L.E., Lunacsek O.E., Szymanski M.D., Woods C.R., Zahn M., Russell A. (2007). Use of combination vaccines is associated with improved coverage rates. Pediatr Infect Dis J, 26(6): 496-500.

Masuda, M., Betancourt L., Matsuzawa T., Kashimoto T., Takao T., Shimonishi Y., Horiguchi Y. (2000). Activation of rho through a cross-link with polyamines catalyzed by Bordetella dermonecrotizing toxin. EMBO J, 19(4): 521-530.

Matsuzawa, T., A. Fukui, Kashimoto T., Nagao K., Oka K., Miyake M., Horiguchi Y. (2004). Bordetella dermonecrotic toxin undergoes proteolytic processing to be translocated from a dynamin-related endosome into the cytoplasm in an acidification-independent manner. J Biol Chem 279(4): 2866-2872.

Mattoo S., Cotter P. A., and Miller J. F. (2000). Differential roles of Bordetella virulence factors as inducers and modulators of the host immune response, p. 254. Proc. 100th Gen. Meet. Am. Soc. Microbiol. American Society for Microbiology, Washington, D.C.

Mattoo S., Yuk M. H., Huang L. L., Miller J. F. (2004). Regulation of type III secretion in Bordetella. Mol. Microbiol, 52:1201–1214.

Mattoo S. & Cherry J. D. (2005). Molecular pathogenesis , epidemiology , and clinical manifestations of respiratory Infections due to *Bordetella pertussis* and other *Bordetella* subspecies. Society, 18(2): 326-382.

McGuirk, P., McCann C., Mills, K. H. (2002). Pathogen-specific T regulatory 1 cells induced in the respiratory tract by a bacterial molecule that stimulates interleukin 10 production by dendritic cells: a novel strategy for evasion of protective T helper type 1 responses by *Bordetella pertussis*. J Exp Med, 195(2): 221-231.

Meade B. D., Kind P. D., Ewell J. B., McGrath P. P., Manclark C. R. (1984). In vitro inhibition of murine macrophage migration by *Bordetella pertussis* lymphocytosis-promoting factor. Infect. Immun, 45:718–725.

Menozzi F. D., Gantiez C., Locht C. (1991). Interaction of the *Bordetella pertussis* filamentous hemagglutinin with heparin. FEMS Microbiol Lett, 62: 59–64.

Mertsola J., Van Der Meeren O., He Q., Linko-Parvinen A., Ramakrishnan G., Mannermaa L., Soila M., Pulkkinen M., Jacquet J.M. (2010). Decennial administration of a reduced antigen content diphtheria and tetanus toxoids and acellular pertussis vaccine in young adults. Clin Infect Dis, 51(6):656-62.

Meyer C. U., Zepp, F., Decker M., Lee M., Chang S. J., Ward J., Yoder S., Bogaert H., Edwards K. M. (2007). Cellular immunity in adolescents and adults following acellular pertussis vaccine administration. Clin Vaccine Immunol, 14(3): 288-92.

Mills K. H., Barnard A., Watkins J., Redhead K. (1993). Cell-mediated immunity to Bordetella pertussis: role of Th1 cells in bacterial clearance in a murine respiratory infection model. Infection and immunity, 61(2): 399-410.

Mills K. H., Ryan M., Ryan E., Mahon B. P. (1998). A murine model in which protection correlates with pertussis vaccine efficacy in children reveals complementary roles for humoral and cell-mediated immunity in protection against *Bordetella pertussis*. Infection and immunity, 66(2): 594-602.

Mills K. (2001). Immunity to *Bordetella pertussis*. Microbes and Infection, 3(8): 655-677.

Mink, C. M., O'Brien C. H., Wassilak S., Deforest A., Meade, B. D. (1994). Isotype and antigen specificity of pertussis agglutinins following whole-cell pertussis vaccination and infection with Bordetella pertussis. Infect Immun, 62(3): 1118-1120.

Mobberley-Schuman P. S., Connelly B., Weiss A. (2003). Phagocytosis of Bordetella pertussis incubated with convalescent serum. J. Infect. Dis, 187: 1646–1653.

Mooi F. R., van der Heide H. G., ter Avest A. R., Welinder K. G., Livey I., van der Zeijst B. A., and Gaastra W. (1987). Characterization of fimbrial subunits from *Bordetella* species. Microb Pathog, 2: 473–484.

Mooi F.R, van Oirschot H., Heuvelman K., van der Heide H.G., Gaastra W., Willems R.J. (1998). Polymorphism in the *Bordetella pertussis* virulence factors P.69/pertactin and pertussis toxin in The Netherlands: temporal trends and evidence for vaccine-driven evolution. Infect Immun, 66(2): 670-5.

Mooi F.R., Hallander H., Wirsing von König C.H., Hoet B., Guiso N. (2000). Epidemiological Typing of *Bordetella pertussis* Isolates: Recommendations for a Standard Methodology. Eur J Clin Microbiol Infect Dis, 19: 174–181.

Mooi, F. R., van Loo I. H., King A.J. (2001). Adaptation of *Bordetella pertussis* to vaccination: a cause for its reemergence?. Emerg Infect Dis 7(3 Suppl): 526-528.

Mooi F.R., He Q., Guiso N. (2007). Phylogeny, Evolution, and Epidemiology of Bordetellae (Locht C., Ed.). *Bordetella*: Molecular Microbiology (pp. 17-45). Norfolk, U.K.: Horizon Bioscience.

Mountzouros K.T., Kimura A., Cowell J.L. (1992). A bactericidal monoclonal antibody specific for the lipooligosaccharide of Bordetella pertussis reduces colonization of the respiratory tract of mice after aerosol infection with *B. pertussis*. Infect Immun, 60(12): 5316-8.

Music S. (2005). Towards universal childhood immunization, why we must and how we might. Vaccine, 23: 5474-5477.

Musser J.M., Bernis D.A., Ishikawa H., Selander R.K. (1987). Clonal diversity and host distribution in *Bordetella bronchiseptica*. J Bacteriol, 169: 2793-2803.

Musser J.M., Hewlett E.L., Peppler M.S., Selander R.K. (1986). Genetic diversity and relationships in populations of *Bordetella* spp. J Bacteriol, 166:230-237.

Müller F. M., Hoppe J. E., & Wirsing von König C. H. (1997). Laboratory diagnosis of pertussis: state of the art in 1997. Journal of clinical microbiology, 35(10): 2435-43.

Packard, E. R., R. Parton, Coote, J. G., Fry, N. K. (2004). Sequence variation and conservation in virulence-related genes of *Bordetella pertussis* isolates from the UK. J Med Microbiol, 53(Pt 5): 355-365.

Parkhill J., Sebaihia M., Preston A., Murphy L.D., Thomson N., Harris D.E., Holden M.T., Churcher C.M., Bentley S.D., Mungall K.L., Cerdeño-Tárraga A.M., Temple L., James K., Harris B., Quail M.A., Achtman M., Atkin R., Baker S., Basham D., Bason N., Cherevach I., Chillingworth T., Collins M., Cronin A., Davis P., Doggett J., Feltwell T., Goble A., Hamlin N., Hauser H., Holroyd S., Jagels K., Leather S., Moule S., Norberczak H., O'Neil S., Ormond D., Price C., Rabbinowitsch E., Rutter S., Sanders M., Saunders D., Seeger K., Sharp S., Simmonds M., Skelton J., Squares R., Squares S., Stevens K., Unwin L., Whitehead S., Barrell B.G., Maskell D.J. (2003). Comparative analysis of the genome sequences of *Bordetella pertussis*, *Bordetella parapertussis* and *Bordetella bronchiseptica*. Nat. Genet, 35(1): 32-40.

Peppler M. S. (1984). Two physically and serologically distinct lipopolysaccharide profiles in strains of *Bordetella pertussis* and their phenotype variants. Infect. Immun, 43:224–232.

Pillemer L., Burrell J.I., Ross O.A. (1947). Separation and Immunologic Evaluation of Soluble Pertussal Antigens. Science, 106(2741):36-7.

Pradel E., Guiso N., Menozzi F. D., Locht, C. (2000). Bordetella pertussis TonB, a Bvg-independent virulence determinant. Infect Immun, 68(4): 1919-1927.

Prasad S. M., Yin Y., Rodzinski E., Tuomanen E. I., Masure H. R. (1993). Identification of a carbohydrate recognition domain in filamentous hemagglutinin from *Bordetella pertussis*. Infect Immun, 61: 2780–2785.

Preston N.W. (1985). Essential immunogens in human pertussis, the role of fimbriae. Dev Biol Stand, 61:137-141.

Preston A., Allen A.G., Cadisch J., Thomas R., Stevens K., Churcher C.M., Badcock K.L., Parkhill J., Barrell B., Maskell D.J. (1999). Genetic basis for lipopolysaccharide O-antigen biosynthesis in Bordetellae. Infect Immun 67: 3763-3767.

Preston A., Petersen B.O., Duus J.O., Kubler-Kielb J., Ben-Menachem G., Li J., Vinogradov E. (2006). Complete structures of Bordetella bronchiseptica bordetella parapertussis lipopolysaccharides. J Biol Chem, 281: 18135-18144.

Preston A., Temple L., Sebaihia M., Parkhill J., Maskell D. J. (2007). Speciation in the genus *Bordetella* as deduced from comparative genome analyses. (Locht C., Ed.). *Bordetella*: Molecular Microbiology (pp. 1-15). Norfolk, U.K.: Horizon Bioscience.

Raetz C.R., Whitfield C. (2002). Lipopolysaccharide endotoxins. Annu Rev Biochem, 71: 635-700.

Redhead K., Watkins J., Barnard A., Mills K. H. (1993). Effective immunization against *Bordetella pertussis* respiratory infection in mice is dependent on induction of cell-mediated immunity. Infection and immunity, 61(8): 3190-8.

Relyveld E., Oato N. H., Guerin N., Coursaget P., Huet M., Gupta R. K. (1991). Determination of circulating antibodies directed to pertussis toxin and of agglutinogens in children vaccinated with either the whole cell or component pertussis vaccine in France, Japan and Senegal. Vaccine, 9(11): 843-850.

Reischl U., Lehn N., Sanden G.N., Loeffelholz M.J. (2001). Real-time PCR assay targeting IS481 of *Bordetella pertussis* and molecular basis for detecting *Bordetella holmesii*. J Clin Microbiol, 39: 1963-1966.

Reizenstein E., Hallander H. O., Blackwelder W. C., Kuhn I., Ljungman M., Mollby R. (1995). Comparison of five calculation modes for antibody ELISA procedures using pertussis serology as a model. J Immunol Methods, 183(2): 279-290.

Rennels M.B., Deloria M.A., Pichichero M.E., Losonsky G.A., Englund J.A., Meade B.D., Anderson E.L., Steinhoff M.C., Edwards K.M. (2000). Extensive swelling after booster doses of acellular pertussis-tetanus-diphtheria vaccines. Pediatrics, 105(1):e12.

Robinson A., Gorringe A. R., Funnell S. G., Fernandez M. (1989). Serospecific protection of mice against intranasal infection with *Bordetella pertussis*. Vaccine, 7(4): 321-324.

Roduit C., Bozzotti P., Mielcarek N., Lambert P. H., Del Giudice G., Locht C., Siegrist C. A. (2002). Immunogenicity and protective efficacy of neonatal vaccination against *Bordetella pertussis* in a murine model: evidence for early control of pertussis. Infection and Immunity, 70(7): 3521-3528.

Ryan, M., McCarthy L., Rappuoli R., Mahon B. P., Mills, K. H. (1998). Pertussis toxin potentiates Th1 and Th2 responses to co-injected antigen: adjuvant action is associated with enhanced regulatory cytokine production and expression of the co-stimulatory molecules B7-1, B7-2 and CD28. Int Immunol, 10(5): 651-662.

Sadosky, A. B., J. W. Wilson, H. M. Steinman, and A. H. Shuman. (1994). The iron superoxide dismutase of *Legionella pneumophila* is essential for viability. J. Bacteriol, 176:3790–3799.

Sato Y., Arai H., Suzuki, K. (1974). Leukocytosis-promoting factor of *Bordetella pertussis*. 3. Its identity with protective antigen. Infect Immun, 9(5): 801-810.

Sato, H., & Sato, Y. (1984). Bordetella pertussis infection in mice: correlation of specific antibodies against two antigens, pertussis toxin, and filamentous hemagglutinin with mouse protectivity in an intracerebral or aerosol challenge system. Infect Immun, 46(2): 415-421.

Saukkonen K., Cabellos C., Burroughs M., Prasad S., Tuomanen E. (1991). Integrin-mediated localization of *Bordetella pertussis* within macrophages: role in pulmonary colonization. J Exp Med, 173(5): 1143-1149.

Schellekens J., von Konig C.H., Gardner P. (2005). Pertussis sources of infection and routes of transmission in the vaccination era. Pediatr Infect Dis J, 24:S19-S24

Schmidt G., Goehring U. M., Schirmer J., Lerm M., Aktories K. (1999). Identification of the c-terminal part of Bordetella dermonecrotic toxin as a transglutaminase for rho GTPases. J Biol Chem, 274(45): 31875-31881.

Schmitt-Grohé S., Stehr K., Cherry J.D., Heininger U., Uberall M.A., Laussucq S., Eckhardt T. (1997). Minor adverse events in a comparative efficacy trial in Germany in infants receiving either the Lederle/Takeda acellular pertussis component DTP (DTaP) vaccine, the Lederle whole-cell component DTP (DTP) or DT vaccine. The Pertussis Vaccine Study Group. Dev Biol Stand, 89:113-8.

Shahin R. D., Brennan M. J., Li Z. M., Meade B. D., Manclark, C. R. (1990). Characterization of the protective capacity and immunogenicity of the 69-kD outer membrane protein of *Bordetella pertussis*. J Exp Med, 171(1): 63-73.

Shumilla J. A., Lacaille V., Hornell T. M., Huang J., Narasimhan S., Relman D. A., Mellins E. D. (2004). *Bordetella pertussis* infection of primary human monocytes alters HLA-DR expression. Infect Immun, 72(3): 1450-1462.

Skowronski D.M., DeSerres G., MacDonald D., Wu W., Shaw C., Macnabb J., Champagne S., Patrick D.M., Halperin S.A. (2002). The changing age and seasonal profile of pertussis in Canada. J Infect Dis, 185: 1448-1453.

Sørensen H. P., & Mortensen, K. K. (2005). Advanced genetic strategies for recombinant protein expression in *Escherichia coli*. Journal of biotechnology, 115(2): 113-28.

Stanbridge, T. N., Preston N. W. (1974). Experimental pertussis infection in the marmoset: type specificity of active immunity. J Hyg (Lond) 72(2): 213-228.

Stibitz S., Aaronson W., Monack D., Falkow S. (1989). Phase variation in *Bordetella pertussis* by frameshift mutation in a gene for a novel two-component system. Nature, 338(6212): 266-269.

Storsaeter J., Hallander H. O., Gustafsson L., and Olin P. (1998). Levels of anti-pertussis antibodies related to protection after household exposure to *Bordetella pertussis*. Vaccine, 16:1907–1916.

Storsaeter J., Wolter J., Locht C. (2007). Pertussis Vaccines (Locht C., Ed.). *Bordetella*: Molecular Microbiology (pp. 245-288). Norfolk, U.K.: Horizon Bioscience.

Sugai T., Mori M., Nakazawa M., Ichino M., Naruto T., Kobayashi N., Kobayashi Y., Minami M., Yokota S. (2005). A CpG-containing oligodeoxynucleotide as an efficient adjuvant counterbalancing the Th1/Th2 immune response in diphtheria-tetanus-pertussis vaccine. Vaccine, 23(46-47): 5450-6.

Tamura M., Nogimori K., Murai S., Yajima M., Ito K., Katada T., Ui M., Ishii S. (1982). Subunit structure of islet-activating protein, pertussis toxin, in conformity with the A-B model. Biochemistry, 21: 5516–5522.

Tanaka M., Vitek C. R., Pascual F.B., Bisgard K.M., Tate J.E., Murphy., T.V. (2003). Trends in pertussis among infants in the United States, 1980-1999. JAMA, 290: 2968-2975.

Taranger J., Trollfors B., Lagergård T., Sundh V., Bryla D.A., Schneerson R., Robbins J.B. (2000). Correlation between pertussis toxin IgG antibodies in postvaccination sera and subsequent protection against pertussis. J Infect Dis, 181(3):1010-3.

Tefon B. E., Maass S., Ozcengiz E., Becher D., Hecker M., Ozcengiz, G. (2011). A comprehensive analysis of Bordetella pertussis surface proteome and identification of new immunogenic proteins. Vaccine, 29(19): 3583-95.

Tiwari T., Murphy T. V., Moran J. (2005). Recommended antimicrobial agents for the treatment and postexposure prohylaxis of pertussis, 2005 CDC Guidelines. MMWR Recomm Rep, 54: 1-16.

Tondella M. L., Carlone G. M., Messonnier N., Quinn C. P., Meade B. D., Burns D. L., Cherry J. D. (2009). International *Bordetella pertussis* assay standardization and harmonization meeting report. Centers for Disease Control and Prevention, Atlanta, Georgia, United States, 19-20 July 2007. Vaccine, 27(6): 803-14.

Torvaldsen S., McIntyre P.B. (2003). Effect of the preschool pertussis booster on national notifications of disease in Australia. Pediatr Infect Dis J, 22:956–959.

Tsang, R. S., A. K. Lau, Sill, M. L. Halperin S. A., Van Caeseele P., Jamieson F., Martin I. E. (2004). Polymorphisms of the fimbria fim3 gene of *Bordetella pertussis* strains isolated in Canada. J Clin Microbiol 42(11): 5364-5367.

Uhl M. A., Miller J. F. (1994). Autophosphorylation and phosphotransfer in the *Bordetella pertussis* BvgAS signal transduction cascade. Proc Natl Acad Sci USA, 91:1163–1167.

Uhl M. A., Miller J. F. (1996). Integration of multiple domains in a two-component sensor protein: the *Bordetella pertussis* BvgAS phospho- relay. EMBO J, 15: 1028–1036.

van Amersfoorth S. C., Schouls L. M., van der Heide, H. G., Advani A., Hallander H. O., Bondeson K., von Konig C. H., Riffelmann M., Vahrenholz C., Guiso N., Caro V., Njamkepo E., He Q., Mertsola J., Mooi F. R. (2005). Analysis of *Bordetella pertussis* populations in European countries with different vaccination policies. J Clin Microbiol, 43(6): 2837-2843.

van den Akker W. M. (1998). Lipopolysaccharide expression within the genus *Bordetella*: influence of temperature and phase variation. Microbiology, 144: 1527–1535.

van den Berg B. M., David S., Beekhuizen H., Mooi F. R., van Furth R. (2000). Protection and humoral immune responses against *Bordetella pertussis* infection in mice immunized with acellular or cellular pertussis immunogens. Vaccine, 19(9-10): 1118-28.

van der Zee A., Mooi F., Van Embden J., Musser J. (1997). Molecular evolution and host adaptation of *Bordetella* spp., phylogenetic analysis using multilocus enzyme electrophoresis and typing with three insertion sequences. J Bacteriol, 179: 6609-6617.

van Loo I. H., van der Heide G. J., Nagelkerke J. D., Verhoef J., Mooi F. R. (1999). Temporal trends in the population structure of *Bordetella pertussis* during 1949–1996 in a highly vaccinated population. J Infect Dis, 179(4): 915-923.

van Loo I.H, Mooi F.R. (2002). Changes in the dutch *Bordetella pertussis* population in the first 20 years after the introduction of whole-cell vaccines. Microbiology. 2002 148(7): 2011-8.

Villagran M. E., Marin C., Rodriguez-Gonzalez I., De Diego J. A., & Sanchez-Moreno M. (2005). Use of an iron superoxide dismutase excreted by *Trypanosoma cruzi* in the diagnosis of Chagas disease: seroprevalence in rural zones of the state of Queretaro, Mexico. [Research Support, Non-U.S. Gov't]. Am J Trop Med Hyg, 73(3): 510-516.

Villaverde, A., & Carrió, M. M. (2003). Protein aggregation in recombinant bacteria: biological role of inclusion bodies. Biotechnology letters, 25(17): 1385-95.

von Konig C.H., Halperin S., Riffelman M., Guiso N. (2002). Pertussis of Adults and Infants. Lancet Infect Dis, 2: 744-750

Ward J.I., Cherry J.D., Chang S.J., Partridge S., Lee H., Treanor J., Greenberg D.P., Keitel W., Barenkamp S., Bernstein D.I., Edelman R., Edwards K. (2005). Efficacy of an acellular pertussis vaccine among adolescents and adults. New Eng J Med, 353: 1555-1563.

Watanabe M., Takimoto H., Kumazawa Y., Amano K. (1990). Biological properties of lipopolysaccharides from *Bordetella* species. J. Gen. Microbiol, 136:489–493.

Watanabe M. and Nagai M. (2003). Role of systemic and mucosal immune responses in reciprocal protection against *Bordetella pertussis* and *Bordetella parapertussis* in a murine model of respiratory infection. Infect. Immun. 71: 733–738.

Weber C., Boursaux-Eude C., Coralie G., Caro V., Guiso N. (2001). Polymorphism of *Bordetella pertussis* isolates circulating for the last 10 years in France, where a single effective whole-cell vaccine has been used for more than 30 years. J Clin Microbiol, 39(12): 4396-403.

Weihl C., Riley H. D., Lapin J. H. (1963). Extracted pertussis antigen. A clinical appraisal. Am J Dis Child, 106: 210-215.

Weingart C. L., Keitel W. A., Edwards K. M., Weiss A.A. (2000). Characterization of bactericidal immune responses following vaccination with acellular pertussis vaccines in adults. Infection and immunity, 68(12):7175-9.

Weiss A. A., Johnson F. D., Burns D. L. (1993). Molecular characterization of an operon required for pertussis toxin secretion. Proc. Natl. Acad. Sci. USA, 90: 2970–2974.

Weiss A.A., Mobberley P.S., Fernandez R.C., Mink C.M. (1999). Characterization of human bactericidal antibodies to *Bordetella pertussis*. Infect Immun, 67(3):1424-31.

Weiss, A. A., Patton A. K., Millen S. H., Chang S. J., Ward J. I., Bernstein D. I. (2004). Acellular pertussis vaccines and complement killing of *Bordetella pertussis*. Infection and immunity, 72(12): 7346-7351.

Wendelboe A. M., Van Rie A., Salmaso S., Englund J. A. (2005). Duration of immunity against pertussis after natural infection or vaccination. Pediatr Infect Dis J 24(5 Suppl): S58-61.

Willems R. J., van der Heide H. G., Mooi F. R. (1992). Characterization of a *Bordetella pertussis* fimbrial gene cluster which is located directly downstream of the filamentous haemagglutinin gene. Mol Microbiol, 6(18): 2661-71.

Wilson R., Read R., Thomas M., Rutman A., Harrison K., Lund V., Cookson B., Goldman W., Lambert H., Cole P. (1991). Effects of *Bordetella pertussis* infection on human respiratory epithelium in vivo and in vitro. Infect. Immun, 59:337–345.

Wolff J., Cook G. H., Goldhammer A. R., and Berkowitz S. A. (1980). Calmodulin activates prokaryotic adenylate cyclase. Proc. Natl. Acad. Sci. USA, 77:3841–3844.

Wood N. and McIntyre P. (2008). Pertussis: review of epidemiology, diagnosis, management and prevention. Paediatric Respiratory Reviews, 9(3): 201-212.

Woods C. R., Versalovic J., Koeuth T., Lupski J. R. (1993). Whole-Cell Repetitive Element Sequence-Based Polymerase Chain Reaction Allows Rapid Assessment of Clonal Relationships of Bacterial Isolates. Clin Microbiol, 31(7): 1927-1931.

Woolfrey B.F. and Moody J.A. (1991). Human infections associated with *Bordetella bronchiseptica*. Clin Microbiol Rev, 4: 243-255.

World Health Organization. (2005). Pertussis vaccines. WHO position paper. W.E.R. 80: 31-93.

Wu C. H. H., J. J. Tsai-Wu Y. T. Huang C. Y. Lin G. G., Lioua F. J. Lee. (1998). Identification and subcellular localization of a novel Cu,Zn superoxide dismutase of *Mycobacterium tuberculosis*. FEBS Lett, 439:192–196.

Xing D. K., Das R. G., Williams L., Canthaboo C., Tremmil J., Corbel M. J. (1999). An aerosol challenge model of *Bordetella pertussis* infection as a potential bioassay for acellular pertussis vaccines. Vaccine, 17(6): 565-76.

Zepp F., Knuf M., Habermehl P., Mannhardt-Laakmann W., Howe B., Friedland L.R. (2006). Safety of reduced-antigen-content tetanus-diphtheria-acellular pertussis vaccine in adolescents as a sixth consecutive dose of acellular pertussis-containing vaccine. J Pediatr, 149(5):603-610.

Zepp F., Heininger U., Mertsola J., Bernatowska E., Guiso N., Roord J., Tozzi A. E., Van Damme P. (2011). Rationale for pertussis booster vaccination throughout life in Europe. The Lancet infectious diseases, 11(7): 557-570.

Zhang J. M., Cowell J. L., Steven A. C., Manclark, C. R. (1985). Purification of serotype 2 fimbriae of Bordetella pertussis and their identification as a mouse protective antigen. Dev Biol Stand, 61: 173-185.

Zhang X, Goebel EM, Rodríguez ME, Preston A, Harvill ET. (2009). Infect Immun, 77(11): 5050-5058.

APPENDIX A

CONTENTS 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

CONTENTS OF BUFFERS AND SOLUTIONS

Agarose Gel (1%)

Agarose	0.5 g
TAE buffer (1X)	50 ml

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

STE Buffer

10,3% Sucrose

25 mM EDTA pH:8.0

25 mM Tris-HCl pH:8.0

2mg/ml Lyzozyme

TBS (1X) (1000ml)

Tris	2.42 g
NaCl	29,2 g

T-Cell Buffer

5% FBS (Fecal Bovine Serum) in washing solution

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

Blocking Solution

5% BSA in washing solution

Dialysis Buffer (pH:8.0)

50 mM NaH_2PO4

500 mM NaCl

4 M Urea

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

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

X-Gal	20 mg
Dimethylformamide	1 ml

- Stored at -20° C and protected from light

IPTG (Isopropyl-β-D-thiogalactoside)

IPTG	100 mg
Distilled water	1 ml

- Filtered and stored at at -20° C

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
Chloroform	Merck
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
Molecular Biology Grade Water	AppliChem
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
*Xho*I	MBI Fermentas
Lysozyme	AppliChem
T4 DNA Ligase	MBI Fermentas
Taq DNA Polymerase	MBI Fermentas