ANALYSIS OF CROSS-IMMUNE REACTION BETWEEN STRAINS OF *BORDETELLA PERTUSSIS*

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ABSTRACT

ANALYSIS OF CROSS-IMMUNE REACTION BETWEEN STRAINS OF *BORDETELLA PERTUSSİS*

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Bordetella pertussis is the causative agent of whooping cough which is a worldwide acute respiratory disease that predominantly involves infants. Whooping cough is one of the ten most common causes of death from infectious diseases worldwide. The increased coverage of the primary pertussis vaccination (DaBT-IPA-Hib) decreased the incidence of disease in Turkey dramatically. However, in spite of the incidence decline, the circulation of *B. pertussis* has not yet been eliminated, and a change in the clinical spectrum and age-related incidence of the disease has been observed. On the other hand, in view of the moderate changes that have been observed in the genomic sequences of certain virulance factors over time, there are concerns about the gradual loss of the efficacy of the current pertussis vaccines as a result of antigenic drift and continuous selection of the least vaccine-sensitive clones.

Proteomics deals with whole protein content (proteome) of cells as a function of space and time. Gel-based approach in proteomics involves two dimensional gel electrophoresis (2-DE) followed by peptide mass fingerprinting (PMF) employing matrix-assisted laser desorption/ionization (MALDI)-mass spectrometry (MS). Immunoproteomics which is a combination of gel based proteomics and Western blot analysis determines tumor-specific antigens as well as immunoreactive proteins of pathogens by combining proteomics with Western blot technique. Although immunoproteomics is a rather new research tool, it has been quite effective to determine the virulence factors of various pathogenic microorganisms.

The present study aims at comparing immunoproteome of the standard *B. pertussis* strain "Tahoma I" with those of two other strains, namely "Saadet" and "Nursel", which are the local isolates that have been preferred as the vaccine strains for many years in our country for their ability to provide a better protection. Of a total of 38 immunogenic proteins identified, 14 were shown to be the novel antigens for *B. pertussis*. Among 14 proteins, one was detected as immunogenic in only Tohama I strain where two proteins were specific for Nursel strain. Among the strains compared, Saadet strain had the highest antigenic variety, than the others.

Key words: Immunoproteome, Comparative immunoproteome*, Bordetella pertussis*, Vaccine candidates.

ÖZ

BORDETELLA PERTUSSIS SUŞLARINDA ÇAPRAZ İMMUNOPROTEOM ANALİZLERİ

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Etkeni *Bordetella pertussis* olan boğmaca, tüm dünyada görülen akut bir solunum yolu enfeksiyonudur. Hastalık en çok yeni doğanlarda görülür ve ölüme neden olabilir. Boğmaca, hala dünya çapında en çok ölüme sebebiyet veren 10 bulaşıcı hastalıktan biri olarak kabul edilmektedir. Türkiye'de uygulanan boğmaca aşısı (DaBT-IPA-Hib), hastalığın görülme sıklığını büyük ölçüde düşürmüştür. Fakat *B. pertussis* bakterisinin populasyondaki dolaşımı ortadan kaldırılamamış, hastalığın klinik spektrumunda yaşa bağlı hastalık görülme sıklığında değişiklikler saptanmıştır. Bunun yanısıra, bakterinin virülans faktörlerini kodlayan genlerde zamanla orta dereceli değişiklikler olduğunu ve aşıya dirençli klonların seçilmekte olduğunu gösteren çeşitli araştırma raporları yayınlanmaktadır. Bu durum, kullanılan aşının zamanla etkinliğini yitireceği endişesini uyandırmaktadır.

Proteomik, hücrelerin belirli bir zamanda ve mekanda içerdiği tüm proteinlerin (proteomunun) bir arada incelenmesini sağlayan bir teknolojidir. Proteomiğin

“jeli temel alan” yaklaşımında, proteomic analizler, iki boyutlu jel elektroforezini (2-DE) takiben “matrix-assisted laser desorption/ionization” (MALDI)-kütle spektrometrisi (MS) ile peptid kütle parmak izlerinin (PMF) elde edilmesini içerir. Bu yaklaşımın Western blot analizleriyle kombinasyonunu içeren immunoproteomik ise kanser araştırmalarında tümöre özgül antijenleri, enfeksiyon hastalıklarına ilişkin araştırmalarda ise patojenik bakterilerin immünojenik proteinlerini belirlemede etkin bir şekilde kullanılan nispeten yeni bir araştırma alanıdır.

Bu çalışmada B. pertussis’ in standard suşu olan “Tahoma I” suşuna ait immunoproteom, yerel birer izolat olan ve ülkemizde uzun yıllar aşı suşu olarak tercih edilmiş “Saadet” ve “Nursel” isimli suşlardan elde edilen immunoproteomlarla karşılaştırılmıştır. Tespit edilen toplam 38 imünojenik protein arasından 14 tanesi *B. pertussis* suşları için ilk kez tanımlanmıştır. Bu proteinlerden biri sadece Tohama I suşuna özgüyken, iki adeti sadece Nursel suşunda gösterilmiştir. Karşılaştırılan suşlar arasında Saadet suşunun en yüksek antijenik çeşitliliğe sahip olduğu bulunmuştur.

Anahtar Kelimeler: İmmünoproteom, Karşılaştırmalı immünoproteomik, *Bordetella pertussis*, Aşı adayları

To my Family,

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CHAPTER 1

INTRODUCTION

1.1. Proteome and Proteomics

"Proteins expressed by the genome of an organism is called proteome" as defined by Pennington *et al*. (1997). Formerly DNA sequencing had been considered as a sole tool for building and understanding of an organism's molecular processes. But nowadays, sequence data cannot be used alone, because of the expression of several thousand different proteins and increasing the number of these proteins with vigorous posttranslational modifications. Besides DNA sequencing cannot provide the information for stability, location, variety and amount of these proteins in cellular and subcellular level (Pennington *et al*., 1997). This type of information can only be provided by proteomics.

A proteome study can be separated into 2 stages. First is "constitutive proteome"; which involves determining and mapping of all the proteins expressed by a particular organism under their optimal conditions. Protein databases and 2-DE reference maps have been developed and easily reachable through World Wide Web. In second stage of the proteome research, changes in posttranslational modifications and subcellular localization of the proteins, or quantitative up or down regulations of the protein expression are investigated under different physiological conditions (Pennington *et al*., 1997). Though second phase is very important in some studies, the design of the experiment and steps that should be taken is

directly dependent on the research subject (Holzmuller *et al.*, 2009). Proteomics area is branching out everyday with the new developing technologies, which holds the promise of new and more precise methods. Some of these technologies including two-dimensional gel electrophoresis (2-D), have historically been the most widely used tools in of proteomic analyse, as well as some newer approaches such as liquid phase separations combined with mass spectrometry, and protein microarrays. It is evident that each method has its own strengths and weaknesses and no single method will be optimal in all applications. However, the continuing development of innovative strategies for protein separation and analysis is providing a wealth of new tools for multi-dimensional protein profiling (Steel at al. 2005).

1.2. Immunoproteomics

Need for immunoproteomics starts from coevolution of mammalians and pathogenic bacteria. While pathogens evolve, mammalian immune system had become a guard against bacterial cell components, viral nucleic acid or uncontrolled malignant cell growth (Matzinger *et al.*, 1994). Immune system of the mammals requires a mechanism that distinguishes infected or malignant cells from the healthy ones in order to protect the organism. Early studies characterized this phenomena as the self-nonself paradigm, and distinction between these two states was broadly defined as the function of the immune system. Infections and transformation of a cell into malignant form have been informed to immune system by peptides, which burdens them a crucial role in immune responses. The tools of proteomics, in particular mass spectrometry, are ideally suited to study these immune responses at a molecular level (Purcell *et al.* 2004).

The classical proteomic workhorse is 2-D PAGE, which was first described by O'Farrel in 1975. This technique can be used to separate complex mixtures

of proteins. Up to thousands of proteins can be resolved in one single experiment. In fact, this high resolution was the starting point of looking at biological systems in a proteome-wide manner. By combining 2-D PAGE with immunoblotting and MS analyses, antigenic proteins (immunoproteome) can be isolated and identified. This approach is also known as serological proteome analysis; shortly "SERPA" (Klade *et al*., 2001; Tjalsma *et al*., 2008).

SERPA is a novel technique combining proteome and Western blot analyses. It was regarded as a powerful tool to screen and identify autoantigens or autoantibodies. The method was first applied in carcinoma studies for the identification of associated biomarkers of carcinoma, including hepatocellular carcinoma, lung cancer, renal cell carcinoma, breast cancer, prostate cancer. Recently, it has been widely used in exploitation of bacteria and identification of disease associated proteins (Liu et al. 2009).

Immunoproteomics is thus the combination of any proteomic technology with an immunological data presentation. Its development is vital in these days where it is increasingly becoming urgent to identify disease biomarkers and pathogenic targets for the development of new drugs and vaccines (Steel *et al*., 2005). It has been used for many different pathogenic bacteria, such as *Helicobacter pylori* (Backert *et al*., 2005, Nilsson *et al*., 2006), *Staphylococcus aureus* (Vytvytska *et al*., 2002), *Bacillus anthracis* (Ariel *et al*., 2002, DelVecchio *et al*., 2006), *Shigella flexneri* (Peng *et al*., 2004), *Francisella tularensis* (Havlosava *et al*., 2002), *Corynebacterium diphtheriae* (Hansmeier *et al*., 2006), *Streptococcus pyogenes* (Rodriguez-Ortega *et al*., 2006), *Chlamydia pneumoniae* (Bunk *et al*., 2008), *Haemophilus influenzae* (Langen *et al*., 2000, Thoren *et al*., 2002) and *Neisseria meningitidis* (LinksHsu *et al*., 2008) to gather information about the immunogenic components.

1.3. Bacterial Pathogens and Comparative Immunoproteomics

Recently, the most important driving force for researchers to study bacterial pathogens is to understand host-pathogen relations, to develop new approaches for their diagnostics and treatments, and to improve vaccines against them. Their research focuses not only in the newly emerging infectious diseases, but also on reemerged old ones. The availability of proteomics and immunoproteomics provides an unprecedented opportunity on these issues (Drake *et al*. 2005).

Comparative immunoprotemics has constitutively used as an identification tool for commonly shared and excluded immunoreactive protein expression of the pathogenic agents. Findings in the literature have revealed coordinately regulated gene expression (Hess *et al*. 2005, Sudheesh *et al*. 2002), novel virulence antigens for vaccines (Altındiş, *et al*. 2009), the difference between the virulence of the members of the same species (Shin *et al*. 2006) and regulation of diseases with antigens (Haas *et al*. 2002).

Comparative immunoproteomics has been used for different pathogenic bacteria, such as *Lactococcus garvieae* (Shin *et al*., 2006), *Helicobacter pylori* (Mini *et al*., 2006) and *Pseudomonas aeruginosa* (Pedersan *et al*., 2005) to gather information about the immunogenic components, identifications of clinical biomarkers for monitoring diseases.

1.4. Steps in Immunoproteome Work

1.4.1. Isoelectric Focusing on Immobilized pH Gradient

Isoelectric focusing (IEF) ensures the partition of the proteins according to their isoelectric points (p*I*), by electrostatic field (O' Farrell, 1975). Isoelectric focusing is performed in a pH gradient. Proteins are amphoteric molecules with their acidic and basic groups that can be affected from the pH environment and become protonated or deprotonated. Net charge of a protein can be calculated from the charges of R groups (amino acid side chains) and amino- and carboxyl-termini. Isoelectric point (p*I*) is the specific pH where the net charge of a protein is zero. If pH value is below protein's p*I,* it will be charged positively, but if the value increases above the p*I* of the protein, then the protein's charge will be negative.

The presence of pH gradient and electric field are essential for an IEF technique. Electrode solutions are applied between the gel and electrodes; acidic proteins with positive net charges will migrate toward the cathode, becoming progressively less positively charged as it moves through the pH gradient until it reaches its p*I*. On the contrary, basic proteins with negative net charges will migrate toward anode, becoming less negatively charged as it moves through the pH gradient until it reaches a zero net charge. In a case of a protein diffuses away from its p*I*, it suddenly gains or looses charge and moves back to its p*I* position. This is called "focusing" effect of IEF, which concentrates proteins at their p*I*s.

Proteins in the mixture should be in the same condition with respect to their configuration, aggregation and intramolecular interaction manner. That is the reason for performing IEF under denaturing conditions. Complete

solubilization and denaturation is achieved by using a mixture of detergent, urea and ampholites.

In practice, there are two ways to establish a pH gradient in a gel;

- the carrier ampholytes are used to form a pH gradient under electrical field
- immobilized pH gradients establishes the texture with the buffering groups which are part of the medium.

Regarding the first entry, carrier ampholytes should posses some properties that are essential for the process, such as high buffering capacity, solubility, good and regular electric conductivity at the p*I*. Ampholytes should lack a biological effect and should be of low molecular weight. Carrier ampholyte-based technique has been used in a number of 2-DE. However, there are several limitations such as reproducibility of the first dimension, as most commonly seen (Westermeier and Naven, 2002) and the difficulty with in handling soft polyacrylamide gel rods.

Immobilized pH gradients (IPG) have overcome the above-mentioned difficulties (Bjellqvist *et al.,* 1982). Reproducible and more reliable data could be obtained with the availability of commercial products, pre-manufactured gel strips and dedicated IPG instruments. Higher protein loads could be achieved and film-supported gel strips become easy to handle.

After the first dimension is completed by either carrier ampholyte-generated system or automated IPG systems, a second dimension on separation is achieved according to protein size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

1.4.2. Visualization of Protein Spots and Image Analysis

One of the important part of the proteome work is to visualize the spots that have been separated by 2-DE. There are various visualization methods and stains varying in sensitivity, linearity and dynamic range. The highest sensitivity is achieved with radioactivity/fluorography detection. Non-radioactive methods include Silver staining and Coomassie Brilliant Blue staining. Ammoniacal silver staining is a very sensitive protein staining method, which can detect proteins at or below nanogram quantities (Hoving *et al.,* 2000).

Following staining, evaluation and comparison of the complex 2-DE patterns seems to be very difficult and nearly impossible. Therefore, the gel images have to be converted into digital data with a scanner, camera or more sophisticated gel image documentation systems. This high resolution image is then evaluated with the aid of the image analysis software (e.g. PDQuest, Biorad; Delta2D, Decodon; Phoretix, Nonlinear; Melanie, GeneBio).

1.4.3. Immunoblotting (Western Blotting)

Blotting is the transfer of macromolecules into immobilizing membranes for specific and sensitive detection using monoclonal or polyclonal antibodies. The electrophoretic transfer of electrophoretically separated proteins into a membrane with sebsequent immunodetection was introduced by Towbin et al. (1979). Proteins are too big to elute quantitatively from the gel by diffusion or capillar forces, therefore they are transferred electrophoretically. There are different blotting protocols such as dot blot and Western blot. One of the most powerful immunoblotting tecniques is Western blotting. In immunoblotting, prior to protein immobilization on the polyvinylidene fluoride (PVDF) or nitrocellulose membranes (NC), sample

proteins are separated using SDS-PAGE providing information about molecular weight and the potential existence of different isoforms of the proteins under study. PVDF membranes have a higher binding capacity for proteins than NC membrane, but NC binds to small proteins.

After the protein components are sufficiently separated by 2-D PAGE, they can be transferred to a PVDF or NC membrane. The reason for the usage of SDS-PAGE is because all proteins have been solubilized and migrated in the same direction, and the epitopes are easier accessible due to the denaturating effect of SDS. The electric current is applied at 90 degrees to the gel for the transfer process and the proteins migrate out of the gel onto the membrane. There are several ways to perform electrophoretic transfer; like tank blotting, semidry blotting and semiwet blotting. Once the proteins are separated and bound to the membrane support, Western blotting can begin. Western blotting can be used to detect target antigens in a pool of proteins by using a polyclonal or monoclonal antibody specific to those proteins (Towbin *et al.*, 2001)

Membrane is blocked by bovine serum albumin (BSA), non-fat dry milk (NFDM) or skim milk in order to reduce non-specific protein interactions between the membrane and the antibody. The primary antibody is specific for the protein of interest and at appropriate concentrations, should not bind any of the other proteins on the membrane. After rinsing the membrane to remove unbound primary antibody, a secondary antibody is introduced to the membrane which binds to the primary antibody. This secondary antibody is typically contains radioactive label or is linked to an enzyme that allows for visual identification by producing fluorescence. The enzyme substrate is incubated with the membrane so that the positions of membrane-bound secondary antibodies will emit light. Bands or spots, if 2-D gel is used,

corresponding to the detected protein of interest will appear as dark regions on the developed film.

1.4.4. Mass Spectrometry Analysis

In proteome analysis a number of approaches were developed for addressing protein identification, characterization and quantification. Methods of protein identification have included peptide sequencing, amino acid composition determination and more recently the use of mass spectrometry, which applies to molecular mass identification and sequencing (Celis *et al.,* 2000). Edman sequencing was the principal method of protein identification, and was a start to expand proteomics area into the dominating field we know today. Although Edman sequencing was used with considerable success for routine protein identification, the method was relatively slow and insensitive (Pappin *et al*., 1996). The evolution of ionization technologies sources and employment of MS by researchers has impacted sensitivity significantly and mass analyzer development continues to extent the boundaries of mass accuracy, resolution and functionality (Westermeier *et al*., 2008).

Mass spectrometry-based proteomics is a sensitive analytical tool for investigating pathogens in a broad proteome characterization, protein identification and quantification manner. (Mottaz and Brewer, 2008). The structural information such as peptide masses and amino acid sequences of a protein can be obtained by MS. Hence, identification of the protein can be accomplished using these data, by searching nucleotide and protein databases. Identification of proteins by mass spectrometry requires going through three major steps: (i) sample preparation, (ii) sample ionization and (iii) mass analysis.

Protein extraction from a gel is a difficult and inefficient way of sample preparation. Therefore *in situ* digestion, which is a method including digest of proteins using proteases (e.g. with trypsin) "in gel", is more efficient at sample recovery than other common methods (Andersen and Mann, 2000). Furthermore, more information can be obtained from the conversion of a protein into fragments, than from a whole protein itself.

There are two main soft ionization technologies available, namely Matrix-assisted Laser Desorption/Ionization (MALDI) (Karas and Hillenkamp, 1988) which is a pulsed ionization technique utilizing energy from a laser in the presence of light absorbing matrix, and Electrospray Ionization (ESI) (Fenn *et al.*, 1989) in which the ionizer can be used with large molecules with high sensitivity. In both methods, peptides are converted into ions by the addition or loss of one or more protons. Both methods allow the formation of ions without major loss of sample integrity. This is important since mass information about proteins and peptides is obtained in their native state.

Mass analyzers in a mass spectrometer converts the protein or peptides as sample molecules into gas phase molecular ions, providing separation. Resolution of the molecular ions on the basis of their mass-charge ratio (*m/z*) in a vacuum is established by mass spectrometer. A time-of-flight (TOF) is one of the simplest mass analyzer, although there are other types of techniques available, such as quadrupole, ion trap etc. TOF measures the *m/z* (mass to charge) ratio of charged ions based upon their time required for it to transverse the length of a flight tube, in an electric or magnetic field. From the MS scan, software must be used to obtain amino acid sequence of the peptide after eliminating the backgrounds. The information obtained is then used to search DNA and protein databases (SWISS-PROT, FASTA; MOWSE, ProFound, PepFrag, PepSea etc.) which are available in the World Wide Web (Table 1).

Table 1. 2-D gel databases and other databases that can be used in proteome analysis. (Pennigton *et al*. 1997, http://www.expasy.ch/ch2d/2d-index.html)

Database	Description	Address
2-D gel databases		
Biobase	Keratinocytes, bladder cancer, etc. (Danish Centre for Genome Research)	http://biobase.dk/cgi-bin/celis
EC02DBASE	*E. coli* (in NCBI repository)	ftp://ncbi.nlm.nih.gov/repository/ECOZDBASE
Heart-ZDPACE	Human myocardial(Berlin, Germany)	http://www.chemie.fu-berlin.de/user/pleiss/
HSC-2DPAGE	Human heart(Heart Science Centre, Harefield, UK)	http://www.harefield.nthameames.nhs.uk/nhli/protein/index.html.
LSB	Human, mouse and rat liver, etc. (Large Scale Biology, USA)	http://lsbc.com/2dmaps/patterns.html
QUEST	Ref52 cells, mouse embryo, yeast (Cold Spring Harbor Lab., USA)	http://siva.cshl.org/
SWISS-2DPAGE	16 human maps including: liver, plasma, lymphocytes. Also, *Mus musculus*, *E. coli* (University Hospital, Geneva, Switzerland)	http://expasy.hcuge.ch/ch2d/ch2d-top.html
SIENA-2DPAGE	Breast ductal carcinoma and histologically normal tissue, Amniotic fluid. Also,*Chlamydia trachomatis*, *Caenorhabditis elegans* (University of Siena, Italy)	http://www.bio-mol.unisi.it/2d/2d.html
Yeast	*S. cerevisiae*, *S. pombe*, etc. (Coteborg University, Sweden)	http://yeast-2dpage.gmm.g-u.se
Yeast	*S. cerevisiae* (Proteome Inc., USA)	http://ww.proteome.com/YPDhome.html
GelBank	*Bordetella spp*.	http://gelbank.anl.gov
Protein sequence databases		
PDD	Protein Disease Database	http://www-pdd.ncifcrf.gov/
PIR	Protein Identification Resource - protein sequences	http://www.gdb.org/Dan/proteins/pir.html
ProDom	Protein Domains and families	http://ww.sanger.ac.uk/-esr/prodom.html
PROSITE	Dictionary of Protein Sites and patterns	http://expasy.hcuge.ch/sprot/prosite.html
SWISS-PROT	SWISS-PROT annotated protein sequences	http://expasy.hcuge.ch/sprot/sprot-top.html
YPD	Yeast Protein Database	http://www.proteome.com
3-D Protein Structure Databases		
PDB	Brookhaven Protein Data Bank	http://wiw.pdb.bnl.gov/
SCOP	Structural Classification Of Proteins	http://scop.nuc-lmbcam.ac.uk/scop
SWISS-3DIMACE	3-D images of proteins (and other molecules)	http://expasy.hcuge.ch/sw3d/sw3d-top.ht-ml

1.5. The Genus *Bordetella*

The genus *Bordetella* encompasses a group of Gram-negative, small coccobacilli, which are obligate aerobes and fail to ferment carbohydrates. This genus currently contains nine species. Pathogenic members of this group have adapted to live in close association with mammalians. Three of the nine species are well known human pathogens, *B. pertussis*, *B. parapertussis* and *B. bronchiseptica*, respectively, colonizing in the respiratory tract, noninvasively (Gerlach *et al.*, 2001, Weiss, 2006, Mattoo *et al.*, 2005). *B. pertussis* and *B. parapertussis* are causative agents of whooping cough (pertussis) in humans. Whooping cough caused by *B. pertussis* is predominantly involves infants with characteristic violent, spasmodic and recurrent cough. *B. parapertussis*, which has also been isolated from sheep causes a milder whooping caugh-like disease in humans (Van der Zee *et al.*, 1996, Mattoo *et al.*, 2005). *B. bronchiseptica* is not a typical human pathogen, which seldomly causes cough illnesses in humans; in particular immunocompromised patients (Wolfe *et al.*, 2005). *B. hinzii,* affects poultry and laboratory mice by infecting and colonizing in respiratory tract (Cookson *et al.*, 1994, Hayashimoto *et al.*, 2008). *B. hinzii* also affect immunocompromised patients causing fetal septicemia. If immunorepression is occuring because of cystic fibrosis, bacteraemia and respiratory tract infection may be seen (Kattar *et al.*, 2000, Gadea *et al.*, 2000). *B. holmesii* causes septisemia in the young adults and is occasionally found in sputum (Weyant *et al.*, 1995). *B. trematum* cause ear and wound infections in human (Vandamme *et al.*, 1996). *B. avium* has never been found in humans and generally causes disease in birds, particularly coryza or rhinotracheitis in poultry. *B. petrii* is one of the recent identified species which can grow in anaerobic conditions. In this respect, it is unique for this genus and isolated from it's natural environment (von Wintzingerode *et al.*, 2005). *B. ansorpii* is

the most recently discovered pathogen in the genus *Bordetella* (Ko *et al.*, 2005).

The genomes of the ancient species of the *Bordetella* genus; *B. pertussis*, *B. parapertussis* and B. *bronchiseptica* have been sequenced in the initial *Bordetella* genome project (Preston *et al.*, 2004). The genomes differ considerably in size; the genome of *B. bronchiseptica* and *B. parapertussis* being bigger than that of *B. pertussis* with approximately more than 4400 kilobase pairs (kbp) (Table 2).The discrepancy between the number of functional genes in each genome is likely to be greater than this because *B. pertussis* and *B. parapertussis* contains large numbers of pseudogenes, whereas *B. bronchiseptica* containes relatively few. Moreover the genes lost by these two species are likely to be those involved in survival in the environment or infection of different host and thus rendered obsolete by their restriction to a single niche, the human respiratory tract. Evolutionary tree of *Bordetella* species is shown in Figure 1 (Preston *et al.*, 2007, Bjornstad *et al.*, 2005).

Table 2. General overview of *B.pertussis, B. parapertussis* and *B. brochiseptica* genomes (Preston *et al.*, 2004).

	B. pertussis	***B.parapertussis***	***B. bronchiseptica***
Genome Size (bp)	**4.0861.886**	**4.773.551**	**5.338.400**
Number of Genes	**3.816**	**4.404**	**5.007**
Pseudogenes (% of total genes)	**358 (9.4%)**	**220 (5%)**	**18 (0,4%)**

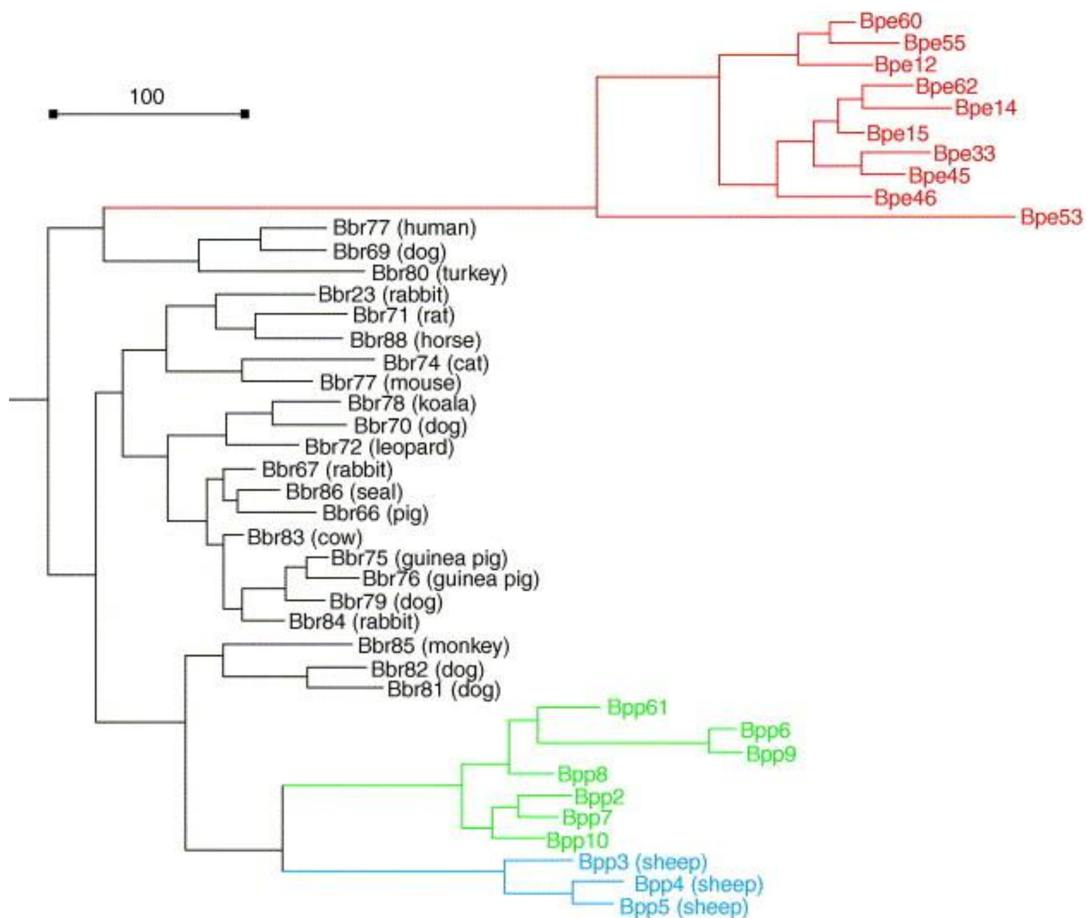

Figure 1. Phylogeny of the *Bordetellae* based on comparative genome hybridization data. The scale bar represents 100 evolutionary events (from Cummings *et al*., 2004). Bpe=*Bordetella pertussis*, Bbr=*B. bronchiseptica* and Bpp=*B. parapertussis*. Non-human strains are also labeled by the host species(Preston *et al*., 2007, Bjornstad *et al*., 2005).

1.6. Pertussis (Whooping Cough)

Whooping cough (pertussis) is an acute and one of the most contagious respiratory infectious diseases, caused by *B. pertussis.* Aerosol droplets from cough, sneeze or direct contact to respiratory tract secretions of a pertussis patient make the disease contagious, particularly in the first 2 weeks of the illness. The disease is characterized by paroxysmal cough with or without whoops, leukocytosis, hypoglysemia, histamine sensitivity, immobilization and/or damage of the cilia of the cilliated epithelium of the respiratory tract,

alveolitis and bronchopnemonia (Khelef *et al.*, 1992, Watanabe and Nagai, 2004, Locht *et al.*, 2007,).

Whooping cough can be divided into 3 phases; catarrhal, paroxysmal and convalescent. Catarrhal phase represents beginning of the disease. In this phase, local tissue damage and symptoms imitating common cold (cough, fever and runny nose) is detected because of the localized infection in the respiratory tract. Catarrhal phase takes about a week, progressing into paroxysmal phase which is more severe. The characteristic cough arises in the paroxysmal phase where the "whoop" sound is heard while inhaling. Chinese call this disease as "Cough of One Hundred Days", because of the persistent and episodic coughs. Progressive improvement of the symptoms in patients means that they are entering convalescent phase. While children older than 1 year and with a good health care do not face mortality, infants are severely affected. Unfortunately, even a physician cannot diagnose pertussis before the paroxysmal cough begins. Since antibiotic treatment is helpful solely if started before "whoops" had developed, it is often too late to benefit from antibiotics. Toxins are responsible from the damage in the respiratory tract where antibiotics are not capable of reversing the effects of toxins. However in case of early diagnosis, erythromycin and related antibiotics are most frequently used for the treatment (Cherry *et al.*, 1999)

The disease is caused by a number of virulence factors, expressed by the bacteria, including; filamentous hemagglutinin (FHA), fimbrial adhesins, pertactin, dermonecrotic toxin (DNT), tracheal cytotoxin (TCT), adenylate cyclase-hemolysin (ACH) and pertussis toxin (PT) (Khelef *et al.*, 1992). *B. pertussis* and *B. parapertussis* are responsible for the outbreaks of pertussis in human. The responsibility of *B. parapertussis* has been known for at least 23 years, because of the close nature to *B. pertussis*. The DNA hybridization studies and isozyme comparisons between *B. pertussis* and *B. parapertussis*

have been performed. Although both species produce number of common virulence factors, *B. parapertussis* carries the genes encoding petussis toxin (PTX), but do not express PTX because of a mutation in the promoter region. However "Both *B. pertussis* and *B. parapertussis* can induce mild or severe disease and no clinician in the world could distinguish between the two infections by clinical symptoms only", as stated by Novotny (1990).

1.7. *Bordetella pertussis*

Taxonomy of *B. pertussis* was described by Bergey *et al.* (1923). The relevant classification is as follows:
Kingdom: Bacteria
Phylum: Proteobacteria
Class: Beta Proteobacteria
Order: Burkholderiales
Family: Alcaligenaceae
Genus: *Bordetella*
Species: *Bordetella pertussis*

The cells of *B. pertussis* are very small, gram-negative, non-spore forming, aerobic coccobacilli that appear singly or in pairs (Figure 2).

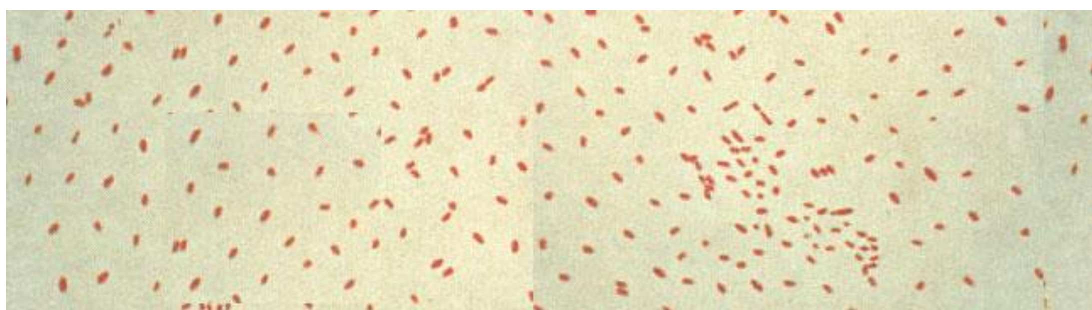

Figure 2. Gram stained cells of *B. pertussis* (http://textbookofbacteriology.net/pertussis.html).

Metabolism of *B. pertussis* is respiratory and taxonomically, this is why *Bordetella* is placed among the "gram negative aerobic rods and cocci" (Kerr and Matthews, 2000). The bacteria are nutritionally fastidious and usually cultivated on rich media supplemented with blood. It can also be grown in synthetic medium which contains buffer, salts, an amino acid energy source, and growth factors such as nicotinamide (for which there is a strict requirement). Even on blood agar, the organism grows slowly and requires 3-6 days to form pinpoint colonies.

1.8. Virulence Factors of *Bordetella Pertussis*

Like many other bacterial pathogens, the expression of the virulence factors (Figure 3) in *B. pertussis* is controlled by growth conditions. Two important phenomena in the regulation of the virulence genes are *phase variation* and *phenotypic modulation*. Phase variation indicates a reversible alteration in the genotype caused by frameshift mutations in which the virulent bacteria simultaneously lose the ability to synthesize toxins and other factors associated with pathogenicity. The other phenomenon termed phenotypic modulation implies repression of the expression of virulence factors, the tracheal cytotoxin at lower temperature (25°C) or in the presence of *in vitro* modulators like $SO_4{}^{2}$, $ClO_4{}^{-}$ and nicotinic acid. The responsible locus for both phenomena is *bvg* (*Bordetella* *v*irulence *g*ene) locus (Lacey, 1960; Babu *et al.*, 2002).

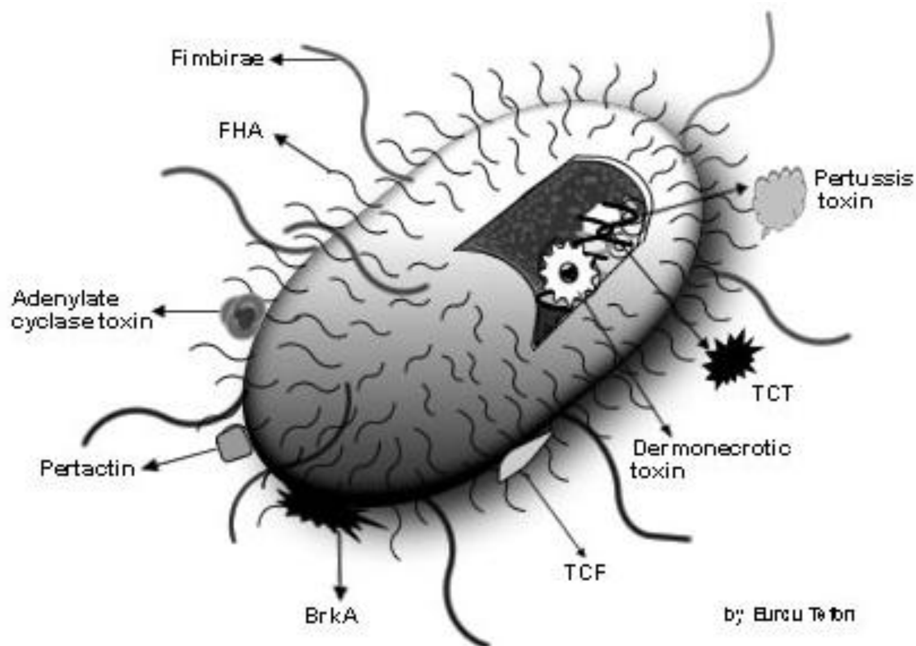

Figure 3. Virulence factors of *B. pertussis*.

1.8.1. BVG System

Like all organisms, bacteria must be able to sense their environment and control their behavior appropriately to survive. The most common mechanism used is the two-component regulatory system (TCS) (Nixon *et al.*, 1986, Ninfa *et al.*, 1986, Hess *et al.*, 1989). These are sophisticated signaling systems marked by a highly modular design that has been adapted and integrated into a wide variety of cellular signaling circuits. To date, researchers have found hundreds of such systems in eubacteria, archaea, and a few eukaryotic organisms. TCS coding genes in bacteria expression of which positively autoregulated include *phoPQ* in *Salmonella*, *cpxAR* in *Escherichia coli*, *misRS* in *Neisseria meningitidis*, and *bvgAS* in *Bordetella* (Scarlato *et al*. 1990, Soncini *et al*. 1995, De Wulf et al 1999, Tzeng et al 2006).

Thus, the BVG system of *B. pertussis* is one of the members of two component signal transduction systems (TCS) in a bacteria and encoded by *bvgAS* locus. BvgAS is composed of a transmembrane, polydomain integral membrane sensor kinase, BvgS, and a DNA binding response regulator, BvgA (Uhl and Miller, 1995). BvgS and BvgA function as dimers which are common for regulatory proteins of this family (Cotter *et al*., 2003, Corinne *et al*., 2006). The bvgAS locus is a regulation point for expression of virulence genes in both positive and negative manner (Stibitz, 2007).

BvgAS has an ability to activate/inactivate the three distinct phenotypic phases which are bvg^+, bvg intermediate (bvg_i) and bvg^-, by changing gene expression profile of the bacterium. Activation of the transcription of a number of vir-activated genes (vags) starts with phosphorylation of BvgA by BvgS. Then activated BvgA binds to the promoters of *vag*s sites and activates the transcription. An additional class of genes termed *vrg* (vir-repressed genes) is repressed by the products of *bvgAS* locus. The repression of these genes is mediated via a cytoplasmic repressor protein called BvgR, which is also activated by BvgAS. Thus, Bvg^+ phase occurs during which BvgAS is fully activated. Hence, active bacteria entering a host can cause infection. Since BvgAS is environmentally responsive, several studies have demonstrated *in vitro* that BvgAS expression can also be activated by growth at 37° C in the relative absence of $MgSO_4$ or nicotinic acid. Bvg^- phase is the second distinct phenotypic phase where BvgAS locus is completely inactive and seen under the conditions of $MgSO_4$ or nicotinic acid availability regardless of temperature. Under Bvg^- phase, BvgAS is unable to activate the transcription of *vag* genes and repression of *vrg* genes. Bvg-intermediate (Bvg_i) is characterized by low expression of *vag*s and the lack of *vrg*s expression. Bvg_i expression can be obtained under conditions of intermediate temperatures or in medium containing low levels of $MgSO_4$ (Parkhill *et al*., 2003, Corinne *et al*. 2006).

Several studies revealed that *bvg* locus-regulated gene expression by phase variation can be divided into four classes. Virulence factors of *Bordetella* spp. fall into first two classes. Class 1 factors that are expressed maximally in the Bvg^+ phase is a toxin class since the respective genes encode toxins, such as *cyaA-E* coding adenylate cyclase toxin and *ptxA-E* coding pertussis toxin. (Gross *et al*. 1988, Steffen *et al*. 1996, Julio *et al*. 2005). Class 2 factors which are expressed in both Bvg^+ and Bvg_i phases contain adhesion-encoding genes such as *fhaB* for coding filamentous hemagglutinin (Relman *et al*. 1989, Domenighini *et al*. 1990, Jacob-Dubuisson et al 2000). Class 3 ones that are expressed exclusively in the Bvg_i phase only has one component demonstrated so far; *bipA*. This gene was found to encode a protein in *B. pertussis* that show sequence similarity to the adhesin intimin from enteropathogenic and enterohemorrhagic *E. coli* and the invasin protein of *Yersinia* spp. (Stockbauer *et al*., 2001). Class 4 ones are the most different group of these four, and unlike the other 3 classes, genes representing this class are repressed by BvgAS and are expressed only in the Bvg^- phase.

1.8.2. Adhesins

Some of the microbial pathogens, such as *B. pertussis* has perfectly evolved to colonize on mucosal surfaces in the respiratory tract. Specially, one of the *B. pertussis*'s distinguishable property is that it has a tendency to adhare to cilliated epithelial cells of human respiratory tract. The increasing degree of colonization of the pathogen is achieved by producing specific adhesins for adherance. Adhesins are vital virulence factors which are produced under the control of the BvgA/S system and found in surface of the bacteria (Tuomanen *et al*., 1983; Jacob-Dubuisson and Locht, 2007). These include fimbriae (FIM), filamentous hemagglutinin (FHA), pertactin (PRN), tracheal colonization factor (TCF) and serum resistance protein (Brk).

1.8.2.1. Fimbriae (FIM)

Like many other muchosal pathogens, *B. pertussis* produces long, thin adhesive peritrichous structures that radiates from the outer membrane, the fimbriae (FIM) (Blom et al 1994). Fimbriae are polymers formed by the assembly of a large number of subunits.

B. pertussis produces fimbriae of two serotypes, serotype 2 and serotype 3, composed of the major fimbrial subunits Fim2 (22.5 kDa) and Fim3 (22 kDa) respectively. The *B. pertussis* genome also harbors a homologous *fimX* gene, the product of which has not been identified. In addition to the major subunits, the fimbriae also contain the minor 40 kDa subunit FimD, which serves as the adhesin (Locht, 1999).

Many years ago, it was observed that anti-fimbrial antibodies in the sera of infected or vaccinated children agglutinate the bacteria. Therefore, the fimbriae were initially called agglutinogens. In addition, a correlation was found between the presence of agglutinating antibodies and protection, which has led to the inclusion of Fim2 and Fim3 as antigens in some of the new acellular pertussis vaccines (Locht, 1999).

1.8.2.2. Filamentous Hemagglutinin (FHA)

Filamentous hemagglutinin is highly immunogenic and found in large quantities in *B. pertussis*. There are the hallmarks for FHA to be assigned as a component in acellular pertussis vaccine. FHA is an elongated, monomeric and horsenail shaped protein which is located at the surface of the bacteria, but can be secreted at the same time. FHA takes a major part in the pathogenesis of *B. pertussis*, by binding to the tracheal and laryngeal

epithelium in human and also animal model systems (Makhov *et al.*, 1994, Inatsuka *et al.*, 2005, Mattoo and Cherry, 2005)

FHA is synthesized as a 367 kDa precursor as encoded by constituted from the *fha*B gene and exported into the periplasm via the Sec machinery (Hodak *et al.*, 2006). It is then processed to a 220 kDa mature protein, which is mainly cell surface-bound, but can be released from the *B. pertussis* cells by the protease SphB1. The protein is highly immunogenic, with two main immunodominant regions (Piatti *et al.*, 1999).

FHA has long been considered to be the major *B. pertussis* adhesin. However, it functions in conjunction with other adhesins, such as the fimbriae, pertactin and even pertussis toxin. Some of these adhesins may even substitute for FHA or may be redundant, especially in strains lacking FHA. During infection, in both humans and animal models, FHA induces a strong antibody response, both systemically and mucosally, and vaccination with purified FHA confers protection against respiratory challenge in mice. For this reason, FHA has been included in new, second-generation vaccines against whooping cough. FHA also has an adjuvant activity for antigens delivered with it by the nasal route. These properties can be exploited to develop systems for presenting heterologous antigens to the respiratory mucosa (Locht, 1999).

1.8.2.3. Pertactin (PRN)

The first member of autotransporter family protein identified and characterized in *Bordetella* is pertactin. It is a surface protein, also called P69. PRN’s appearent molecular weight is 69 kDa in SDS-PAGE, but actually closer to 60 kDa. Pertactin derives from a precursor called P93, encoded by the structural gene named *prn*. Precursor undergoes to two proteolytic

cleavages after the transportation to periplasm via signal-peptide dependent secretion mechanism. First cleavage is 34 amino acid signal peptide and the second is 30kDa C-terminal region (Charles et al 1994). This C-terminal region remains anchored in the outer membrane and is essential for the secretion of pertactin. This mechanism can be seen in several gram negative bacteria, which are known to be autotransporters (Locht, 1999). PRN belongs to autotransporter type I (Jacob-Dubuisson *et al.*, 2004).

1.8.2.4. Tracheal Colonization Factor (TCF)

A relatively recently identified virulence factor with a structure similarity to pertactin and filamentous hemagglutinin is the tracheal colonization factor (TCF). This protein is encoded by the *tcfA* gene and plays an important role in colonization in the trancheal mucosal epithelial. Its sequence reveals several proline-rich regions, like FHA, pertactin and BrkA, and also contains an Arg-Gly-Asp sequence. Also, like pertactin and Brk, the C-terminal end of TCF is an autotransporter domain, suggesting a similar secretion mechanism. TCF can be found in two forms, similar to autotransportes, cell-membrane integrated form or secreted form with apperent masses of 90 and 60 kDa (Fernandez and Weiss, 1994, Locht, 1999).

1.8.2.5. Serum Resistance Protein (BrkA)

B. pertussis produces other autotransporters with adhesive properties such as Serum Resistance Protein (BrkA). Serum resistance or resistance to killing protein in *B. pertussis* is *bvg* regulated and the *Bordetella* resistance to killing (*brk*) locus mediates much of the resistance. BrkA is a virulence factor of *B. pertussis* that confers serum resistance to killing by the classical pathway of complement system and is involved in adherence and invasion (Fernandez *et al.*, 1998). BrkA is an autotransporter, an outer membrane

protein that mediate their own export across the bacterial outer membrane in gram-negative bacteria. It is expressed as a 103 kDa precursor that is processed during secretion to yield a 73 kDa N-terminal passenger-domain and a 30 kDa C-terminal transporter domain (Lambert *et al*., 1993, Fernandez and Weiss 1994, Passerini de Rossi *et al*. 1999). BrkA appears to be produced only by *B. pertussis* since the gene for this protein in *B. parapertussis* is a pseudogene.

1.8.3. Toxins

Considering pertussis toxin's influence in the disease of pertussis, the disease has long been used to referred as "toxin mediated". But now it is clear that *Bordetella* expresses various virulence factors, causes infection and production of clinical disease (Hewlett and Donato, 2007). Major virulence protein toxins are, pertussis toxin (PT), adenylate cyclase toxin (ACT), dermonecrotic toxin (DNT), tracheal cytotoxins (TCT) and enterotoxins.

1.8.3.1. Pertussis Toxin (PTX)

Pertussis Toxin (PTX) is a major colonizing factor and an exotoxin, which remains cell-bound as well as released into the extracellular environment (Babu *et al*., 2002). PTX is one of the major components of acellular pertussis vaccine with FHA. PTX is the main toxin of *B. pertussis* with a molecular weight of 117 kDa. This hexameric and globular protein toxin is a member of the ADP-ribosylating protein family, having several biological activities. PTX is composed of five dissimilar subunits, S1 to S5, which assemble into two functionally distinct moieties (Hazes *et al*. 1996). The enzymatically active "A" component consists of the S1 subunit. The "B" or

binding domain, also referred as B-oligomer, binds to eukaryotic cells and mediates translocation of the S1 subunit into the cell (Farizo *et al.*, 2002).

The action of PTX on the immune system appears rather complex as it was shown to induce lymphocyte mitogenesis and IgE synthesis, but also to promote Th1-type inflammatory responses in experimental autoimmune diseases (Tonon *et al.*, 2002). Moreover, PTX induces high levels of antibody after infection or vaccination with classical whole cell vaccine and the toxin provides full protection against challenge in mouse models. For this reason, inactivated PTX is the major protective antigen in all new acellular vaccines available today (Locht, 1999). PTX is secreted only by *B. pertussis*, the genes of PTX are being present but not expressed because of the inactivation in the promoter region, in non-pertusis species (*B. parapertussis and B. brochiseptica*) (Arico and Rappuoli, 1987).

1.8.3.2. Adenylate Cyclase Toxin (ACT)

Adenylate cyclase (ACT) is an important invasive toxin secreted by *B. pertussis* as encoded by *cyaA*. It has both adenylate cyclase and hemolytic activities. ACT is a single polypeptide which has an apparent molecular weight of 177 kDa. It is made up of two major domains, one catalytic and one binding domain. Catalytic domain, which is located at first 400 amino acids, is composed of an active site and calmodulin-binding sites. Binding domain is composed of calmodulin-binding repeats. 2 major domains are connected with a hydrophobic region and at the C-terminal a signal sequence is found which is required for biological activity and secretion (Locht *et al.*, 1999, Hewlett and Donato, 2007,). It is able to enter many eukaryotic cells, where it is activated by eukaryotic calmodulin and subsequently catalyses the conversion of endogenous ATP to cAMP. This uncontrolled increase in cAMP concentration leads to paralysis of the killing

functions of phagocyte and immune effector cells (Kerr and Matthews, 2000).

1.8.3.3. Dermonecrotic Toxin (DNT)

Dermonecrotic toxin (DNT), also called lethal toxin, is a single chain polypeptide and has a hypothetical molecular mass of 160 kDa with 1464 amino acids. It induces inflammation, vasoconstriction and dermonecrotic lesions around the areas which *B. pertussis* colonize in the respiratory tract. Based on biological activity and protein structure, DNT belongs to the family of dermonecrosis, which affects regulation of cell growth or division (Babu *et al.*, 2002). This heat-labile toxin induced localized necrotic lesions in mice and other laboratory animals when injected intradermally and is lethal for mice at low doses when administered intravenously (Mattoo and Cherry, 2005). The protein is encoded by the *bvg*-regulated *dnt* gene and a member of a A/B toxin family. Structure of the toxin contains a binding domain at its N-terminal and an active domain at its C-terminal with molecular weights of 30 and 24 kDa. (Kashimoto et al 1999). A part of the toxin should be expressed on the outer membrane of the bacteria, even though the localization of the toxin had been mentioned as cytoplasm. At low doses, subcutaneous injection of DNT of *B. pertussis* causes necrotic lesions into mice and is lethal at high doses (Kerr *et al.*, 2000).

1.8.3.4. Tracheal Cytotoxin (TCT)

The characteristic whooping cough caused by *B. pertussis* can be attributed to the elaboration of the tracheal cytotoxin (TCT). Unlike other virulence factors or toxins from the genus *Bordetella* monomeric subunit of the peptidoglycan is not expressed by a gene and not regulated by *bvg*. TCT is a disaccharide tetrapeptide with a molecular weight of 921 000 Da and with a

chemical composition of N-acetylglucosamine-1,6-anhydro-N-acetylmuramic acid–L-alanine-Dglutamic acid-diaminopimelic acid-D-alanine (Goldman *et al*. 1990). It cannot be classified as a classical exotoxin though it is secreted to the extracellular fluid by the bacteria (Babu *et al*., 2002).

TCT decreases ciliated epithelial cells of the respiratory tract by extrusing the ciliated cells from the epithelial surface. *In vitro*, DNA synthesis in tracheal epithelial cells is inhibited by TCT and it induces the production of intracellular interleukin-1 and of nitric oxide, the most likely triggering of TCT-mediated cytopathy. Much less is known about TCT, relative to PTX (Locht, 1999).

1.8.3.5. Lipopolysaccharides (LPS)

The B. pertussis lipopolysaccharide (LPS) may act in synergy with other toxins, such as TCT or pertussis toxin. By itself, it has endotoxin activities, similar to those of LPS from enteric bacteria. But structurally, *Bordetella* LPS molecules differ from the well known smooth-type LPS expressed by members of the family Enterobacteriaceae. Specifically, *B. pertussis* LPS lacks a repetitive O-antigenic structure and in order to acquire serum resistance, the bacteria uses a combination of O-antigen deficient LPS and BrkA (Mattoo and Cherry *et al*., 2007). LPS affects spleen cell cultures in a pyrogenic and mithogenic way and is lethal in galactosamine-sensitized mice. However, the precise role in pathogenesis of these structural differences are not clear, it induces the production of tumor necrotic factors and activates macrophages. Two LPS classes, LPSI and LPSII are present in *B. pertussis*. LPSI to LPSII ratio may differ between species of the same genus and strains of the same strain (Locht, 1999).

1.9. Vaccines Against *B. pertussis*

Within the decade following the first description of *B. pertussis* by Bordet and Gengou in 1906, vaccines made up of whole cells were developed. The first whole cell vaccine which was made up of dead *B. pertussis* organisms were tested in children in Tunisia and Denmark (Howson *et al.*, 1991).

In the 1930s, many candidate pertussis vaccines were developed and used for the treatment and prevention of pertussis in limited studies. These initial vaccines were prepared by many different methods; the products included whole-cell vaccines that contained culture media, whole cells that were washed, mixed vaccines that contained other bacteria from the upper respiratory tract flora as well as *B. pertussis* cells, fractionated vaccines (extracted vaccines), "detoxified vaccines," and vaccine enriched with "toxic factors." Beginning in the mid-1940s, routine immunizations of children with pertussis vaccines was started in the United States, and this continued until the replacement of whole-cell vaccines with acellular pertussis (aP) vaccine in the late 1990s. Initial vaccines were monocomponent whole-cell pertussis vaccines, but by 1947 combination vaccines with diphtheria and tetanus toxoids (DTP) were available and recommended. During the 1950s, other countries also started performing routine pertussis immunization. The mass-vaccination enabled high coverage, which maintained for 50 years (Mattoo and Cherry, 2005). Whole cell pertussis vaccine was used in these vaccination and the studies showed that neither the mentioned vaccine, nor infection with *B. pertussis* does not protect against *B. parapertussis* infections (Neimark, 1961; Khelef, 1993).

Pertussis is one of the ten most common causes of death from infectious disease worldwide. The immunization of infants with either whole-cell or acellular pertussis (aP) vaccines has been remarkably successful in reducing

severe disease complications and deaths in young children. In those countries where the data are available, pertussis vaccination has reduced the numbers of notified cases from peak years by more than 95% (König, 2002). Despite high vaccination coverage, *B pertussis* remains endemic and reports of increasing incidence in the USA, Canada, Australia, France, the UK, and Poland have been accumulating since the 1980s. News media announced a global resurgence of whooping cough in April 2002 following a session on pertussis at the 12th European Congress of Clinical Microbiology and Infectious Diseases in Milan, Italy. Subsequently the European Union sent an alert to member states (Crowcroft *et al.*, 2002). The rise in the incidence of whooping cough has been accompanied by a shift to older age groups and calling for a reappraisal of clinical management in adolescents and adults and a reinforcement of vaccination strategies (Águas *et al.*, 2006). These strategies which are not mutually exclusive include universal adult and/or adolescent vaccination, indirect protection of infants by immunisation of parents and possibly others in close contact with the newborn such as grandparents and healthcare workers, early infant immunisation (from birth to 1 month of age) and maternal immunisation (Wood and McIntyre, 2008).

Pertussis vaccine schedules differ significantly around the world but none currently starts at earlier than 6 weeks of age (Table 2). The WHO recommends a schedule of 6, 10 and 14 weeks for primary immunisation against pertussis. A reduction in the adult/adolescent reservoir could be achieved by administering a booster dose of acellular vaccine, DTpa. Universal vaccination for adolescents is recommended in several countries (Table 2) (Wood and McIntyre, 2008).

Table 3. Pertussis vaccination schedules in selected countries. (http://www.who.int/immunization/en/, Wood et al. 2008)

Country/ Organization	Type of vaccine	Primary immunization schedule	Adolescent dTp booster recommended
WHO	DTPw	6,10,14 months	No
Australia	DTPa-combination	2,4,6 months	Yes
Brazil	DTPw-combination	2,4,6 months	No
Canada	DTPa-combination	2,4,6 months	Yes
Egypt	DTPw-combination	2,4,6 months	No
France	DTPa-combination	2,3,4 months	Yes
Germany	DTPa-combination	3,4,5 months	Yes
Italy	DTPa-combination	3,5,11 months	No
South Africa	DTPw-combination	6,10,14 months	No
Sweden	DTPa-combination	3,5,12 months	Yes
UK	DTPa-combination	2,3,4 months	No
USA	DTPa-combination	2,4,6 months	Yes

1.9.1. Vaccine Manufactured Against *Bordetella pertussis* in Turkey

After participation in the"Expanded Program on Immunization" (EPI) of the World Health Organisation (WHO) in 1974, the immunization was accelerated with Turkey's "National Vaccination Campaign" between 1980 and 1985. The program focused on prevention of 6 diseases namely; whooping cough, diptheria, tetanus, tuberculosis, polio and rubeola (Özmert *et al*. 2008).

The manufacture of whooping cough vaccine was first started at 1937 in Turkey as Diptheria+Pertussis (DB) and after 1968, it was manufactured as as Diptheria+Pertussis+Tetanus (DBT) till 1996 at which the local production of human vaccines was quitted as a government policy. Since then, all human vaccines in use in the country have been the imported ones.

Since 1968, there was no changes in the vaccination schedule, i.e. primer vaccination dates and first enhanced dose, as seen in Table 3 for DBT. A major improvement was made in 2008 by converting the whole cell pertussis vaccine into acellular pertussis vaccine. According to the EPI plan in 2006, *Haemophilus influenza B*. (Hib) and in 2008 inactive polio (IPA) vaccination has started and combined to DBT, resulting in a DaBT-IPA-Hib combined vaccine (Özmert *et al*. 2008).

In Turkey, whole cell pertussis vaccine was being administered in the 2nd, 3rd, and 4th months of life, in combination with a booster dose administered between the 16th and 24th months. If the booster is delayed, it could be administered into children younger than six years old. After alteration of whole cell vaccine into acellular pertussis vaccine, administration dates were changed as 2nd, 4th and 6th months, and the booster dose range became narrowed between 18th and 24th months (Table 3) (Özmert *et al*. 2008).

Table 4. Current vaccination program in Turkey (as of January 2008) (Özmert *et al*. 2008)

		End of Month				Month		Grade	
	Birth	1	2	4	6	12	18-24	1.	8.
BCG			I						
HepB	I	II			III				
DaBT-IPA-Hib			I	II	III		R*		
OPA					I		II		
KKK						I		R*	
Td									R*

BCG; Tuberculosis. HepB; Hepatitis B. DaBT-IPA-Hib; Diptheria, Acellular pertussis, Tetanus, İnactive Polio, Haemophilus influenza B. OPA; Oral Polio, KKK; Rubeola, Rubella, Epidemic Parositis. Td; Diptheria-Tetanus for adults

*Enhanced Dose

Whole cell vaccines were replaced with aceullular pertussis vaccine in the late 1990s. Acellular vaccines were started to be used in Turkey in 1998s for private immunization, but as stated earlier, systematic vaccination was

started in the country in 2008. DaBT vaccination increased to cover population from 20–30% to 83%. This increased coverage of the primary pertussis vaccination decreased the incidence of disease in Turkey dramatically, from 21 cases per 100.000 in 1970 to 0.55 per 100.000 in 2004 (Figure 4). According to the available data of the Ministry of Health, Turkey appears to be reaching the WHO target, with a pertussis incidence of <1 case per 100.000, except for East Anatolia. However, pertussis still affects all age groups, especially adolescents, adults, and young infants in Turkey, and occurs endemically with 2- to 5-year cycles of increased disease incidence. It was noticed that the number of cases increased in 1997, 2000 and 2004 despite increased coverage. The incidence of reported pertussis among adolescents and adults has increased over the past decade in Turkey. Even though up to 6.5% of the cases were ≥15 years of age until 2005, 16.9% of them were included in this age group in 2005. A large number of schoolchildren, adolescents, and adults are susceptible to pertussis infection (Vatansever *et al.*, 2005; Kurugöl, 2009).

In spite of the incidence decline, the circulation of *B. pertussis* has not been eliminated, and a change in the clinical spectrum and age-related incidence of the disease has been observed. Developing effective strategies for reducing the burden of pertussis must be made on a country by country basis, with an assessment of local circumstances (Vatansever *et al.*, 2005).

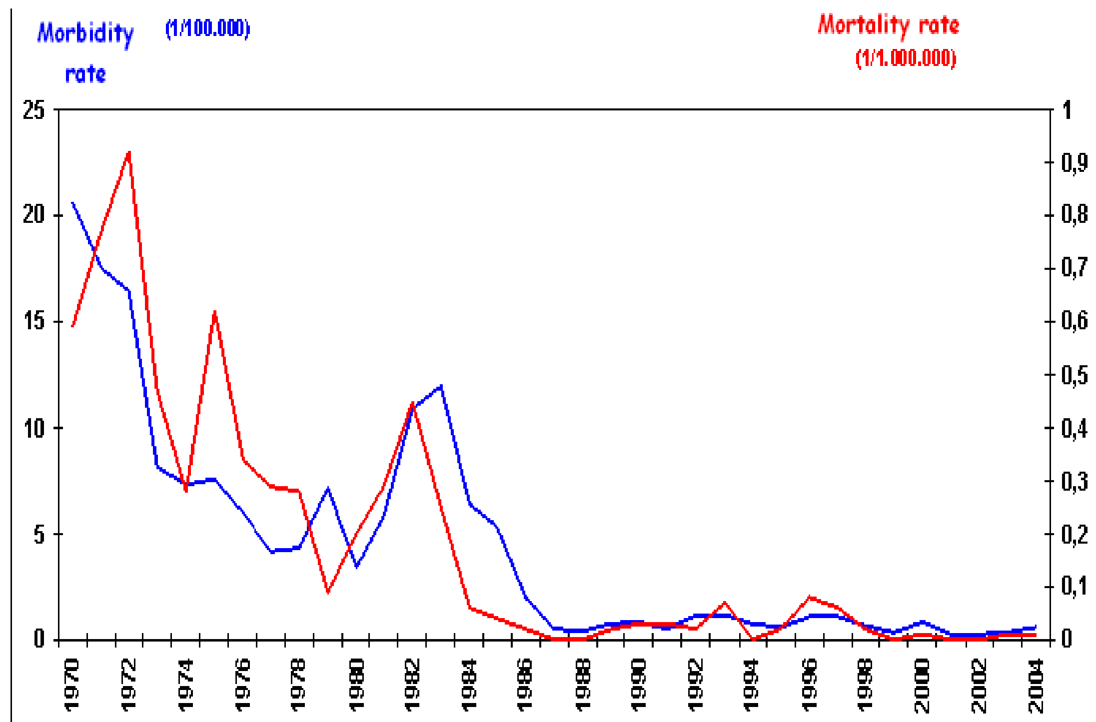

Figure 4. Pertussis morbidity and mortality rates in Turkey, 1970-2004 (http://www.saglik.gov.tr/extras/istatistikler/temel2004/tablo-39.htm)

1.10. Aim of the Study

In spite of the extensive pertussis vaccination programs worldwide, remaining non-immunized children and older individuals with waning immunity may serve as reservoirs for the infection, occasionally transmitting *B. pertussis* to unimmunized young infants. Pertussis which has long been considered to be only a disease of childhood is now being reported in adolescents and adults. It often goes underrecognized in such individuals because their disease manifestations are frequently atypical. Contraindication to the use of whole cell pertussis vaccines in individuals over 7 years of age as well as the short-lived duration of protection afforded is well known. The licensed adolescent and adult formulations of acellular pertussis vaccine combined with an adult formulation of diphtheria and tetanus toxoids (Tdap) are recorded to be safe, immunogenic, and well tolerated. On the other hand, in view of the moderate changes that have been observed in the

genomic sequences of the bacterial PRN and PTX over time, there are concerns about the gradual loss of the efficacy of the current pertussis vaccines as a result of antigenic drift and continuous selection of the least vaccine-sensitive clones.

Tohama I is the strain of *B. pertussis* that has been well recognized and used for vaccine manufacture (cellular DTP and acellular DaPT) worldwide. Saadet and Nursel strains are the local isolates of *B*. pertussis which had been preferred over the standard strain Tohama I as the vaccine strains and used effectively for many years for local production of cellular pertussis vaccine. However, for the reasons explained above new and more protective pertussis vaccines are still needed. The protective power of vaccine may be increased by preparing vaccines against specific epitopes.

The aim of the present study is to persue an immunoproteomic comparison between the standard and local strains of *B. pertussis* in order to identify common and specific immunogenic proteins, with the hope of finding out yet undiscovered novel antigens as the candidates of third-generation pertussis vaccines.

CHAPTER 2

MATERIALS AND METHODS

2.1. Materials

The materials that were used in this study are listed in the Appendix B. All chemicals were of analytical grade and were obtained from the commercial sources at the highest grade of purity available.

2.2. Bacteral Strains

The strains of *B. pertussis* Tohama I, Saadet and Nursel were used in this study. While Tohama I is a standard strain of *B. pertussis* and used for both vaccine manufacture and research worldwide, Saadet and Nursel are two local *B. pertussis* strains used for many years in local cellular vaccine manufacture in our country. All *B. pertussis* strains were kindly provided by Dr. Erkan Özcengiz (VBR Vaccine Res. Co., Ankara).

2.3. Culture Media and Growth

B. pertussis strains (Sato *et al.*, 1972) were grown for 60 h in Cohen-Wheeler agar medium, then stored at 4° C and subcultured at 2 week intervals. For long term maintanence, the colonies were collected from the surface of the agar, then resuspended in phosphate buffered saline (PBS) to an OD_{630} of 0.6 (corresponding to ca. 10^9 cells/ml). After covering with 30% glycerol, the cells were stored at – 80° C. When necessary, the strains were

also grown on modified Morse and Bray liquid medium (Sato et al., 1974) for 60 h at 37° C, by shaking at 180 rpm (Appendix C).

2.4. Total Soluble Protein Extraction

B. pertussis Tohama, Saadet and Nursel strains were grown on Cohen-Wheeler agar medium for 60 h at 37°C. For the preparation of whole cell lysate, the cellular mass were collected using an inoculation loop and suspended in cold TE buffer (0.2 mM EDTA, 2 mM Tris-HCl) and centrifuged at 10000rpm for 10 min at room temperature (Eppendorf, Germany). The pellet was resuspended in lysozyme (in 0.04 M TE buffer) and incubated at 37°C for 30 min. After centrifugation at 10000rpm for 10 min at room temperature, the pellet was treated with 8 M urea, vortexed at 4°C for 20 min, and recentrifuged after which the pellet was discarded. Protein concentration was determined by Bradford technique (Ramagli et al. 1985) (Appendix A). Protein samples were stored at -20°C at most for 10 days till usage.

2.5. Determination of Protein Concentration

To determine total protein concentration, the modified Bradford assay described by Ramagli and Rodrigez (1985) was used. 5X Bradford reagents (containing 500 mg Coomassie Brillant Blue G-250, 250 ml of 96% ethanol and 500 ml of 85% ortho-phosphoric acid; completed to a 1 l with dH_2O) was diluted 1:3 with dH_2O and filtered at least three times using Whatman No. 1 filter paper. For determination of the total protein concentration, bacterial cell extract was dissolved in 500 µl of rehydration buffer (containing 8 M urea, 2 M thiourea, 28 mM DTT, 2% (w/v) CHAPS, 0.5% (v/v) ampholyte pH 3-10). The suspension was mixed and incubated at room temperature for 1 h and then centrifuged at 12000 xg for 5 min. To 20 µl of aliquots of the

supernatant, 80 µl of 0.1 N HCl were added to protonate samples and mixed thoroughly. To this mixture, 3.5 mL of 1:3 diluted 5 X Bradford reagent was added, incubated at room temperature for 10 min and absorbance was measured at 595 nm. Bovine Serum Albumin (BSA) fraction number V was used as a standard for the construction of calibration curves.

2.6. 2-Dimensional Gel Electrophoresis

2.6.1. Rehydration of IPG Strips

For passive rehydration, commercially available IPG strips (17 cm, pH 3–10, Bio-Rad) were rehydrated with 400 µl of rehydration buffer (8 M urea, 2 M thiourea, 2% w/v CHAPS, 28 mM DTT and 0,5% v/v ampholyte 3-10) containing 350 µg protein sample for 16 h at room temperature (20-25°C) with mineral oil coverage**.**

2.6.2. Isoelectric Focusing

Rehydrated strips were taken to the Isoelectric focusing (IEF), which was performed by using the Protean IEF Cell (Bio-Rad, USA), with mineral oil coverage. The following voltage profile was used for IEF; 1 h 100 V; 1 h 300 V; 1h 600 V; 1 h 1000 V; 2 h 3000 V; 2 h 5000 V followed by a linear increase to 8000 V for 50,000 Vh. The final phase of 8000 V was terminated after 72 000 Vh.

2.6.3. SDS-PAGE

The IPG strips were equilibrated twice 15 min each in equilibration buffer (6 M urea, 50mM Tris-HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS), while first

equilibration buffer includes 100mg DTT (Dithiothreitol)/10ml, second buffer contains 250mg IAA (Iodoacetamide)/10ml (Gorg et al.,2004).

2.6.3.1. Preparation of SDS-PAGE Gels

Equilibrated IPG strips were placed on the 20cmx20cm, continuous 12% acrylamide/bis-acrylamide gels in a Bio-Rad Cell system (Bio-Rad, USA). Approximately 25 mA was used per gel.

2.6.3.2. Commassie Blue Staining and Imaging

After electrophoresis, colloidal Coomassie blue was used to stain and visualize the protein spots, according to Neuhof *et al.* (1988) (Appendix A). Coomassie stained gels were scanned by using a scanner (HP Scanjet 4070 Photosmart scanner, USA) or stored at 4˚C for further testing.

Gel images were installed into 2D image analysis software Delta2D version 3.3 (Decodon, Germany). This program was used for determination of spot pattern, spots to be cut for verification of the pattern and cross-reaction differentiation analyses after Western blotting.

2.7. Antisera Preparation Against *B. pertussis* Strains

For antisera preparation against *B. pertussis* strains, BALB/c mice were subjected to subcutaneous immunization. *B. pertussis* Tohama, Saadet and Nursel strains were grown on Cohen-Wheeler agar for 60 h at 37˚C. All the strains were suspended in 0.85 % saline solution separately. The concentrations of these suspensions were adjusted to 0.6 (corresponding to ca. $4x10^{10}$ bacteria/ml) at OD600, then inactivated at 56° C for 30 min. 6-10 mice received two subcutaneous injections of 0.5 ml per animal at two week

intervals between the first and second injections. Their sera were collected and pooled 14 days after the second injection and stored at -20˚C.

2.8. Western Blotting

Non-stained 2-D gels were transferred to nitrocellulose membrane (0,2 µm, BioRad, USA) for 2h at 400mA using transfer buffer (25 mM Tris, 192 mM glycine, 2% w/v SDS and 20% v/v methanol) by using semi dry blotting system (Cleaver Scientific Ltd.). NC membrane is blocked, after the transfer has been completed, by incubating in 10% skim milk, prepared with TBS solution (20 mM Tris including 5M NaCl), at 37˚C for 2h in constant rotation. Membrane was incubated for 1h at room temperature with gentle shaking in anti-*B. pertussis* strain based primary antibody, antisera collected from, immunizied mice which has been immunized by inactivated whole cells of *B. pertussis* strains, at a dilution of 1:200 in 5% skim milk solubilized in TBS, after rinsed with 20% Tween-TBS (TTBS) for 10 minutes. The membrane was rinsed for 10 min with 20% Tween-TBS (TTBS) again, and incubated with rabbit anti-mouse IgG-alkaline phosphatase (Sigma), with a dilution factor of 1: 15,000 in TBS containing 5% skim milk for 1 h. Development of the color was started by adding the substrate (AP Conjugate Substrate Kit, Bio-Rad) after the membrane was washed with TBS for 10 min.

2.8.1.Dot Blotting

In order to confirm the immunogenic reaction of the spots after the Western Blot analysis, the serum reactive protein spots were cut from the stained 2-DE gels and placed into a 96 well plate. Spots were destained with destaining solution (10 % Methanol, 20 % Acetic acid, 70 % dH_2O) until the dye is removed. Spots were first washed with dH_2O and secondly with 10% SDS, two times each for 10 min. The protein gel spots were placed onto the NC

membrane for Western blotting. For Western analysis, the modified method of Towbin et al. (1979) was used.

2.9. Mass Spectrometry

The identification of protein spots were accomplished by using mass spectrometry according to the established protocols. Proteins were cut form the gel and sent to the Greifswald University (Germany) for their MALDI-TOF/MS Analysis. Briefly, protein spots were excised from stained 2-D gels, destained and digested with trypsin (Promega, Madison, WI, USA) and for extraction of peptides, the gel pieces were covered with 60 µl 0.1% trifluoroacetic acid in 50% CH_3CN and incubated for 30 min at 40°C. Peptide solutions were mixed with an equal volume of saturated α-cyano-3-hydroxycinnamic acid solution in 50% acetonitrile-0.1% trifluoroacetic acid (v/v) and applied to a sample plate for MALDI-TOF-MS. Mass analyses were carried out on the Proteome-Analyzer 4700 (Applied Biosystems, Foster City, CA, USA). The three most abundant peptides in each MS spectrum were chosen for MS/MS experiment. The resulting sequence data were included for the database search to increase the reliability of protein identification. Mass accuracy was usually in a range between 10 and 30 ppm.

2.10. Protein Identification

Amino acid sequences for B. pertussis proteins were obtained from organism's genome project [Sanger Institute] web site (http://www.sanger.ac.uk/Projects/B_pertussis/). The peak lists of each protein spot were analyzed with the aid of "PMF" and "MS/MS Ion Search" engines of MASCOT software (http://www.matrixscience.com/). The searches considered oxidation of methionine and modification of cysteine by carbamidomethylation as well as partial cleavage leaving one internal

cleavage site. Of the results given by the MASCOT software, those having a probability score value higher than 53 were considered for successful protein identification. To find out putative functions, protein accession numbers of the identified spots were searched in the website for B. pertussis. We used 39 the artificial network-based B-cell epitope prediction server ABCpred (Saha *et al.*, 2006) to predict the epitopes of the identified proteins of *B. pertussis* (http://www.imtech.res.in/raghava/abcpred/index.html).

CHAPTER 3

RESULTS AND DISCUSSION

3.1. Theoretical map

In a 2-D based proteome work, the first thing to be done after extracting the protein mixture from an organism is to determine the optimal p*I* range for IEF step. A theoretical 2-D gel map of *B. pertussis* has been constituted using genomic data from gelbank database (http://gelbank.anl.gov/proteomes/insilico/257313.asp) (Figure 5). This theoretical map, revealed the presence of 3436 records of proteins in a p*I* range of 3 to 13 for the 2-D gel based proteome analysis of *B. pertussis*. The p*I* range for *B. pertussis* proteins is between pH 3.6 (for putative outer membrane protein) and 12.7 (for 50S ribosomal protein L34). In this p*I* range, the molecular weights of proteins vary from 4,270 Da (for entericidin anti-toxin precursor) to 437,306 Da (for adhesin). In the theoretical map, most of the proteins were seen in the area where the p*I* value is between 4 and 12. However, commercially available IPG strips are only available in a pH range of 4 to 7, 3 to 6, and 3 to 10. According to the theoretical map (Figure 5), a p*I* range between 3 and 10 was chosen as the analytical window to cover most of the proteins.

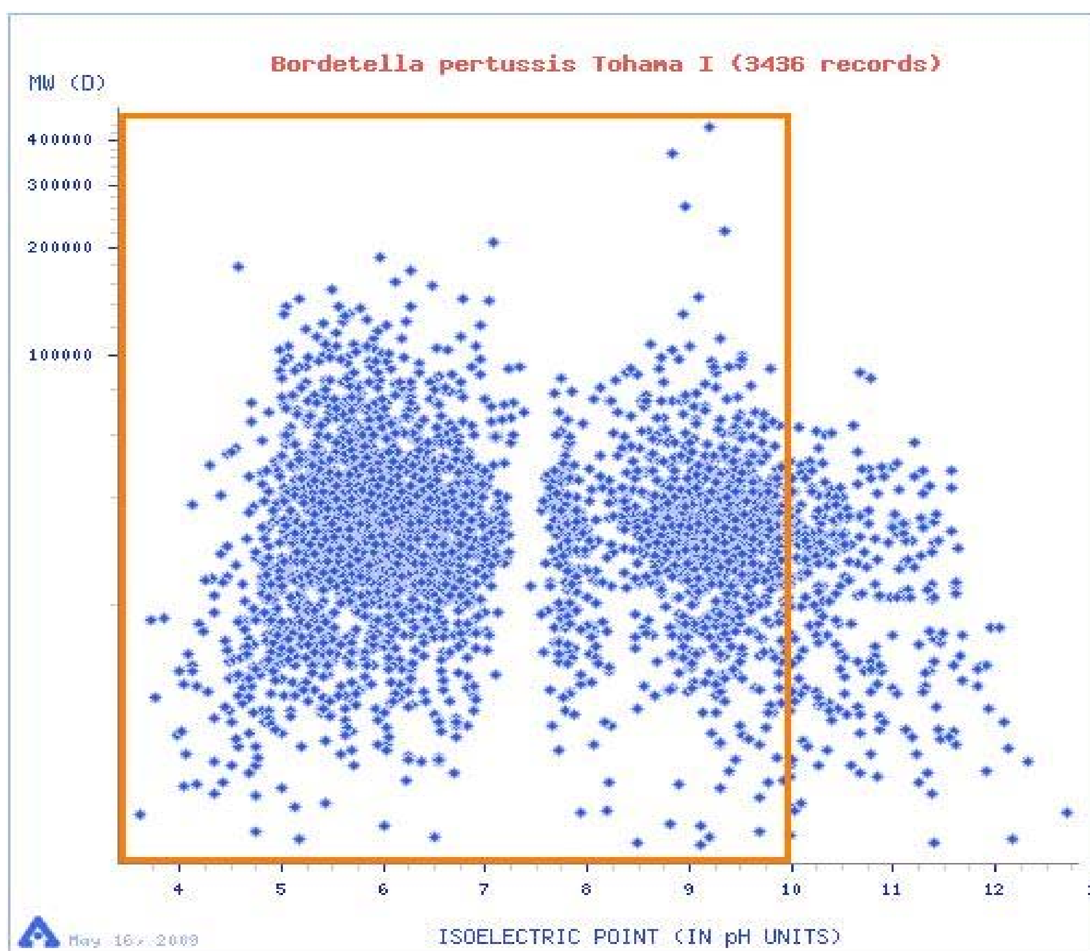

Figure 5. A theoretical map of *B. pertussis* Tohama I strain proteome; predicting a 2-D gel using isoelectric point (in pH units) vs. molecular weight (in Dalton). The orange colored rectangle indicates the margins of the master gels that were used in this study.

The theoretical map shown in Figure 5 is specific for *B. pertussis* Tohama I strain. Since patterns of Saadet and Nursel strains of *B. pertussis* do theoretically correspond to that of Tohama I strain, same working window (pH 3-10) was used for all strains of the bacteria.

3.2. Master gels of *B. pertussis* Strains

Master gels have been prepared by growing the cells to the exponential phase under normal physiological conditions, in order to view the main protein components of *B. pertussis* strains. Exponential phase of *B. pertussis*

batch cultures occured between 48 to 72 hours, therefore the cells were collected in this growth period. The protein extracts of the three strains were prepared separately. At the end of 2D-PAGE, soluble proteins of Tohama I, Saadet and Nursel strains that are visualized in the 2-D gels have been restricted to the pH range of commercially available IPG strips in between pH 3-10. For each of the strain, there were 3 biological and 3 technical replicates to minimize the errors and the same amount of protein (350µl) was used for all replicates. The three master 2-D gels belonging to the *B. pertussis* strains, Tohama I, Saadet and Nursel are shown in Figures 6-8, respectively. All of these master gel patterns were used as templates for visualization of immunogenic proteins.

To visualize the separated proteins, colloidal CBB G250 was used to stain the gels and more than 600 spots were seen in total soluble proteome of each strain. The stained gels were digitized by using a scanner and spot pattern analysis was accomplished by using the 2-D image software Delta 2D version 3.4 (Decodon, Germany).

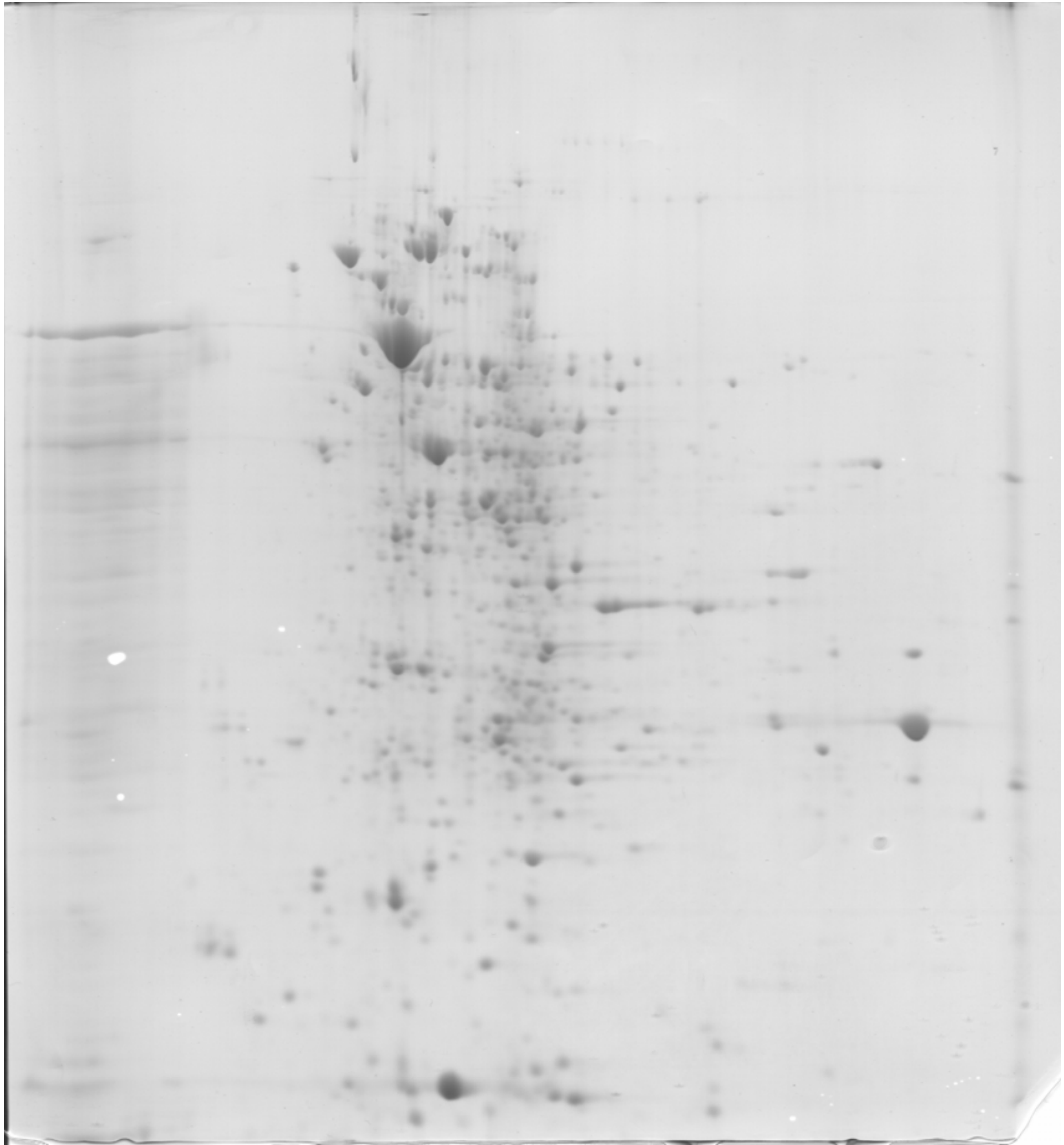

Figure 6. 2-D master gel of the total soluble proteome of *B. pertussis* Tohama I.

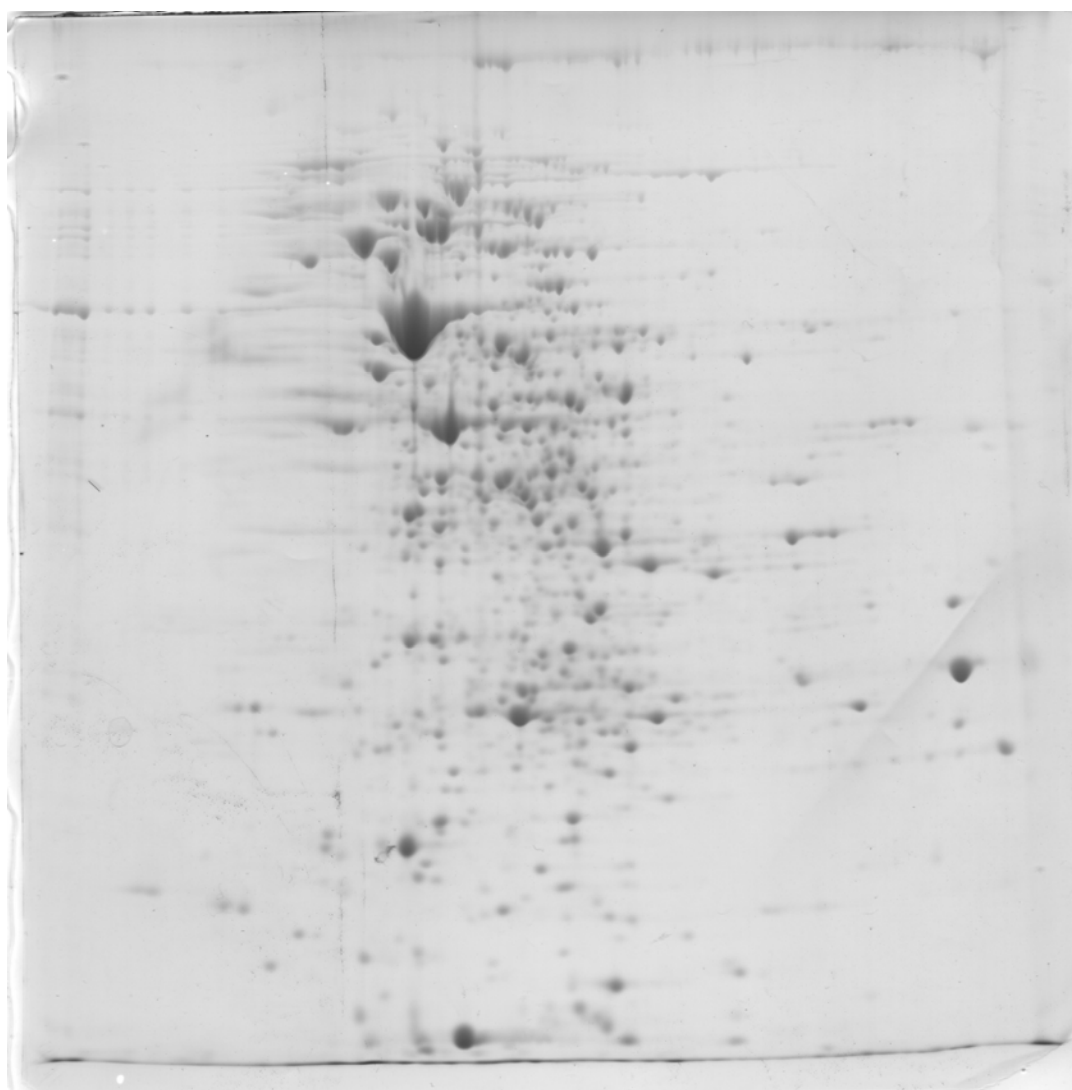

Figure 7. 2-D master gel of the total soluble proteome of *B. pertussis* Saadet.

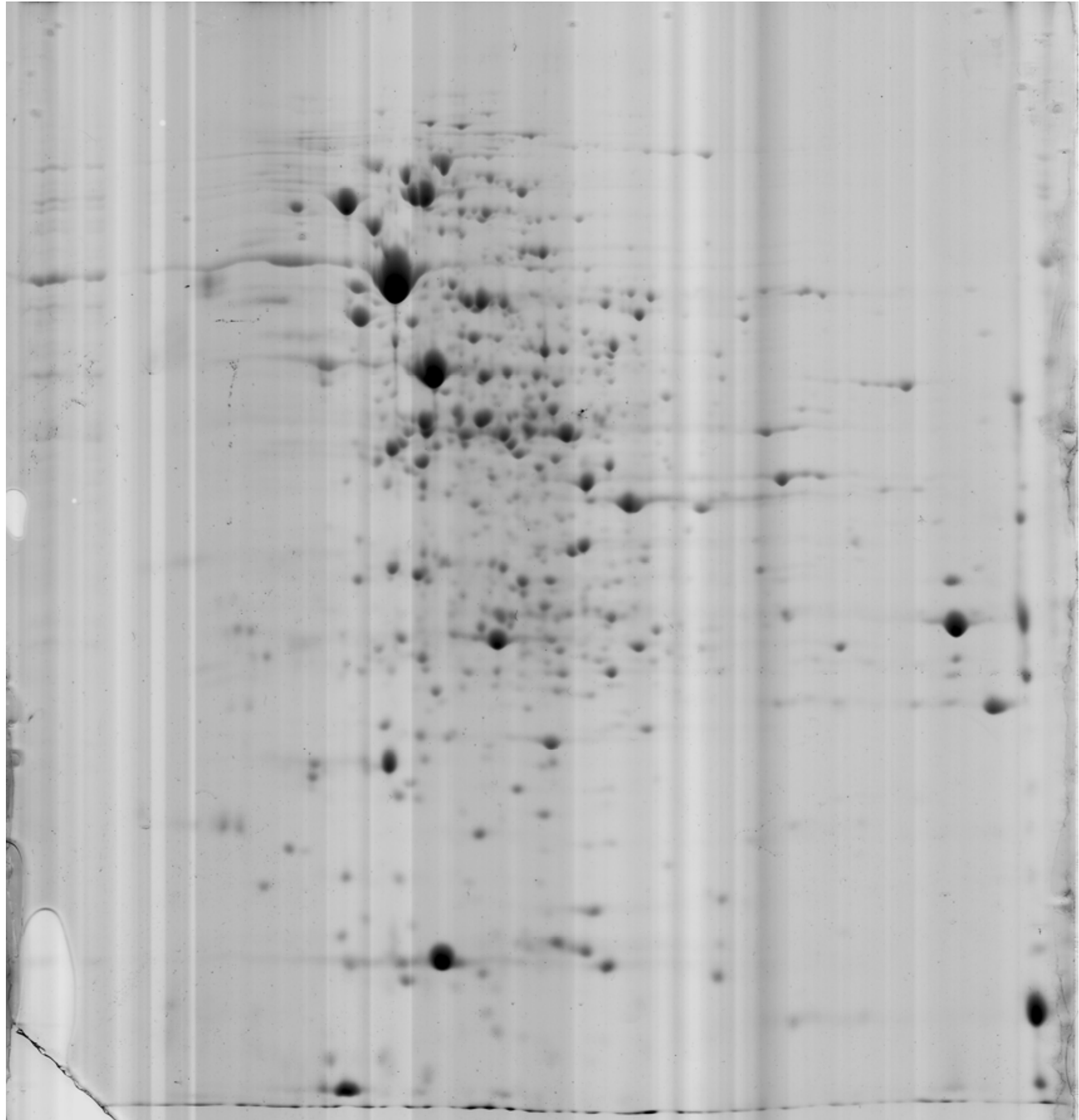

Figure 8. 2-D master gel of the total soluble proteome of *B. pertussis* Nursel.

3.2.1. Multicolor Imaging of Expressed Patterns from Three Different Strains of *B. pertussis*

Delta 2-D image analysis software version 3.4 was used to overlap soluble proteins contained in 2-DE master gels and thus comparing soluble proteins of three different strains. After loading the gel images to the program, different color codes have been given to the master gels of strains and exact matching could be established. A different color was seen when different

master gel spots have overlapped, making the comparison possible. The images of the colored gels are seen in Figure 9.

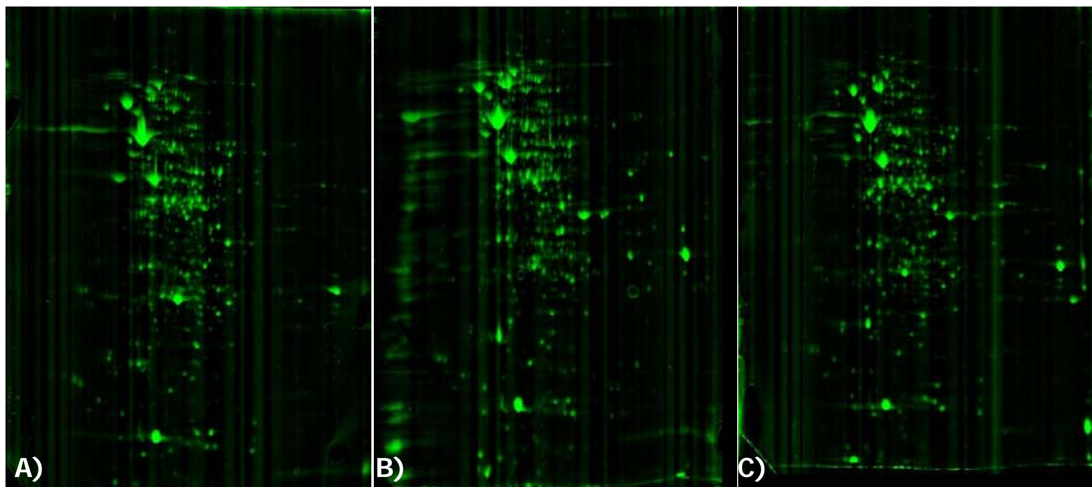

Figure 9. 2-DE colored gel images of *B. pertussis* **A)** Tohama I, **B)** Saadet and **C)** Nursel strains that were used for comparison.

3.3. Immunoblotting and Identification of Cross-Reactive Proteins from *B. pertussis* Strains

Immunogenic protein detection was made by performing Western Blot analysis. The antibodies which have been used for detection in these analyses were formed in mice immunized by subcutaneous injection of the experimental whole-cell vaccines. In immunogenic protein detection, the most crucial point is the kind of anti-sera used in Western Blotting. For this reason, we decided to classify our Western results as based on the anti-sera used.

Tohama I is a worldwide known strain of *B. pertussis*, which is studied all over the world and most of the proteins have recently been identified using proteomics approaches as described in many papers, such as those of Bottero *et al.* (2007), Vidakovics *et al.* (2007) and Serra *et al.* (2008).

Saadet and Nursel strains, which are domestic strains from Turkey, had been isolated at 1948 in Refik Saydam Central Institute for Hygiene, Department of Vaccine and Sera Production and Research, where they have been used for whole cell vaccine manufacture in the country for several years.

3.3.1. Cross-Reactivities with Anti-Tohama I Serum

Total soluble proteomes of *B. pertussis* Tahoma I, Saadet and Nursel strains have been resolved by 2-DE. After being stained with colloidal CBB G250, over 600 spots could be detected on the gels within the p*I* range 3–10. Western blotting of the 2-D gels using antisera obtained from Tohama I-immununized mice revealed a total of 61 immunoreactive protein spots (Figure 10).

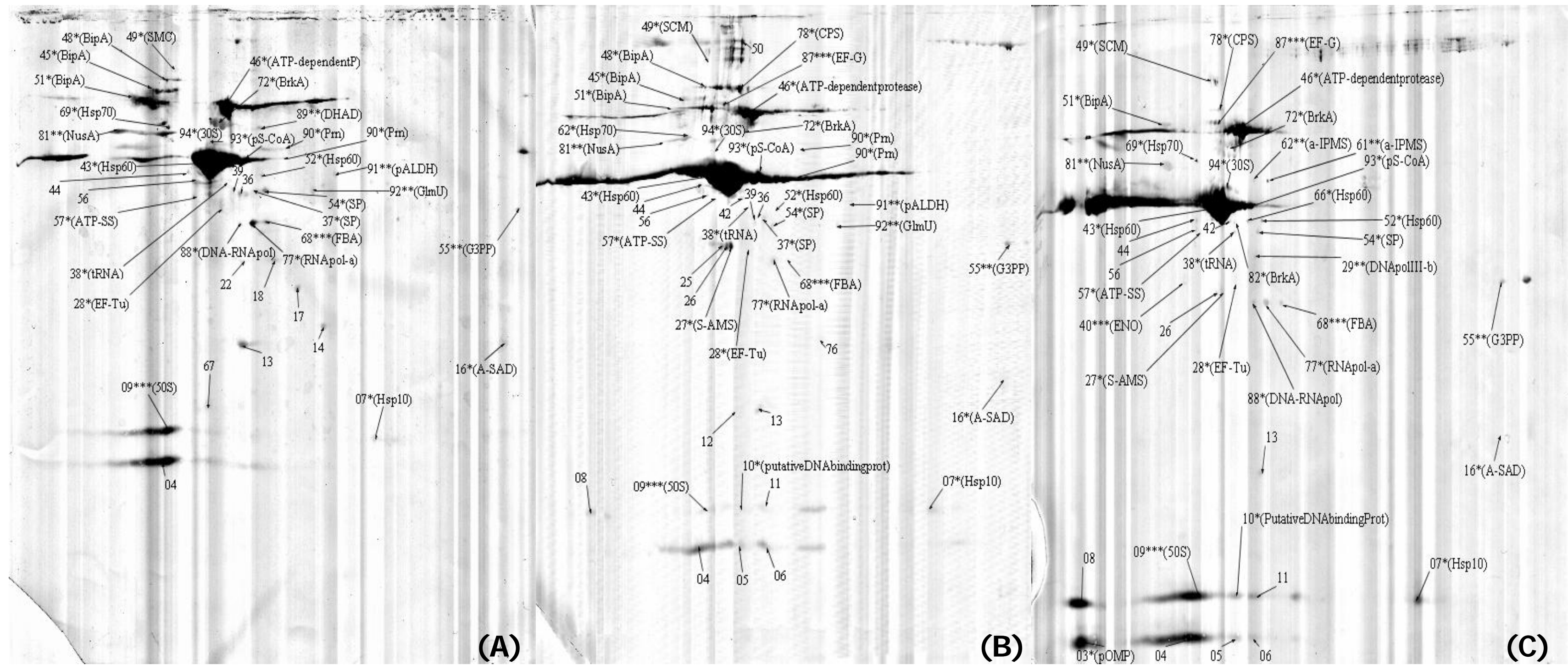

Figure 10. 2-D Western blot analysis of the total soluble proteome of *B. pertussis* **(A)** Tohama I, **(B)** Saadet, **(C)** Nursel against Anti-Tohama I serum. (*Proteins that were identified in the study of Altındiş *et al*. 2009, **Novel proteins found to be immunogenic in B. pertussis for the first time, *** Proteins that were identified for the first time in B. pertussis but previously identified in some other pathogen.)

Table 5. Immunogenic proteins of the total soluble proteome of *B. pertussis* Tohama I, Saadet and Nursel strains as identified against anti-Tohama I antibodies.

Spot No	Protein name/ function	Gene locus	Mass (kDa)	p*I*	Epitope predicton	Subcellular localization[a]	Signal peptide[b]	Strains		
								Tohama I	Saadet	Nursel
03	Putative outer membrane protein (pOMP)*	BB2614	20920.96	9.69	RKRREFQEDFNRRRNE	OM	+	-	-	+
04	Unidentified protein							+	+	+
05	Unidentified protein							-	+	+
06	Unidentified protein							-	+	+
07	Heat shock protein 10 (Hsp10)*	BPP0869	10265.62	5.39	AVGPGKKTEDGKILPV	C	-	+	+	+
08	Unidentified protein							-	+	+
09	50S ribosomal protein L7/L12 (50S)***	BPP0013	12787,92	4.9	KDLVDGAPKPVKEALP	ND	+	+	+	+
10	Putative DNA-binding protein*	BB2935	18512.33	6.19	MFMAQYTEEWNALDSI	ND	-	-	+	+
11	Unidentified protein							-	+	+
12	Unidentified protein							-	+	-
13	Unidentified protein							+	+	+
14	Unidentified protein							+	-	-
16	Aspartate-semialdehyde dehydrogenase (A-SAD)*	BPP1945	40340.78	5.08	AGTQWAKVVPNTKED T	ND	-	+	+	+
17	Unidentified protein							+	-	-
18	Unidentified protein							+	-	-
22	Unidentified protein							+	-	-
25	Unidentified protein							-	+	-
26	Unidentified protein							-	+	+
27	S-adenosylmethionine synthetase (S-AMS)*	BP3071	41976,16	5,12	TSESVSEGHPDKVADQ	C	-	-	+	+

Table 5. Continued

28	Elongation factor Tu (EF-Tu)*	BP3611	42889,12	5,34	PGSINPHTDFTAEVYI	C	-	+	+	+
29	DNA polymerase III, beta chain (DNApolIII-b)**	BP0490	41231,42	5,49	TLPILANILMRKEGNK	C	-	-	-	+
36	Unidentified protein							+	+	-
37,54	Serine protease (SP)*	BP2434	5210,42	7.79	PLTIGDPKTLKKGQWV	P	+	+	+	+
38	Glutamyl-tRNA amidotransferase subunit A (tRNA)*	BP0372	53383,87	5.37	KMLAGYASPFDATVVE	ND	-	+	+	+
39	Unidentified protein							+	+	-
40	Enolase (ENO)***	BPP3252	45885,05	4,72	KYPIISIEDGMAENDW	C	-	-	-	+
42	Unidentified protein							-	+	+
43,52, 66	Heat shock protein 60 (Hsp60)*	BP3495	53383,87	5,09	TGLKGDTADQNAGIKL	C	-	+	+	+
44	Unidentified protein							+	+	+
45,48, 51	Putative Outer Membrane Ligand Binding protein (BipA)*	BP1112	137111,3 1	6,26	ADTVDTTPPAVPTITD	OM	+	+	+	+
46	ATP-dependent protease, ATPase subunit*	BP1198	96274,57	5,37	RMEIDSKPEVMDRLDR	C	-	+	+	+
49	Putative chromosome partition protein (SCM)*	BP3558	130596,0 1	5,02	RELAEMPDEWRKASW L	C	-	-	+	-
50	Unidentified protein							-	+	-
55	Glycerol-3-phosphate-binding periplasmic protein (G3PP)**	BP1281	47667,19	8,67	HSMEGALGDRVNGLVE	P	+	+	+	+
57	ATP synthase subunit B (ATP-SS)*	BP3284	50498,80	4.94	SMIEQARREAEAERAR	ND	-	+	+	+
61,62	2-isopropylmalate synthase (a-IPMS)**	BP0131	63034,60	5,36	PVRIMDYHEHAIGTDT	C	-	-	-	+
66	Heat shock protein 60 (Hsp60)*	BP3495	53383,87	5,09	TGLKGDTADQNAGIKL	C	-	-	-	+
67	Unidentified protein							+	-	-
68	Fructose-biphosphate aldolase (FBA)***	BPP1192	38338,33	5,58	VEEIQEAIKFGVRKIN	C	-	+	+	+
69	Heat shock protein 70 (Hsp70)*	BP0074	71110,52	5,04	KIYYVTADTFTAAANS	C	-	+	+	+

Table 5. Continued

72,82	Serum resistance protein (BrkA)*	BP3494	103314,7	6.62	YSLAEDPKTHVWSLQR	OM	+	+	+	+
76	Unidentified protein							-	+	-
77	RNA polymerase alpha subunit (RNApol-a)*	BP3642	36136,08	5,6	NELLKTPNLGRKSLNE	C	-	+	+	+
78	Carbamoyl-phosphate synthase large chain (CPS)*	BP1453	118066,5 2	5,25	VEKIIEREKPDALLPT	ND	+	-	+	+
81	N Utilization substance protein A (NusA)**	BPP1861	54964,33	4,55	TILIATINSSTANCEP	C	-	+	+	+
87	Elongation Factor G (EF-G)**	BP3610	77066,84	5,15	AGEIQPMLCGTAFKNK	C	-	-	+	+
88	DNA-directed RNA polymerase alpha chain (DNA-RNA)*	BPP0057	36136,07	5,6	NELLKTPNLGRKSLNE	C	-	+	-	+
89	Dihydroxy-acid dehydratase (DHAD)**	BPP1861	54964,33	4,55	AELSTDELAEIAGLQE	C	-	+	-	-
90	Pertactin (Prn)*	BP1054	68644,38	6,92	TLTGGADAQGDIVATE	OM	+	+	+	-
91	Putative adenylate dehydrogenase (pALDH)**	BPP0040	51676,37	6,25	TWKRTSPMERAAILRK	C	-	+	+	-
92	Bifunctional protein glmU (GlmU)**	BPP4229	48355,33	6,61	VVGPYARLRPGADLGE	C	-	+	+	-
93	Putative substrate-CoA ligase (pS-CoA)*	BP0624	54538,05	5,62	CVALLAPPTPDAMVCL	C	-	+	+	+
94	30S ribosomal protein S1 (30S)*	BP0950	62979,00	5,10	KQLGEDPWVGLARRYP	C	-	+	+	+

[a] Predicted location of proteins by PSORTb version 2.0.4. C: Cytoplasm, OM: Outer membrane and ND: Not determined

[b] Signal peptide protein sequence are predicted using SignalP prediction program.

*, ** and *** as in the legend of Figure 10.

When immune reactions were demonstrated by using Tohama I proteins against anti-Tohama I antibodies, 39 out of 61 immunogenic protein spots were seen as shown in the Figure 10 (A). Among 39 immunogenic protein spots; 5 corresponded to three well-known antigens of the organism; pertactin (Spots 90), heat shock protein 60 (spot 43 and 52) and serum resistance protein (spot 72). The other 13 proteins have been detected and identified as immune reactive in the previous studies in our laboratory (Altındiş *et al.* 2009). 11 new proteins were identified in this work which corresponded to 8 gene products (ORFs). 13 out of 39 protein spots could not be identified, as seen in Table 5.

When Saadet proteins were subjected to anti-Tohama I antibodies, a total of 47 protein spots were detected as immunogenic in Western blot analysis, as seen in Figure 10 (B). 20 protein spots were already identified as immunogenic earlier in our laboratory (Altındiş *et al.* 2009). Among the other 27 proteins, 11 spots corresponded to 8 Bordetellae protein entries which comprised 2 known and 6 novel antigens, as listed in Table 5. 12 antigenic spots could not be identified.

When Nursel proteins were reacted with anti-Tohama I antibodies, a total of 42 protein spots were immunogenic (Figure 10, C). 21 of these spots were detected earlier as immunogenic proteins including heat shock protein 60 (spot no. 43, 52 and 66) and serum resistance protein (spot no. 72), as in Altındiş *et al.* 2009 (Table 5). Among these 21 protein spots, 10 remained undetermined and 11 protein spots corresponded to 10 gene products comprising 2 known and 8 novel antigens, respectively, as in Table 5.

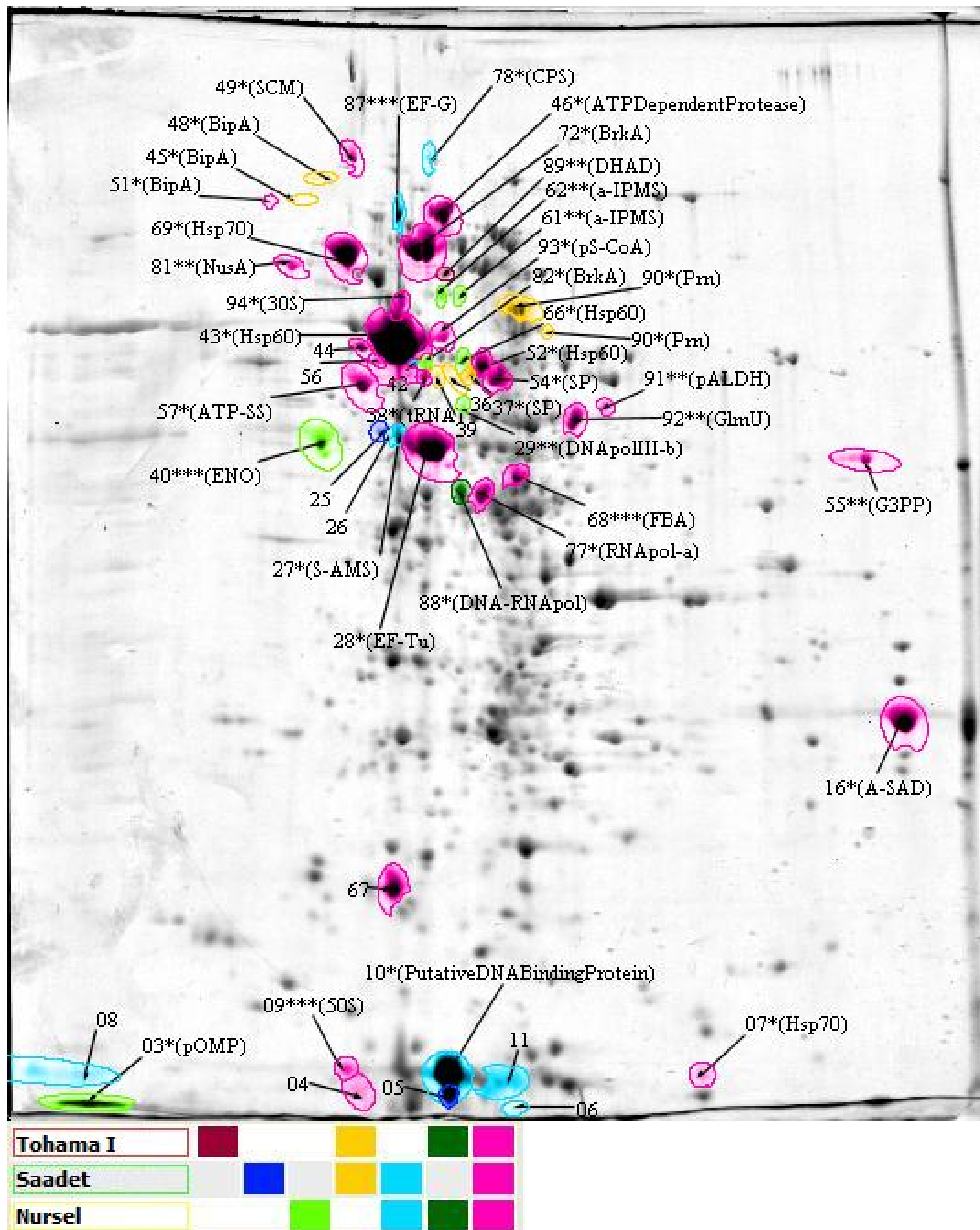

Figure 11. Fused gel for 2-D gel images for three different strains and colour coding according to the immunoreactivity of the proteins against anti-Tohama I serum. Immunogenic proteins form Tohama I strain (red), Saadet strain (dark blue) and Nursel strain (light green) were detected against anti-Tohama I antibodies. The immune reactive proteins that were common to Tohama I - Saadet, Saadet - Nursel and Tohama I - Nursel strains are shown in yellow, light blue and dark green, respectively. Pink color was used to visualize the common immunogenic spots for all three strains. *, ** and *** are as in the legend of Figure 10.

The spots numbered as 14, 17, 18, 22, 25, 67 and 76 were unidentified immunogenic ones that are listed in Table 6. Those detected in only one of the *B. pertussis* strain against anti-Tohama I antibodies are also included in this Table.

Table 6. Immunogenic proteins detected in only one of the *B. pertussis* strains, Tohama I or Saadet or Nursel, against anti-Tohama I antibodies.

Spot no	Protein name/ function	Strain
03	Putative outer membrane protein (pOMP)*	Nursel
14	Unidentified protein	Tohama I
17	Unidentified protein	Tohama I
18	Unidentified protein	Tohama I
22	Unidentified protein	Tohama I
25	Unidentified protein	Saadet
29	DNA polymerase III, beta chain (DNApolIII-b)**	Nursel
40	Enolase (ENO)***	Nursel
49	Putative chromosome partition protein (SCM)*	Saadet
50	Unidentified protein	Saadet
61,62	2-isopropylmalate synthase (a-IPMS)**	Nursel
66	Heat shock protein 60 (Hsp60)*	Nursel
67	Unidentified protein	Tohama I
76	Unidentified protein	Saadet
89	Dihydroxy-acid dehydratase (DHAD)**	Tohama I

*, ** and *** are as in the legend of Figure 10.

The general information about the proteins which were identified in the present study (as displayed in Table 6) are below;

DNA Polymerase III- Beta Chain;

This was a protein identified as immunogenic among Nursel antigens, reacting against anti-Tohama I antibodies and among Saadet antigens reacting against anti-Saadet antibodies (Figure 10, Table 6).

DNA polymerase III (EC 2.7.7.-) is the primary enzyme complex involved in prokaryotic DNA replication. The complex has high processivity, specifically

referring to the replication of the *E.coli* genome, works in conjunction with four other DNA polymerases (Pol I, Pol II, Pol IV, and Pol V). The DNA Pol III holoenzyme also has proofreading capabilities that correct replication mistakes by means of exonuclease activity working 3'->5'. DNA Pol III is a component of the replisome, which is located at the replication fork. One of the components of replisome is Beta units which act as sliding DNA clamps, keeping the polymerase bound to the DNA (Heltzer *et al.*, 2009)

In the literature, the studies have mostly been performed using *E. coli*, focusing on the function of the enzyme and the homodimeric clamps structure (Neuwald *et al.*, 2003). Our study was the first implication of the existence of this protein in immunoproteome of a pathogenic bacterium.

Enolase;

This was a more common protein identified as immunogenic among Nursel antigens, reacting against anti-Tohama I antibodies, among Tohama I and Saadet antigens reacting against anti-Nursel antibodies and among Tohama I antigens reacting against anti-Saadet antibodies (Figure 10, Table 6).

Enolase (EC 4.2.1.11), also known as phosphopyruvate dehydratase, is a metalloenzyme responsible for the catalysis of 2-phosphoglycerate (2-PG) to phosphoenolpyruvate (PEP), the ninth and penultimate step of glycolysis. Enolase is present in all tissues and organisms capable of glycolysis or fermentation. Enolases have been studied and found to be highly conserved from a variety of sources, including bacteria, yeasts, drosophila, amphibians, birds, plants, and humans (Van Der Straeten *et al.*, 1991). In addition to its well-known glycolytic function, enolase comprises function as a toxin in bacteria (Bisseret *et al.*, 1989).

In *Saccharomyces cerevisiae*, enolase and other glycolytic enzymes are the most abundant proteins in the cell. Enolase from *S. cerevisiae* is the most

studied of all enolases with respect both to biochemical and biophysical characteristics and to genetic regulation (Maitra *et al.*, 1971; Holland *et al.*, 1978). In further studies, it has been discovered that enolase has potential as a vaccine candidate for *Streptococcus suis* infections (Feng *et al.*, 2009). Moreover, studies have also been done with several other bacteria; such as, *Streptococcus gordonii* (Kesimer *et al.*, 2009) and *Lactobacillus acidophilus* (Prangli *et al.*, 2009). Present study constitutes the first information on the immunogenicity of cytoplasmic enolase in *B. pertussis* strains.

2- isopropylmalate synthase;

This was a protein identified as immunogenic among Nursel antigens, reacting against anti-Tohama I antibodies, among Tohama I; Saadet and Nursel antigens reacting against anti-Nursel antibodies and among Nursel antigens reacting against anti-Saadet antibodies (Figure 10, Table 6).

2-Isopropylmalate synthase, alpha-isopropylmalate synthase or a-IPMS (EC 2.3.3.13), is an allosteric enzyme that plays a very important role of leucine biosynthesis and pyruvate metabolism in many kinds of microorganisms and plants. The enzyme, encoded by leuA, catalyses the first step of the isopropylmalate (IPM) pathway where a-IPM is formed by aldol condensation of acetyl coenzyme A (CoA) and a-ketoisovalerate (Cole *et al.*, 1973)

a-IPMS is present in various kinds of microorganisms, for example *Alcaligenes eutrophus* (Wiegel & Schlegel, 1977), *Salmonella typhimurium*, *E. coli* (Kohlhaw & Leary, 1969; Leary & Kohlhaw, 1972), *Bacillus subtilis* (Fink, 1993), *Corynebacterium glutamicum* (Patek *et al.*, 1994), *Saccharomyces cerevisiae* (Beltzer *et al.*, 1986; Kohlhaw, 1988) *Bacteroides fragilis*, *Clostridium thermoaceticum*, *Clostridium formicoaceticum*, *Clostridium pasteurianum*, and *Clostridium kluyveri* (Weigel, 1981) and *Mycobacterium*

tuberculosis (Namwat *et al*., 1998; Smittipat & Palittapongarnpim, 2000). Our study constitutes the first report on the immunogenicity of a-IPMS in a pathogenic bacterium.

<u>Dihydroxy Acid Dehydratase;</u>

This was a protein identified as immunogenic only among Tohama I antigens, reacting only against anti-Tohama I antibodies (Figure 10, Table 6).

Dihydroxy acid dehydratase; (DHAD) (EC 4.2.1.9) belongs to the family of lyases, specifically the hydro-lyases, which cleave carbon-oxygen bonds. The systematic name of this enzyme class is 2,3-dihydroxy-acid hydrolyase. DHAD participates in valine, leucine and isoleucine biosynthesis and pantothenate and CoA biosynthesis (Kanamori *et al*., 1968).

DHAD was identified in the cellular proteome of *Penicillum expansum* of the fungal pathogens (Oui *et al*., 2007). The enzyme has been purified and characterized in various mesophiles, including bacteria and eukarya; *Sulfolobus solfataricus*, *E*. *coli* (Kuo *et al*.,1987), *Salmonella typhimurium* (Armstrong *et al*., 1977), *Methanococcus spp*. (Xing and Whitman, 1991), *Phaseolus radiatus* (Satyanarayana and Radhakrishnan, 1964), *Neurospora crassa* (Kiritani *et al*., 1966). The present study constitutes the first report on the immunogenicity of DHAD in a pathogen bacterium.

3.3.2. Cross-Reactivities with Anti-Saadet Serum

Total soluble proteomes of *B*. *pertussis* Tahoma I, Saadet and Nursel strains were resolved by 2-DE. After being stained with colloidal CBB G250, over 600 spots could be detected on the gels within the p*I* range 3–10. Western blotting of the 2-D gels using antisera obtained from Saadet immunized mice revealed a total of 72 different immunoreactive protein spots (Figure 12).

When immune reactions were demonstrated by using Tohama I proteins against anti-Saadet antibodies, 57 out of 72 immunogenic protein spots were seen as shown in the Figure 12 (A). Among 57 immunogenic protein spots; 5 corresponded to three well-known antigens of the organism; pertactin (Spots 90), heat shock protein 60 (spot 43 and 52) and serum resistance protein (spot 72). The other 19 proteins have been detected and identified as immune reactive in the previous studies in our laboratory (Altındiş *et al.* 2009). 9 new proteins were identified in this work which corresponded to 7 gene products (ORFs). 24 out of 57 protein spots could not be identified, as seen in Table 7.

When Saadet proteins were subjected to anti-Saadet antibodies, a total of 62 protein spots were detected as immunogenic in Western blot analysis, as seen in Figure 12 (B). 23 protein spots were already identified as immunogenic earlier in our laboratory (Altındiş *et al*. 2009). Among the other 39 proteins, 8 spots corresponded to 6 Bordetellae protein entries which comprised 2 known and 4 novel antigens, as listed in Table 7. 21 antigenic spots could not be identified.

When Nursel proteins were reacted with anti-Saadet antibodies, a total of 41 protein spots were immunogenic (Figure 12, C). 15 of these spots were detected earlier as immunogenic proteins in Altındiş *et al.* 2009 (Table 7). Among these 41 protein spots, 14 remained undetermined and 7 proteins were identified, as in Table 7.

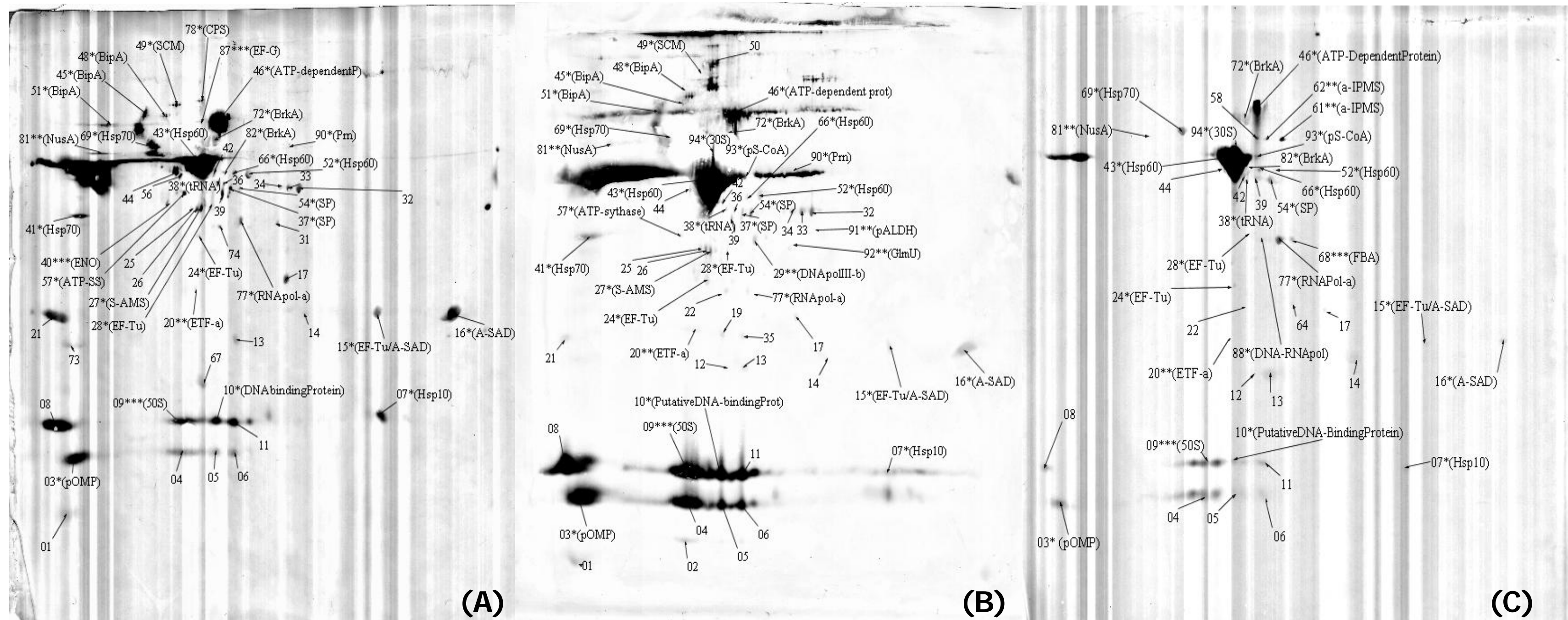

Figure 12. 2-D Western blot analysis of the total soluble proteome of *B. pertussis* **(A)** Tohama I, **(B)** Saadet, **(C)** Nursel against Anti-Saadet I serum. *, ** and *** are as in the legend of Figure 10.

Table 7. Immunogenic proteins of the total soluble proteome of *B. pertussis* Tohama I, Saadet and Nursel strains as identified against anti-Saadet antibodies.

Spot No	Protein name/ function	Gene locus	Mass (kDa)	p*I*	Epitope predicton	Subcellular localization[a]	Signal peptide[b]	Strains		
								Tohama I	Saadet	Nursel
01	Unidentified protein							+	+	-
02	Unidentified protein							-	+	-
03	Putative outer membrane protein (pOMP)*	BB2614	20920.96	9.69	RKRREFQEDFNRRRNE	OM	+	+	+	+
04	Unidentified protein							+	+	+
05	Unidentified protein							+	+	+
06	Unidentified protein							+	+	+
07	Heat shock protein 10 (Hsp10)*	BPP0869	10265.62	5.39	AVGPGKKTEDGKILPV	C	-	+	+	+
08	Unidentified protein							+	+	+
09	50S ribosomal protein L7/L12 (50S)***	BPP0013	12787,92	4.9	KDLVDGAPKPVKEALP	ND	+	+	+	+
10	Putative DNA-binding protein*	BB2935	18512.33	6.19	MFMAQYTEEWNALDSI	ND	-	+	+	+
11	Unidentified protein							+	+	+
12	Unidentified protein							-	+	+
13	Unidentified protein							+	+	+
14	Unidentified protein							+	+	+
15,16	Aspartate-semialdehyde dehydrogenase (A-SAD)*	BPP1945	40340.78	5.08	AGTQWAKVVPNTKEDT	ND	-	+	+	+
17	Unidentified protein							+	-	-
19	Unidentified protein							-	+	-
20	Electron transfer flavoprotein alpha-subunit (ETF-a)**	BP0962	31000,41	5.13	AVGISGAIQHLAGMKD	ND	-	+	+	+
21	Unidentified protein							+	+	-

Table 7. Continued

22	Unidentified protein							-	+	+
24,15,28	Elongation factor-Tu (EF-Tu)*	BP3611	42889,12	5,34	PGSINPHTDFTAEVYI	C	-	+	+	+
25	Unidentified protein							+	+	-
26	Unidentified protein							+	+	-
27	S-adenosylmethionine synthetase (S-AMS)*	BP3071	41976,16	5,12	TSESVSEGHPDKVADQ	C	-	+	+	-
29	DNA polymerase III, beta chain (DNApolIII-b)**	BP0490	41231,42	5,49	TLPILANILMRKEGNK	C	-	-	+	-
31	Unidentified protein							+	-	-
32	Unidentified protein							+	+	-
33	Unidentified protein							+	+	-
34	Unidentified protein							+	+	-
35	Unidentified protein							-	+	-
36	Unidentified protein							+	+	-
37	Serine protease (SP)*	BP2434	5210,42	7.79	PLTIGDPKTLKKGQWV	P	+	+	+	-
38	Glutamyl-tRNA amidotransferase subunit A (tRNA)*	BP0372	53383,87	5.37	KMLAGYASPFDATVVE	ND	-	+	+	+
39	Unidentified protein							+	+	+
40	Enolase (ENO)***	BPP3252	45885,05	4,72	KYPIISIEDGMAENDW	C	-	+	-	-
41	Heat shock protein 70 (Hsp10)*	BP0074	71110,52	5,04	KIYYVTADTFTAAANS	C	-	+	+	-
42	Unidentified protein							+	+	+
43,52,66	Heat shock protein 60 (Hsp60)*	BP3495	53383,87	5,09	TGLKGDTADQNAGIKL	C	-	+	+	+
44	Unidentified protein							+	+	+
45,48,51	Putative outer membrane ligand binding protein (BipA)*	BP1112	137111,31	6,26	ADTVDTTPPAVPTITD	OM	+	+	+	-
46	ATP-dependent protease, ATPase subunit*	BP1198	96274,57	5,37	RMEIDSKPEVMDRLDR	C	-	+	+	+
49	Putative chromosome partition protein (SCM)*	BP3558	130596,01	5,02	RELAEMPDEWRKASWL	C	-	+	+	-
50	Unidentified protein							-	+	-
54	Serine protease (SP)*	BP2434	5210,42	7,79	PLTIGDPKTLKKGQWV	P	+	+	+	+

Table 7. Continued

56	Unidentified protein							+	-	-
57	ATP synthase subunit B (ATP-SS)*	BP3284	50498,80	4.94	SMIEQARREAEAERAR	ND	-	+	+	-
58	Unidentified protein							-	-	+
61,62	2-isopropylmalate synthase (a-IPMS)**	BP0131	63034,60	5,36	PVRIMDYHEHAIGTDT	C	-	-	-	+
64	Unidentified protein							-	-	+
67	Unidentified protein							+	-	-
68	Fructose-biphosphate aldolase (FBA)***	BPP1192	38338,33	5,58	VEEIQEAIKFGVRKIN	C	-	-	-	+
69	Heat shock protein 70 (Hsp70)*	BP0074	71110,52	5,04	KIYYVTADTFTAAANS	C	-	+	+	+
72,82	Serum resistance protein (BrkA)*	BP3494	103314,7	6.62	YSLAEDPKTHVWSLQR	OM	+	+	+	+
73	Unidentified protein							+	-	-
74	Unidentified protein							+	-	-
77	RNA polymerase alpha subunit*	BP3642	36136,08	5,6	NELLKTPNLGRKSLNE	C	-	+	+	+
78	Carbamoyl-phosphate synthase large chain (CPS)*	BP1453	118066,52	5,25	VEKIIEREKPDALLPT	ND	+	+	-	-
81	N Utilization substance protein A (NusA)**	BPP1861	54964,33	4,55	TILIATINSSTANCEP	C	-	+	+	+
87	Elongation Factor G (EF-G)***	BP3610	77066,84	5,15	AGEIQPMLCGTAFKNK	C	-	+	-	-
88	DNA-directed RNA polymerase alpha chain (DNA-RNA)*	BPP0057	36136,07	5,6	NELLKTPNLGRKSLNE	C	-	+	-	-
90	Pertactin (Prn)*	BP1054	68644,38	6,92	TLTGGADAQGDIVATE	OM	+	+	+	-
91	Putative adenylate dehydrogenase (pALDH)**	BPP0040	51676,37	6,25	TWKRTSPMERAAILRK	C	-	-	+	-
92	Bifunctional protein glmU (GlmU)**	BPP4229	48355,33	6,61	VVGPYARLRPGADLGE	C	-	-	+	-
93	Putative substrate-CoA ligase (pS-CoA)*	BP0624	54538,05	5,62	CVALLAPPTPDAMVCL	C	-	-	+	+
94	30S ribosomal protein S1 (30S)*	BP0950	62979,00	5,10	KQLGEDPWVGLARRYP	C	-	+	+	+

[a] Predicted location of proteins by PSORTb version 2.0.4. C: Cytoplasm, OM: Outer membrane and ND: Not determined
[b] Signal peptide protein sequence are predicted using SignalP prediction program.
*, ** and *** as in the legend of Figure 10.

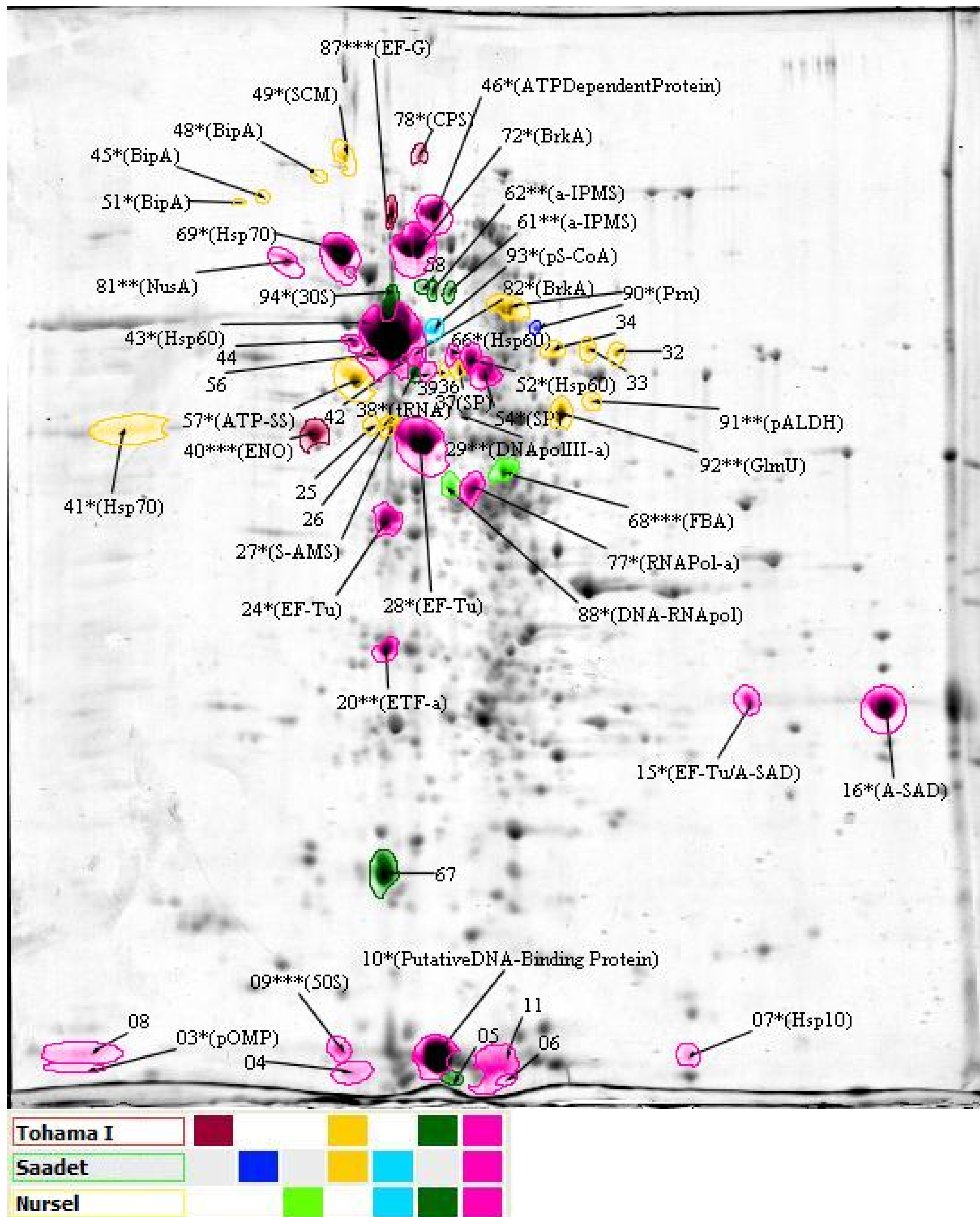

Figure 13. Fused gel for 2-D gel images for three different strains and colour coding according to the immunoreactivity of the proteins against anti-Saadet serum. Immunogenic proteins form Tohama I strain (red), Saadet strain (dark blue) and Nursel strain (light green) were detected against anti-Saadet antibodies. The immune reactive proteins that were common to Tohama I - Saadet, Saadet - Nursel and Tohama I - Nursel strains are shown in yellow, light blue and dark green, respectively. Pink color was used to visualize the common immunogenic spots for all three strains. *, ** and *** are as in the legend of Figure 10.

The spots numbered as 02, 17, 19, 31, 35, 50, 56, 58, 64, 67, 73 and 74 were unidentified immunogenic ones that are listed in Table 8. Those detected in only one of the *B. pertussis* strain against anti-Saadet antibodies are also included in this Table.

Table 8. Immunogenic proteins detected in only one of the *B. pertussis* strains, Tohama I or Saadet or Nursel, against anti-Saadet antibodies.

Spot no	Protein name/ function	Strain
02	Unidentified protein	Saadet
17	Unidentified protein	Tohama I
19	Unidentified protein	Saadet
29	DNA polymerase III, beta chain (DNApolIII-b)**	Saadet
31	Unidentified protein	Tohama I
35	Unidentified protein	Saadet
40	Enolase (ENO)***	Tohama I
50	Unidentified protein	Saadet
56	Unidentified protein	Tohama I
58	Unidentified protein	Nursel
61,62	2-isopropylmalate synthase (a-IPMS)**	Nursel
64	Unidentified protein	Nursel
67	Unidentified protein	Tohama I
68	Fructose-biphosphate aldolase (FBA)***	Nursel
73	Unidentified protein	Tohama I
74	Unidentified protein	Tohama I
78	Carbamoyl-phosphate synthase large chain (CPS)*	Tohama I
87	Elongation factor G (EF-G)***	Tohama I
88	DNA-directed RNA polymerase alpha chain (DNA-RNA)*	Tohama I
91	Putative adenylate dehydrogenase (pALDH)**	Saadet
92	Bifunctional protein glmU (GlmU)**	Saadet

*, ** and *** are as in the legend of Figure 10.

The general information about the proteins which were identified in the present section of the study (as shown in Table 8) are given below of the proteins, DNA polymerase III, beta chain, Enolase and 2-isopropylmalate synthase were already presented in Chapter 3.3.1.

Fructose Bisphosphate Aldolase (FBA);

This was a common protein identified as immunogenic among Nursel antigens, reacting against anti-Saadet antibodies, among Tohama I, Saadet and Nursel antigens reacting against anti-Tohama I antibodies and among Tohama I antigens reacting against anti-Nursel antibodies (Figure12, Table 7).

Fructose bisphosphate aldolase (EC 4.1.2.13) is of the lyase class that catalyzes the cleavage of fructose 1,6-biphosphate to form dihydroxyacetone phosphate and glyceraldehyde 3-phosphate. The enzyme also acts on (3S,4R)-ketose 1-phosphates. The yeast and bacterial enzymes are zinc proteins. There are two classes of fructose-1,6-bisphosphate aldolases class I aldolases, present in animals and plants, and class II aldolases which present in fungi and bacteria. Class I fructose 1,6 bisphosphate aldolases were once thought to be confined to eukaryotic organisms but have since been detected in several bacterial species. Class II aldolase; catalyzes the reversible aldol condensation of dihydroxyacetonephosphate and glyceraldehyde 3-phosphate in the Calvin cycle, glycolysis and gluconeogenesis (Baldwin *et al.*, 1978).

FBA was identified as immunogenic in the proteome analysis of *Streptococcus pneumoniae* by Ling *et al.*, 2004, and *Streptococcus suis* by Wu *et al.*, 2008. This study constitutes the first report on the immunogenicity of FBA in *B. pertussis*.

Elongation Factor G (EF-G);

This was a protein identified as immunogenic among Tohama I antigens, reacting against anti-Saadet antibodies, among Saadet and Nursel antigens reacting against anti-Tohama I antibodies and among Tohama I and Saadet antigens reacting against anti-Nursel antibodies(Figure12, Table 7).

Elongation factor G (EF-G) and elongation factor Tu (EF-Tu) (EC 3.6.1.-) are two GTP-driven elongation factors, most abundant proteins in prokaryotes. They are required to add new amino acid to the growing polypeptide chain, during protein biosynthesis by binding alternately to the the ribosomal GTPase centre located between the 50S and 30S subunits. In the elongation process, EF-Tu catalyzes the binding of each aminoacyl-tRNA to the ribosomal "A" site. Once the peptide bond has been formed, EF-G translocates the peptidyl-tRNA from the "A" site to the "P" site (and moves the mRNA through the ribosome) in preparation for the next catalytic cycle. As multifunctional proteins, they interact with several macromolecules and guanine nucleotides, including GDP, GTP and some ribosomal proteins.

Inhibition of elongation factor G at the level of the ribosome is used as a target of bacteriostatics, such as fusidic acid. Fusidic acid is active against Gram-positive anaerobic activity, and it shows in vitro activity against *Neisseria spp*. and *Moraxella catarrhalis* (Collegon *et al*., 1999).

Immunogenicity of elongation factors was recently shown by using immunoproteomics for *Staphylococcus epidermidis* (Sellman *et al*., 2005), *Anaplasma marginale* (Brown *et al*., 2005), *Mycoplasma hyopneumoniae* (Marcos *et al*., 2006) and *Lactococcus garvieae* (Shin *et al*., 2007). The presence and immunoreactivity of this protein was shown in sporadic forms of *Bacillus spp*. as well(DelVecchio *et al*., 2006). Our study constitutes the first report on the immunogenicity of elongation factor G in *B. pertussis*.

Aldehyde Dehydrogenase (ALDH);

This was a protein identified as immunogenic among Saadet antigens, reacting against anti-Saadet antibodies, among Tohama I and Saadet antigens reacting against anti-Tohama I antibodies and among Saadet antigens reacting against anti-Nursel antibodies (Figure12, Table 7).

Aldehyde dehydrogenases (EC 1.2.1.3) are a group of enzymes that catalyse the oxidation (dehydrogenation) of aldehydes.

It has been known that certain bacteria; *E. coli*, *Klebsella pneumoniae*, *Klebsiella oxytoca*, *Hafnia alvei*, *Proteus mirabilis* and *Pseudomonas aeruginosa* metabolize acetaldehyde by aldehyde dehydrogenase (ALDH) (Nosova *et al.*, 1996). Our study constituted report on the first the existence of this protein in the immunoproteome of a pathogen.

<u>Bifunctional Protein GlmU;</u>

This was a protein identified as immunogenic among Saadet antigens, reacting against anti-Saadet antibodies, among Tohama I and Saadet antigens reacting against anti-Tohama I antibodies and among Saadet antigens reacting against anti-Nursel antibodies (Figure12, Table 7).

Bifunctional Protein GlmU is a bifunctional enzyme catalyzing the acetylation of glucosamine-1-phosphate and uridylylation of N-acetylglucosamine-1-phosphate to produce UDP-GlcNAc. The intermediary metabolite UDP-N-acetylglucosamine (UDP-GlcNAc) is of central importance to all organisms and is an essential precursor for bacterial biosynthesis of peptidoglycan, lipopolysaccharide, enterobacterial common antigen and teichoic acids (Varon *et al.*, 1993).

Up to date, the studies on this protein were mostly done using *E. coli* (Olsen *et al.*, 2001) and *Mycobacterium tuberculosis* (Verma *et al.*, 2009). This study constitutes the first report on the immunogenicity of GlmU in a pathogenic bacterium.

3.3.3. Cross-Reactivities with Anti-Nursel Serum

Total soluble proteomes of *B. pertussis* Tahoma I, Saadet and Nursel strains were resolved by 2-DE. After being stained with colloidal CBB G250, over 600 spots could be detected on the gels within the p*I* range 3–10. Western blotting of the 2-D gels using antisera obtained from Nursel immunized mice revealed a total of 59 different immunoreactive protein spots (Figure 14, Table 9), *in vitro*.

When immune reactions were demonstrated by using Tohama I proteins against anti-Nursel antibodies, 48 out of 59 immunogenic protein spots were seen as shown in the Figure 14 (A). Among 48 immunogenic protein spots; 5 corresponded to three well-known antigens of the organism; pertactin (Spots 90), heat shock protein 60 (spot 43 and 52) and serum resistance protein (spot 72). The other 22 proteins have been detected and identified as immune reactive in the previous studies in our laboratory (Altındiş *et al.* 2009). 11 new proteins were identified in this work which corresponded to 8 gene products (ORFs). 12 out of 48 protein spots could not be identified, as seen in Table 9.

When Saadet proteins were subjected to anti-Nursel antibodies, a total of 44 protein spots were detected as immunogenic in Western blot analysis, as seen in Figure 14 (B). 17 protein spots were already identified as immunogenic earlier in our laboratory (Altındiş *et al*. 2009). Among the other 27 proteins, 14 spots corresponded to 10 Bordetellae protein entries which comprised 2 known and 8 novel antigens, as listed in Table 9. 10 antigenic spots could not be identified.

When Nursel proteins were reacted with anti-Nursel antibodies, a total of 34 protein spots were immunogenic (Figure 14, C). 12 of these spots were detected earlier as immunogenic proteins including heat shock protein 60

(spot no. 43, 52 and 66) and serum resistance protein (spot no. 72), as in Altındiş *et al.* 2009 (Table 5). Among these 34 protein spots, 9 remained undetermined and 9 protein have been identified, as in Table 9.

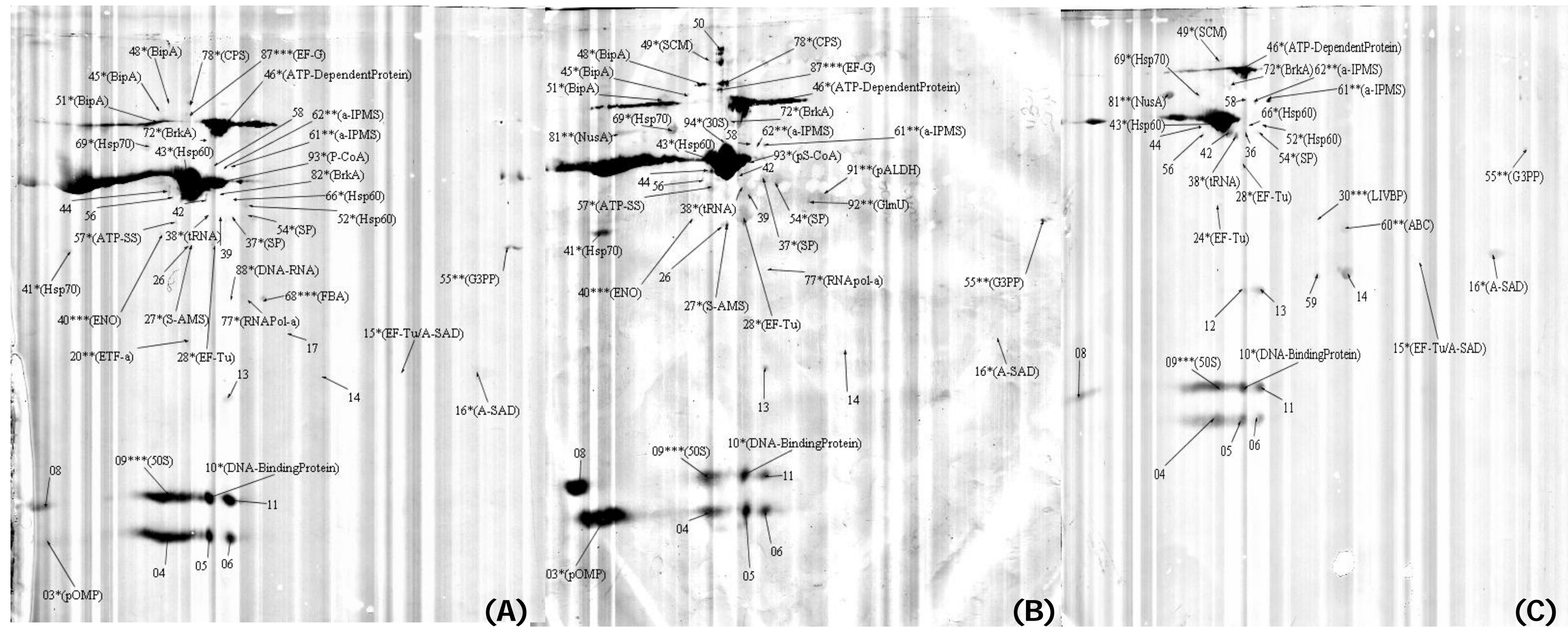

Figure 14. 2-D Western blot analysis of the total soluble proteome of *B. pertussis* **(A)** Tohama I, **(B)** Saadet, **(C)** Nursel against Anti-Nursel I serum. *, ** and *** are as in the legend of Figure 10.

Table 9. Immunogenic proteins of the total soluble proteome of *B. pertussis* Tohama I, Saadet and Nursel strains as identified against anti-Nursel antibodies.

Spot No	Protein name/ function	Gene locus	Mass (kDa)	p*I*	Epitope predicton	Subcellular localization[a]	Signal peptide[b]	Strains		
								Tohama I	Saadet	Nursel
03	Putative outer membrane protein (pOMP)*	BB2614	20920.96	9.69	RKRREFQEDFNRRRNE	OM	+	+	+	-
04	Unidentified protein							+	+	+
05	Unidentified protein							+	+	+
06	Unidentified protein							+	+	+
08	Unidentified protein							+	+	+
09	50S ribosomal protein L7/L12 (50S)***	BPP0013	12787,92	4.9	KDLVDGAPKPVKEALP	ND	+	+	+	+
10	Putative DNA-binding protein*	BB2935	18512.33	6.19	MFMAQYTEEWNALDSI	ND	-	+	+	+
11	Unidentified protein							+	+	+
12	Unidentified protein							-	-	+
13	Unidentified protein							+	+	+
14	Unidentified protein							+	+	+
15,16	Aspartate-semialdehyde dehydrogenase (A-SAD)*	BPP1945	40340.78	5.08	AGTQWAKVVPNTKEDT	ND	-	+	-	+
17	Unidentified protein							+	-	-
20	Electron transfer flavoprotein alpha-subunit (ETF-a)**	BP0962	31000,41	5.13	AVGISGAIQHLAGMKD	ND	-	+	-	-
24,28	Elongation factor-Tu (EF-Tu)*	BP3611	42889,12	5,34	PGSINPHTDFTAEVYI	C	-	-	-	+
26	Unidentified protein							+	+	-
27	S-adenosylmethionine synthetase (S-AMS)*	BP3071	41976,16	5,12	TSESVSEGHPDKVADQ	C	-	+	+	-
30	Leu/Ile/Val binding protein (LIVBP)***	BPP2941	39383,5	6,67	PILIDDGCEPKQAVPA	P	+	-	-	+
36	Unidentified protein							-	-	+
37	Serine protease (SP)*	BP2434	5210,42	7.79	PLTIGDPKTLKKGQWV	P	+	+	+	-

Table 9. Continued

38	Glutamyl-tRNA amidotransferase subunit A (tRNA)*	BP0372	53383,87	5.37	KMLAGYASPFDATVVE	ND	-	+	+	+
39	Unidentified protein							+	+	-
40	Enolase (ENO)**	BPP3252	45885,05	4,72	KYPIISIEDGMAENDW	C	-	+	+	-
41	Heat shock protein 70 (Hsp70)*	BP0074	71110,52	5,04	KIYYVTADTFTAAANS	C	-	+	+	-
42	Unidentified protein							+	+	+
44	Unidentified protein							+	+	+
45,48,51	Putative outer membrane ligand binding protein (BipA)*	BP1112	137111,31	6,26	ADTVDTTPPAVPTITD	OM	+	+	+	-
46	ATP-dependent protease, ATPase subunit*	BP1198	96274,57	5,37	RMEIDSKPEVMDRLDR	C	-	+	+	+
49	Putative chromosome partition protein (SCM)*	BP3558	130596,01	5,02	RELAEMPDEWRKASWL	C	-	-	+	+
50	Unidentified protein							-	+	-
43,52,66	Heat shock protein 60 (Hsp60)*	BP3495	53383,87	5,09	TGLKGDTADQNAGIKL	C	-	+	+	+
54	Serine protease (SP)*	BP2434	5210,42	7.79	PLTIGDPKTLKKGQWV	P	+	+	+	+
55	Glycerol-3-phosphate-binding periplasmic protein (G3PP)**	BP1281	47667,19	8,67	HSMEGALGDRVNGLVE	P	+	+	+	+
56	Unidentified protein							+	+	+
57	ATP synthase subunit B*	BP3284	50498,80	4.94	SMIEQARREAEAERAR	ND	-	+	+	-
58	Unidentified protein							+	+	+
59	Unidentified protein							-	-	+
60	Putative ABC transporter periplasmic amino acid binding protein (ABC)**	BPP3975	36654,53	8,2	IVRAFSAGRCDAFTTD	P	+	-	-	+
61,62	2-isopropylmalate sythase (a-IPMS)**	BP0131	63034,60	5,36	PVRIMDYHEHAIGTDT	C	-	+	+	+
68	Fructose-biphosphate aldolase (FBA)***	BPP1192	38338,33	5,58	VEEIQEAIKFGVRKIN	C	-	+	-	-
69	Heat shock protein 70 (Hsp70)*	BP0074	71110,52	5,04	KIYYVTADTFTAAANS	C	-	+	+	+
72,82	Serum resistance protein (BrkA)*	BP3494	103314,7	6.62	YSLAEDPKTHVWSLQR	OM	+	+	+	+
77	RNA polymerase alpha subunit*	BP3642	36136,08	5,6	NELLKTPNLGRKSLNE	C	-	+	+	-

Table 9. Continued

78	Carbamoyl-phosphate synthase large chain (CPS)*	BP1453	118066,52	5,25	VEKIIEREKPDALLPT	ND	+	+	+	-
81	N Utilization substance protein A (NusA)**	BPP1861	54964,33	4,55	TILIATINSSTANCEP	C	-	-	+	+
87	Elongation factor G (EF-G)***	BP3610	77066,84	5,15	AGEIQPMLCGTAFKNK	C	-	+	+	-
88	DNA-directed RNA polymerase alpha chain (DNA-RNA)*	BPP0057	36136,07	5,6	NELLKTPNLGRKSLNE	C	-	+	-	-
91	Putative adenylate dehydrogenase (pALDH)**	BPP0040	51676,37	6,25	TWKRTSPMERAAILRK	C	-	-	+	-
92	Bifunctional protein glmU (GlmU)**	BPP4229	48355,33	6,61	VVGPYARLRPGADLGE	C	-	-	+	-
93	Putative substrate-CoA ligase (pS-CoA)*	BP0624	54538,05	5,62	CVALLAPPTPDAMVCL	C	-	+	+	-
94	30S ribosomal protein S1 (30S)*	BP0950	62979,00	5,10	KQLGEDPWVGLARRYP	C	-	-	+	-

[a] Predicted location of proteins by PSORTb version 2.0.4. C: Cytoplasm, OM: Outer membrane and ND: Not determined

[b] Signal peptide protein sequence are predicted using SignalP prediction program.

*, ** and *** as in the legend of Figure 10.

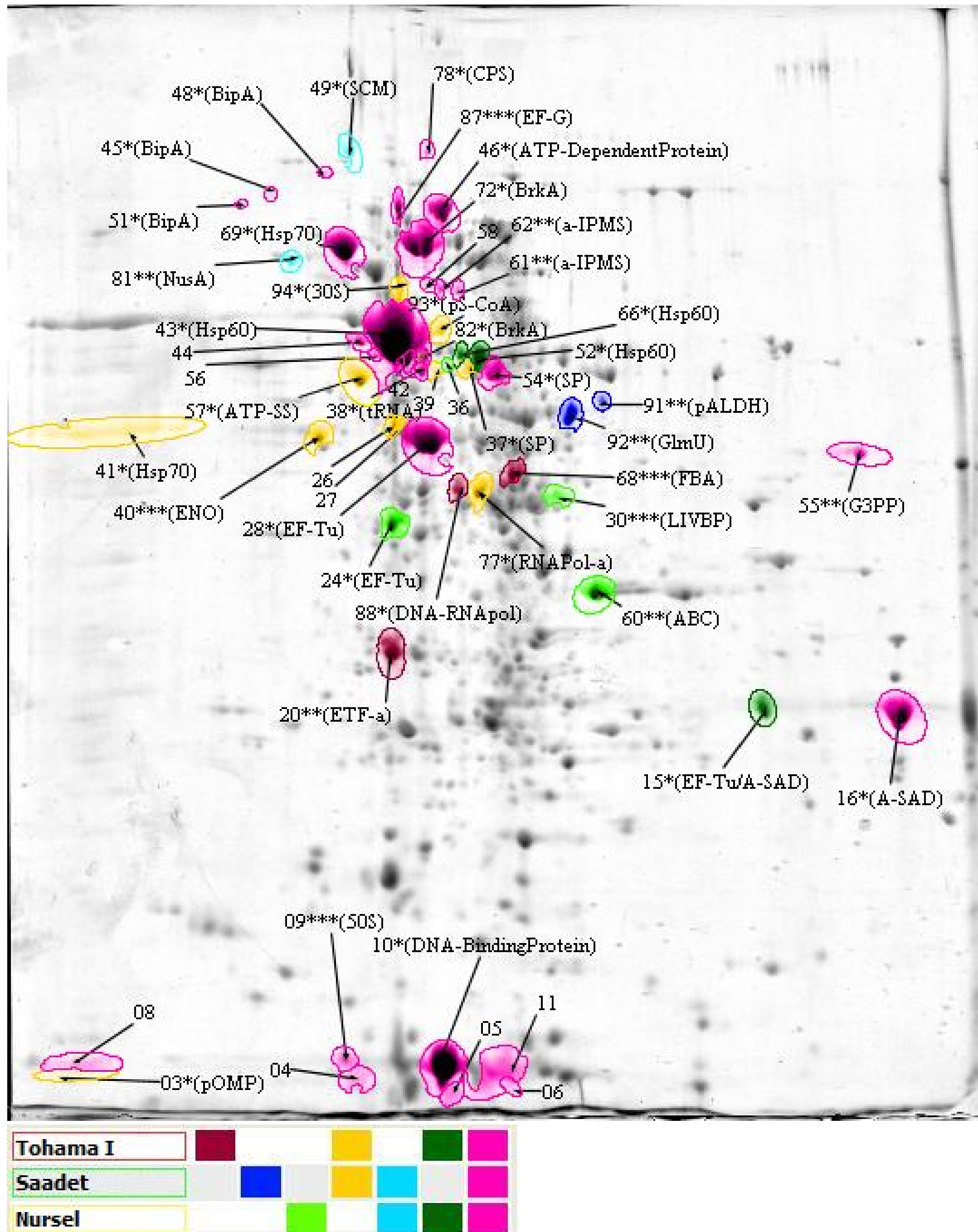

Figure 15. Fused gel for 2-D gel images for three different strains and colour coding according to the immunoreactivity of the proteins against anti-Nursel serum. Immunogenic proteins form Tohama I strain (red), Saadet strain (dark blue) and Nursel strain (light green) were detected against anti-Nursel antibodies. The immune reactive proteins that were common to Tohama I - Saadet, Saadet - Nursel and Tohama I - Nursel strains are shown in yellow, light blue and dark green, respectively. Pink color was used to visualize the common immunogenic spots for all three strains. *, ** and *** are as in the legend of Figure 10.

The spots numbered as 12, 17, 36, 50 and 59 were unidentified immunogenic ones that are listed in Table 10. Those detected in only one of the *B. pertussis* strain against anti-Nursel antibodies are also included in this Table.

Table 10. Immunogenic proteins detected in only one of the *B. pertussis* strains, Tohama I or Saadet or Nursel, against anti-Nursel antibodies.

Spot no	Protein name/ function	Strain
12	Unidentified protein	Nursel
17	Unidentified protein	Tohama I
20	Electron transfer flavoprotein alpha-subunit (ETF-a)**	Tohama I
24	Elongation factor-Tu (EF-Tu)*	Nursel
30	Leu/Ile/Val binding protein (LIVBP)***	Nursel
36	Unidentified protein	Nursel
50	Unidentified protein	Saadet
59	Unidentified protein	Nursel
60	Putative ABC transporter periplasmic amino acid binding protein (ABC)**	Nursel
68	Fructose-biphosphate aldolase (FBA)***	Tohama I
82	Serum resistance protein (BrkA)*	Tohama I
88	DNA-directed RNA polymerase alpha chain (DNA-RNA)*	Tohama I
91	Putative adenylate dehydrogenase (pALDH)**	Saadet
92	Bifunctional protein glmU (GlmU)**	Saadet
94	30S ribosomal protein S1 (30S)*	Saadet

*, ** and *** are as in the legend of Figure 10.

The general information about the proteins which were identified in the present section of the study (as shown in Table 8) are given below of the proteins, fructose-biphosphate aldolase, putative adenylate dehydrogenase and bifunctional protein glmU were already presented in Chapter 3.3.2.

Electron transfer flavoproteins Alpha Subunit (EFT-a);

This was a protein identified as immunogenic among Tohama I antigens, reacting against anti-Nursel antibodies and among Tohama I, Saadet and Nursel antigens reacting against anti-Saadet antibodies (Table 14, Figure 9).

Electron transfer flavoproteins (ETFs) serve as specific electron acceptors for primary dehydrogenases, transferring the electrons to terminal respiratory systems. They can be functionally classified into constitutive, "housekeeping" ETFs, mainly involved in the oxidation of fatty acids (Group I), and ETFs produced by some prokaryotes under specific growth conditions, receiving electrons only from the oxidation of specific substrates (Group II). ETFs are heterodimeric proteins composed of an alpha and beta subunit, and contain an FAD cofactor and AMP. ETF consists of three domains: domains I and II are formed by the N- and C-terminal portions of the alpha subunit, respectively, while domain III is formed by the beta subunit (Tsai *et al.*, 1995; Weidenhaupt *et al.*, 1996).

The studies were performed on ETF's from different bacteria; *E. coli* (Dwyer *et al.*, 1999), *Megasphaera elsdenii* (O'Neill *et al.*, 1998) but our study constitutes the first report on the immunogenicity of ETF alpha chain protein in a bacterial pathogen.

Leu/Ile/Val Binding Protein (LIVBP);

This was a protein identified as immunogenic only among Nursel antigens reacting against anti-Nursel antibodies (Table 14, Figure 9).

The leucine/isoleucine/valine-binding protein (LIVBP) is a components of the leucine specific transport system. This is one of two periplasmic binding protein dependent systems in the high-affinity transport of branched-chain amino acids in bacteria (Sack *et al.*, 1989).

LIVBP serves as the primary high affinity receptor of the *E. coli* ABC-type transporter for the three aliphatic amino acids (Thrakhanov *et al.*, 2005). Moreover, the protein has been found as immunogenic in *Brucella ovis* based on N-terminal sequencing and immunoblotting in the study held by Teixeira-

Gomes *et al*. (1997). Immunogenicity of LIVBP in a strain of *B. pertussis* was revealed for the first time in this work.

ABC Transporter Periplasmic Amino Acid Binding Protein;

This was a protein identified as immunogenic among Nursel antigens reacting only with anti-Nursel antibodies (Table 14, Figure 9).

The ABC transporters (also called traffic ATPases) make up a large superfamily of proteins which share a common function and a common ATP-binding domain. ABC transporters are classified into three major groups: bacterial importers (the periplasmic permeases), eukaryotic transporters, and bacterial exporters. Bacteria rely heavily on the binding protein-dependent ABC-type active transport systems for uptake of nutrients even in severe conditions of limiting or very low concentrations. In *E. coli* alone, there are approximately 50 of these systems for uptake of diverse sets of nutrients, including carbohydrates (monosaccharides and oligosaccharides), amino acids, dipeptides and oligopeptides, polyamines, vitamins, cations, tetrahedral oxyanions, organophosphonate and organosulfonate, choline/taurine, heme, etc. The binding proteins, with masses ranging from 25 to 60 kDa and little amino acid sequence similarity, reside freely in the periplasmic space of gram-negative bacteria (Ames *et al*., 1986, Pearce *et al*., 1992, Fath and Kolter, 1993)

The present study reports periplasmic amino acid binding protein of ABC transporters immunogenic in a pathogenic microbe, for the first time in literature.

3.3.4. Other Novel Immunogenic Proteins Common to More than One Strains of *B. pertussis*

The general information about 2 novel immunogenic proteins common to more than one strains of *B. pertussis* is given below:

Glycerol-3-Phosphate Binding Periplasmic Protein;

It is now well recognized that active transport of small polar molecules in *E. coli* and other Gram-negative bacteria can be mediated by several types of active transport systems. One type is still operating in isolated membrane vesicles and driven by one or both components of the proton motive force. Another type is mediated by periplasmic binding proteins that are the recognition sites of these systems and are essential for the uptake of the particular substrate. GlpT is belongs to the largest family of gradient driven proteins located in periplasm, transporting G3P (Lemieux *et al*. 2004).

The transport systems for G3P were studied in certain bacteria; *Salmonella typhimurium* (Hengge *et al*., 1983), *Yersinia pestis* (Pieper *et al*., 2008), the more extensively in *E. coli,* and in *B. pertussis* in the present work which constituted the first report on the immune reactivity of the protein.

50S Ribosomal Protein L7/L12;

During protein synthesis, the ribosome performs a number of discrete steps which are modulated by certain protein factors. In both prokaryotic and eukaryotic organisms, the 'acidic proteins' from the large ribosomal subunit are involved in the functioning of many of these factors. Ribosomes of *E. coli,* where the acidic proteins are designated L7/L12, it has been demonstrated that the large subunit contains four copies of L7/L12, probably in form of two dimers. Electron microscope studies have shown that L7/L12

is involved in the formation of a morphological feature on the 50S subunit, the so-called L7/L12 stalk (Maassen *et al*., 1983)

50S ribosomal protein L7/L12 have been shown immunoreactive reactions in the bacteria, such as *H. pylori* (Voland *et al*., 2002) and *F. tularensis* (Savitt *et al*., 2009). The protein has been used for development of vaccines against *Brucella abortus* (Luo *et al*., 2006). Our study provided the first information on the immunogenicity of 50S ribosomal protein L7/L12 in *B. pertussis* strains.

3.3.5. Overall Analysis of the Data

The immunoreactive proteins expressed in three strains of *B. pertussis* were investigated against each of the respective anti-sera. Figures 11, 13 and 15 show that the immunogenic proteins are substantially common to the three strains. Certain immunogenic protein spots were detected in the proteome of only one of the *B. pertussis* strains against one anti-serum, as can be clearly seen in tables 6, 8 and 10. Nonetheless, some such protein spots could be detected in other strain by using the same or different anti-serum. This situation can be exemplified by the following findings: enolase, putative adenylate dehydrogenase, bifunctional protein glmU, electron transfer flavoprotein alpha-subunit. This might be explained by either low antigen expression by some strains *in vitro* or certain experimental errors.

The total number of immunogenic protein spots detected from the strains was the highest when anti-Saadet serum was used as the antibody source. At the same time, this serum identified more immunogenic protein spots when reacted with the proteome of Saadet strain. It can therefore be anticipated that immunization with this strain may be more potent as compared with the other strains.

Some yet unidentified protein spots reacting only with their own sera, but not with the sera obtained from other strains might point to their significance as strain-spesific antigens. For instance, unidentified spots labeled as 02, 19 and 35 which obtained from Saadet strain were detected as immunogenic only against anti-Saadet serum. Likewise, unidentified spots labeled as 14, 17, 22 and 67 from Tohama I strain were detected as immunogenic only against anti-Tohama I serum. In a similar way, unidentified spots labeled as 12 and 59 which obtained from Nursel strain were detected as immunogenic only against anti-Nursel serum (Table 6, 8, 10).

Belonging to the above mentioned group there were 3 proteins namely, dihydroxy-acid dehydratase specific only to Tohama and Leu/Ile/Val binding protein as well as putative ABC transporter periplasmic amino acid binding protein specific only to Nursel.

As an example to the power of the comparative immunoproteomics, the present study revealed strain-spesific immunogenic proteins. In our future studies, such protein spots will be separated by 2D gel electrophoresis, identified by their known coordinates, and their immunoreactivity will be studied to provide invaluable information about antigenic drift/shift among the strains of *B. pertussis*. More important than this will be the future immunization studies to be performed with novel, but yet unidentified proteins common to all these strains. Such proteins might be the ideal targets for the design of novel vaccines after the demonstration of their protective potential.

3.3.6. Protein Localizations and Epitope Analysis

The server ABCpred (http://www.imtech.res.in/raghava/abcpred/index.html) was used for B-cell epitope prediction. The accuracy scores of the predicted epitopes of all identified immunogenic proteins were within a meaningful range. PSORTb (http://www.psort.org/psortb/) analysis predicted that 9 of the 14 newly found immunogenic proteins are localized in cytoplasm and 3 protein in the periplasm of the organism. The localization of 2 proteins could not be determined. According to SignalP analysis, 4 of the 14 immunogenic proteins were predicted to have a signal peptide sequence (Table 7).

3.4. Future Prospects

In the present study, 14 novel immunogenic proteins have been identified from three important strains of *B. pertussis*. There were many highly abundant proteins in the 2-DE gels which did not found as immunoreactive. It is therefore rational to suggest that the immunogenic ones may be the significant vaccine candidates. The protective capacities of the immunogenic proteins identified either by alone or in different combinations remain to be determined in further studies. The identified proteins should be cloned, expressed in high yields, purified and their protective potential will be investigated through i*n vivo* experiments

In vitro immunoreactive proteome experiments have been done in this work to mimic *in vivo* immunogenic activity. The antibody response formed in mice against the experimental vaccines used in this work might not exactly represent the circulating pathogenic strains in population and hence antibodies generated in human body. In this respect, Western blot analyses using the patient's sera would contribute to the identification of novel antigens.

In the present study, B-cell response has almost been demonstrated and B-cell epitope predictions were also made, still the antigen-specific T cell response also remains to be determined.

CHAPTER 4

CONCLUSION

- The proteome and immunoproteome of Nursel strain of *B. pertussis* have been studied for the first time, with more than 600 proteins separated in 2-D PAGE within a pH range of 3-10. Thirty four proteins were found to be immunogenic in mice.

- The proteins expressed during immunization of mice with either Tohama I, Saadet and Nursel strains of *B. pertussis* were analysed. Among a total of 14 novel immunogenic proteins; 5 proteins (elongation factor G, fructose bisphosphate aldolase, Leu/Ile/Val binding protein, enolase, 50S ribosomal protein L7/L12) were found to be immunogenic for *B. pertussis* and 8 proteins (dihydroxy acid dehydratese, 2-isopropylmalate sythase, DNA polymerase III-beta chain, bifunctional protein glmU, putative aldehyde dehydrogenase, putative ABC transporter periplasmic amino acid binding protein, glycerol-3-phosphate binding periplasmic protein, electron transfer flavoprotein alpha subunit) were found to be immunogenic proteins for a pathogenic bacterium for the first time in this study.

- Dihydroxy-acid dehydratase was detected as an antigenic protein in Tohama I its own serum, whereas 2 proteins; Lue/Ile/Val binding protein and putative ABC transporter periplasmic amino-acid binding protein were specific for Nursel against anti-Nursel antibodies.

- The use of anti-Saadet serum as the antibody source led to the detection of the highest number of immunogenic protein spots from all three strains when compared to anti-Tohama and anti-Nursel sera. This finding suggested that Saadet strain might provide more potent immunization than the other strains.

- Our findings are expected to facilitate further immunoproteomics, physiological proteomics, reverse vaccinology and recombinant subunit vaccine research in our group and aid in development of new generation vaccines against the pathogen.

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APPENDIX A

COLLOIDAL COOMASSIE BLUE (CCB) STAINING
AND
STOCK SOLUTIONS

1-Coomassie Brilliant Blue (CBB) Stock

Coomassie Brilliant Blue G-250	5 g
dH_2O	100 mL

2-Fixation Buffer

40 % Ethanol	125 mL
10 % Acetic Acid	25 mL
50 % dH_2O	100 mL

The gel is shaked in this solution for 1 to 2 hours

3-Washing

Gels are washed with dH_2O two times for 10 min.

4-CCB dye solution

Ammonium sulfate	100 g
85% phosphoric acid	12 mL
CBB stock solution	20 mL
Distilled water add to	1000mL

5-CCB staining

CCB dye solution	200 mL
Methanol	50 mL

Gels are put in to 200 mL CCB dye solution. 50 mL methanol is added afterwards. Gels are kept in this solution for 24 hours.

6-Washing

The gel is washed with dH_2O until protein spots are clearly visible.

APPENDIX B

CHEMICALS AND THEIR SUPPLIERS

Chemicals	**Supplier**
Acetic acid	Merck
Acetone	Merck
Acrylamide	Sigma
Ammonium sulfate	Merck
Ampholines pH (3-10)	Fluka
Bis-acryamide	Sigma
Bovine Serum Albumin (BSA)	Sigma
$CaCl_2$ H_2O	Merck
CH3CN	Applichem
CHAPS	Merck
Comassie Brillant Blue G 250	Sigma
DTT	Fluka
Ethanol	Merck
Glucose	Merck
Glycerol	Merck
Glycine	Merck
H_3PO_4	Merck
HCI	Merck
IPG strips	BioRad
KH_2PO_4	Merck
Methanol	Merck
$MgSO_4$ $7H_2O$	Merck

Molecular Weight Standard (14,400-116,000)	Fermentas
NaOH	Merck
NH_4Cl	Merck
NH_4HCO_3	Applichem
SDS	Sigma
TEMED	Sigma
TFA	Applichem
Thiamine	Sigma
Thiourea	Fluka
Trichloroacetic acid (TCA)	Merck
Tris- HCI	Sigma
Urea	Fluka

APPENDIX C

CULTURE MEDIA COMPONENTS

Modified Morse Bray Medium

Solution A

Component	**Concentration**
Casamino Acid	10 g/l
Tris	6 g/l
NaCl	2.5 g/l
Starch	1.5 g/l
KH_2PO_4	0.5 g/l
Glutamic Acid	0.2 g/l
$MgCl_2.6H_2O$	0.4 g/l
$CaCl_2$	0.01 g/l

Dissolved in 600 ml of dH_2O. pH was adjusted to 7.4 and volume was made up to 975 ml with dH_2O and the mixture was autoclaved.

Solution B

Component	**Concentration**
Glutation	100 mg/l
L-Prolin	120 mg/l
$FeSO_4.7H_2O$	10 mg/l

L-Cysteine	40 mg/l
Nicotonic Acid	4 mg/l
Ascorbic Acid	20 mg/l

Dissolved in 10 ml of dH_2O and volume was completed to 25 ml with dH_2O. The mixture was filtered through 0.2 μm membrane and added to A (Sato et al., 1974).

Cohen Wheeler Medium

Component	**Concentration**
Agar	30 (g/l)
NaCl	2.5 (g/l)
KH_2PO_4	0.5 (g/l)
$MgCl_2.6H_2O$	0.4 (g/l)
Soluble starch	1.5 (g/l)
Yeast Extract	2.0 (g/l)
Casamino Acid	10 (g/l)
$CaCl_2$ (%1)	1 (cc/)
$FeSO_4.7H_2O$ (%0.5)	2 (cc/l)
L-Cystein	2.5 (cc/l)
$CuSO_4.5H_2O$ (%0.5)	1 (cc/l)
Activated Charcoal	4 (g/l)

The pH was asjusted to 7.3 and the medium was autoclaved (Sato et al., 1972).