IDENTIFICATION OF THE NEW IMMUNOGENIC PROTEINS OF *BORDETELLA PERTUSSIS* BY IMMUNOPROTEOMICS

A THESIS SUBMITTED TO
THE GRADUATE SCHOOL OF NATURAL AND APPLIED SCIENCES
OF
MIDDLE EAST TECHNICAL UNIVERSITY

BY

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IN PARTIAL FULFILLMENT OF THE REQUIREMENTS
FOR
THE DEGREE OF MASTER OF SCIENCE
IN
BIOTECHNOLOGY

APRIL 2007

Approval of the Graduate School of Natural and Applied Sciences

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ABSTRACT

IDENTIFICATION OF THE NEW IMMUNOGENIC PROTEINS OF *BORDETELLA PERTUSSIS* BY IMMUNOPROTEOMICS

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April 2007, 115 pages

The genus *Bordetella* contains several pathogenic species generally associated with upper respiratory tract infections in warm-blooded animals. *Bordetella pertussis* is the etiologic agent of whooping cough. Whooping cough is presently one of the ten most common causes of death from infectious diseases and reported by the World Health Organisation (WHO) to cause 50 million cases and 350000 deaths worldwide per year, mainly among unvaccinated individuals in poor countries.

The term proteome, in analogy to the term genome, was coined to describe the complete set of proteins that an organism has produced under a defined set of conditions. Proteomics has been used to identify novel bacterial vaccine candidates against several human pathogens. Fueled by growing DNA sequence information, the analysis of the proteome becomes a valuable and useful tool for antigen discovery. Much of information about immunogenic component can be derived from proteomics coupled to Western blotting, namely immunoproteomics.

In the present study, we report first immunoproteomics analysis to identify candidate antigens of *B. pertussis* for vaccine development. Different sera from mice, which were immunized or challenged with *B. pertussis*, were analyzed for reactivity by Western blot against whole cell extracts of *B. pertussis* Tohama and Saadet strains separated by 2-DE.

We identified 15 immunogenic proteins of *Bordetella pertussis* as a total (60 kDa chaperonin, heat shock protein, serum resistance protein, putative substrate-CoA ligase, ATP-dependent protease, preprotein translocase secA subunit, S-adenosylmethionine synthetase, elongation factor Tu, RNA polymerase alpha subunit, ketol-acid reductoisomerase, pertactin, lysyl-tRNA synthetase, serum resistance protein, carbamoyl-phosphate synthase large chain, 30S ribosomal protein S1 subunit), 6 of which being identified as immunogenic in a pathogenic microbe (ATP-dependent protease, carbamoyl-phosphate synthase large chain, lysyl-tRNA synthetase, putative chromosome partition protein, preprotein translocase secA subunit, 30S ribosomal protein S1 subunit) and 5 identified as immunogenic for *Bordetella pertussis* (RNA polymerase alpha subunit, S-adenosylmethionine synthatase, putative substrate-CoA ligase, elongation factor Tu, ketol-acid reductoisomerase) for the first time.

Key words: *Bordetella pertussis*, immunoproteomics, 2-DE map, MALDI-TOF-MS, Western Blot

ÖZ

BORDETELLA PERTUSSIS'İN YENİ İMMUNOJENİK PROTEİNLERİNİN İMMUNOPROTEOMİK YÖNTEMLE TANIMLANMASI

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Nisan 2007, 115 sayfa

Bordetella cinsi, sıcakkanlı hayvanlarda üst solunum yolu enfeksiyonlarına neden olan pek çok patojenik bakteri türünü içermektedir. *Bordetella pertussis*, boğmaca hastalığının etyolojik ajanıdır. Halen en fazla ölüme sebep olan on infeksiyoz hastalıktan birisi olan boğmaca, Dünya Sağlık Örgütü (DSÖ) raporlarına göre yoksul ülkelerde aşılanmamış bireyler başta gelmek üzere, dünya çapında yılda 50 milyon vakaya ve 350000 ölüme sebep olmaktadır.

Genom teriminden türetilerek oluşturulan proteom, bir organizmanın tanımlanmış koşullar altında ürettiği tüm proteinleri tarif etmektedir. Proteomik, pek çok insan patojenine karşı yeni bakteriyel aşı adaylarının tanımlanması için kullanılmaktadır. Giderek artan DNA sekans bilgileri ile güçlenen proteom analizleri yeni antijenlerin keşfi için çok kullanışlı ve değerli bir araç haline gelmiştir. Patojenlerin immunojenik yapılarıyla ilgili en kapsamlı bilgi, proteomik teknolojisinin, Western blot analiziyle birleşmesini içeren, immunoproteomik yöntemlerle elde edilebilir.

Çalışmamız, B*. pertussis* için yeni aşıların geliştirilmesinde kullanılabilecek aşı aday antijenlerin tanımlanması için yapılan ilk *B. pertussis* immunoproteomik analizini içermektedir. *B. pertussis* ile immunize edilmiş veya enfekte olmuş farelerden elde edilen farklı serumlar, Western blot tekniği kullanılarak *B. pertussis*'in Tohama ve Saadet suşlarına ait tüm hücre ekstratlarının 2-DE'de ayrılmış proteinlerine karşı Western blot reaktivitelerine göre analiz edilmişlerdir.

İncelenen *Bordetella pertussis* örneklerinde toplam 15 immunojenik protein tanımlanabilmiştir (60 kDa chaperonin, heat shock protein, serum resistance protein, putative substrate-CoA ligase, ATP-dependent protease, preprotein translocase secA subunit, S-adenosylmethionine synthetase, elongation factor Tu, RNA polymerase alpha subunit, ketol-acid reductoisomerase, pertactin, lysyl-tRNA synthetase, serum resistance protein, carbamoyl-phosphate synthase large chain, 30S ribosomal protein S1 subunit). Tanımlanan proteinlerden 6'sı (ATP-dependent protease, carbamoyl-phosphate synthase large chain, lysyl-tRNA synthetase, putative chromosome partition protein, preprotein translocase secA subunit, 30S ribosomal protein S1 subunit) ilk kez patojenik bir organizmada immunojenik proteinler olarak bulunmuştur. Diğer yandan bu çalışmada tanımlanan immunojenik proteinlerinden 5'inin (RNA polymerase alpha subunit, S-adenosylmethionine synthatase, putative substrate-CoA ligase, elongation factor Tu, ketol-acid reductoisomerase) *Bordetella*' da immunojenik olduğu ilk kez bu çalışmada gösterilmiştir.

Anahtar sözcükler: *Bordetella pertussis*, , immunoproteomik, 2-DE haritası, MALDITOF-MS, Western Blot.

To my Grandmothers:

Zehra Çemrek and Nimet Altındiş

ACKNOWLEDGEMENTS

I would like to express my sincerest appreciation to my supervisor, Prof. Dr. Gülay Özcengiz for her guidance, supervision and patience given during this long process.

Secondly, I would also like to thank Dr. Erkan Özcengiz for his kindness, guidance, advices, encouragement and sharing all his work experiences with me.

I also thank my labmate Volkan Yıldırım for his endless patience and invaluable guidance in proteomics technology. I am also grateful to Dr. Pınar Atakan Ablay of Refik Saydam Hygiene Center for her invaluable help during the animal experiments. My special thanks also go to Ass. Prof. Dr. Hamdi Uysal and Dr. Servet Özcan for their encouragement and suggestions.

I would like to express my indebtedness to Prof. Dr. Michael Hecker and Dr. Dörte Becher for making available all the equipment for MALDI-TOF MS analysis in this work. Very special thanks to my labmates Burcu Tefon, Orhan Özcan, Araz Zeyniyev, Bilgin Taskın, Ayşe Koca, Aslıhan Kurt, Erkam Gündogdu, Hande Selamoglu, Didem Coral, Sezer Okay and İsmail Ögülür for their friendship and understanding.

My deepest thanks and special gratitude are extended to Ezgi Doğru for her *şarabi* love.

The last but not least, I would like to extend my gratitude to my precious family, to my parents Zeynep and Cengiz ALTINDİŞ and to my brother Burak ALTINDİŞ for their endless love, kind understanding, patience and endless support during my studies. Of course, thanks to life as well (*gracias a la vida*).

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CHAPTER 1

INTRODUCTION

1. 1. The genus *Bordetella*

The members of the genus *Bordetella* are gram-negative bacteria which are obligate, non-invasive parasites of the respiratory tracts of warm-blooded animals, including birds, with a predilection for the respiratory ciliated epithelium. Whooping cough is a highly contagious, acute respiratory illness of humans that is caused by *Bordetella pertussis*. *B. pertussis* is a strict human pathogen with no known animal or environmental reservoir. As such, transmission of disease is postulated to occur via respiratory droplets. While nine species of *Bordetella* have been identified to date, only three additional members, namely *B. bronchiseptica*, *B. parapertussis*, and *B. holmesii* have been associated with respiratory infections in humans and other mammals. *B. bronchiseptica* infects a wide range of hosts and occasionally causes cough illnesses in humans; in particular, severe infections have been noted in persons who are immunocompromised such as patients with AIDS (Blay et al., 1997, Wolfe et al., 2005). Human adapted *B. parapertussis* (*B. parapertussis*$_{hu}$) causes a milder pertussis-like disease and, like *B. pertussis*, lacks an environmental reservoir. *B. holmesii*, the most recent of the *Bordetella* species associated with human respiratory tract infection, has been found in the blood of young adults and occasionally in the sputum. Little is known about the biology, virulence mechanisms and pathogenic significance of *B. holmesii* whereas *B. pertussis*, *B. bronchiseptica*, and *B. parapertussis* have been extensively studied (Mattoo et al., 2005). On the other hand, *Bordetella avium* is the causative agent of bordetellosis, an avian upper respiratory tract disease to which commercially raised turkeys are particularly susceptible (Spears et al., 2003).

Recently, the genomes of *B. bronchiseptica* RB50 (5,338,400 bp; 5,007 predicted genes), *B. parapertussis* 12822 (4,773,551 bp; 4,404 genes) and *B. pertussis* Tohama (4,086,186 bp; 3,816 genes) were sequenced. The analysis of genomes indicates that *B. parapertussis* and *B. pertussis* are independent derivatives of *B. bronchiseptica*-like ancestors. During the evolution of these two host-restricted species, there was large scale gene loss and inactivation; host adaptation seems to be a consequence of loss, not gain, of function, and differences in virulence may be related to loss of regulatory or control functions (Parkhill et al., 2003).

Table 1. Pathogenicity of *Bordetella* spp (from Preston, 2004)

6Species	Host	Infection	Epidemiology
B. pertussis	Human	Whooping cough in infants (age 1–10 years) through inhalation of infected airborne and persistent cough.	Exclusively a human pathogen. Infection is through inhalation of infected airborne bacterial droplets. Symptoms include a characteristic ' whoop' during coughing episodes. Illness lasts 6–8 weeks; complications rarely arise, but include pneumonia, seizures and encephalopathy, which can be fatal.
B. parapertussis	Human, ovine	Whooping cough, chronic pneumonia Kennel cough (canine),	Asymptomatic carriage in many animal hosts. Rare infections described in humans.
B. bronchiseptica	Mammals	Atrophic rhinitis (swine), Snuffles (rabbits)	
B. avium	Avian	Rhinotracheitis	The *B. avium* genome sequencing project is in progress.
B. hinzii	Human, domestic fowl	Asymptomatic	Rare infections of humans. Probably a commensal of the respiratory tract of fowl.
B. trematum	Human?	Wound infections, otitis media	Rare infections of humans.
B. holmesii	Human?	Septicemia	Rare but increasing number of infections. May cause a pertussis-like illness.
Bordetella petrii	Environmental	?	Recently isolated from river sediment in a bioreactor

1.2. *B. pertussis* and whooping cough

B. pertussis is a very small gram negative aerobic coccobacillus that appears singly or in pairs (Figure 1).

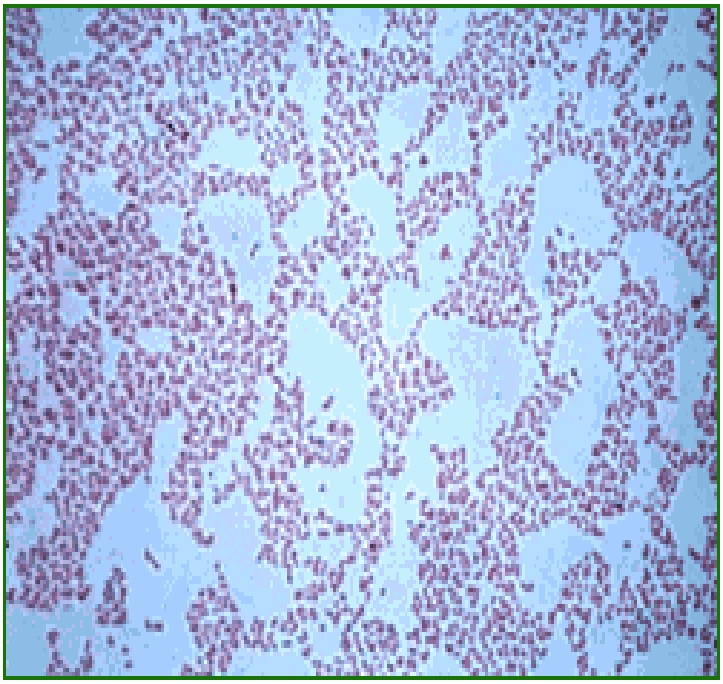

Figure 1. Gram stained cells of *B. pertussis* (http://web.umr.edu/~microbio/BIO221_2001/bordetella_pertussis.html).

Its metabolism is respiratory and taxonomically, *Bordetella* is placed among the "gram negative aerobic rods and cocci" (Kerr et al., 2000). The bacteria are nutritionally fastidious and usually cultivated on rich media supplemented with blood. They 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.

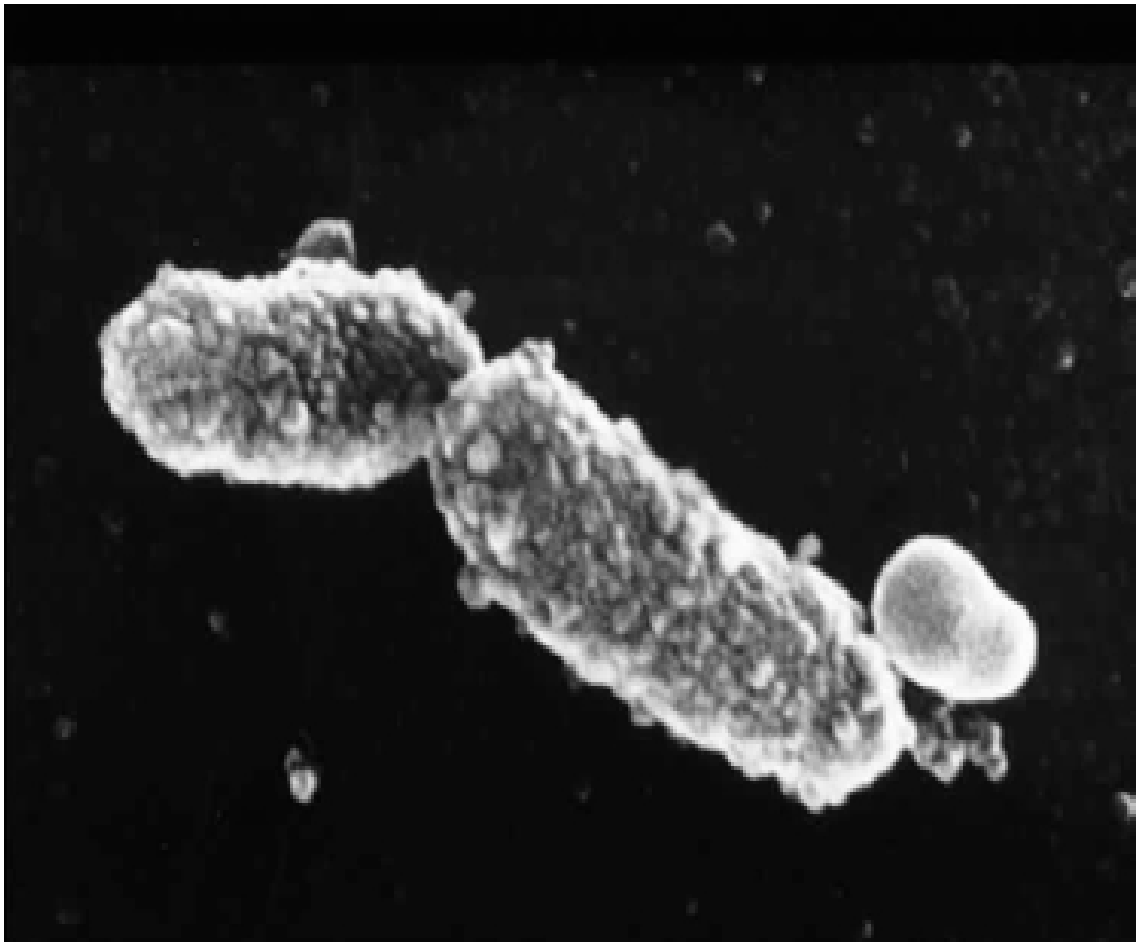

Figure 2. Electron micrograph (x5000) of *B. pertussis* (from König et al., 2002).

The introduction of *B. pertussis* into the respiratory tract in droplets is followed by interaction with ciliated epithelial cells, likely to be dependent on bacterial adhesins, of which at least five have been suggested: filamentous haemagglutinin (FHA), pertactin (PRN), fimbriae, pertussis toxin (PT) and tracheal colonization factor (TCF) (Figure 3). The expression of many of the proteins implicated in *Bordetella* pathogenesis, including these adhesins, is regulated in response to environmental stimuli by a two-component regulatory system encoded by the *bvg* genes. It is likely that the expression of the members of the *bvg* regulon is altered dependent on the different

environments encountered in the respiratory tract.

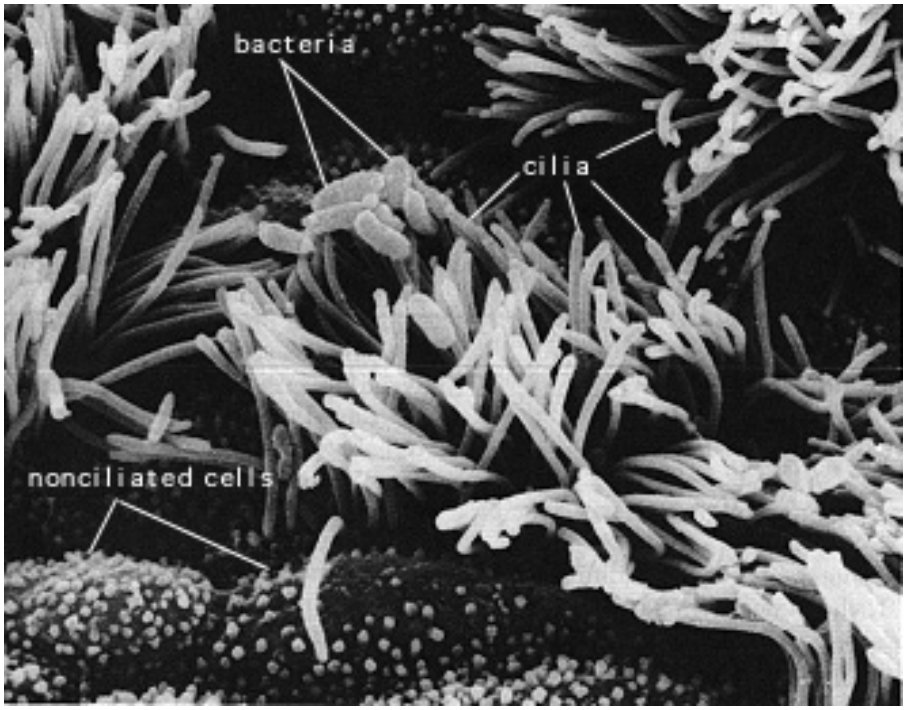

Figure 3. Colonization of tracheal epithelial cells by *B. pertussis* (*http://web.umr.edu/~microbio/BIO221_2001/bordetella_pertussis.html*).

The next step in pathogenesis is thought to be paralysis of the cilia and death of ciliated cells, probably mediated by a synergistic effect of tracheal cytotoxin (TCT) and lipopolysaccharide (LPS) via induction of interleukin-1 (IL-1) and nitric oxide. This leads to defective mucociliary clearance, allowing the bacteria to establish in, and move down, the respiratory tract. Expression of the *bvg* regulated toxins, PT and adenylate cyclase-haemolysin, may damage the respiratory tract further and may also interfere with immune responses. PT has been suggested as a primary cause of the prolonged cough associated with pertussis. At this point the infection is established and the patient exhibits the symptoms characteristic of whooping cough. Depending on the response of the patient, the infection will be cleared over

time, or may progress in some cases to pertussis pneumonia and possibly death (Van Den Berg et al., 1999).

The pathogenesis of pertussis infection may also involve an intracellular stage. The organism can invade and survive within phagocytes (including dendritic cells) and non-phagocytic cells in vitro and can be seen inside alveolar macrophages in children with AIDS. Intracellular survival may explain the prolonged nature of the cough in some cases of whooping cough. Cell mediated immunity is likely to be required to combat intracellular infection, distinct from the antibody responses previously thought to be sufficient for protection (Maskell and Preston, 2002).

The disease has three typical stages, namely catarrhal, spasmodic and convalescent stages. Catarrhal stage has nonspecific symptoms including rhinorrhoea, sore throat, conjunctivitis and non-productive cough. This stage typically lasts two weeks. In the second stage, the cough becomes paroxysmal and episodes of coughing may cause cyanosis or facial discoloration as a result of venous congestion. Episodes typically end with a deep inspiration (whoop) and vomiting. Paroxysms may occur more than 30 times per 24 hours and are more common at night. They occur spontaneously or are precipitated by external stimuli such as noise and cold air. Between coughing episodes, there are few clinical signs unless complications develop. This stage also typically lasts two weeks. During the third stage, the coughing gradually subsides. Relapse may occur if another respiratory infection is acquired. This stage can last from 2 weeks to several months (Sinha and Heath, 2005).

1.3. Virulence factors of *B. pertussis*

A number of virulence factors of *B. pertussis* and potential protective antigens

have been identified and some are at different stages of evaluation as acellular vaccine candidates. They are divided into two groups, adhesins and toxins, respectively (Figure 4).

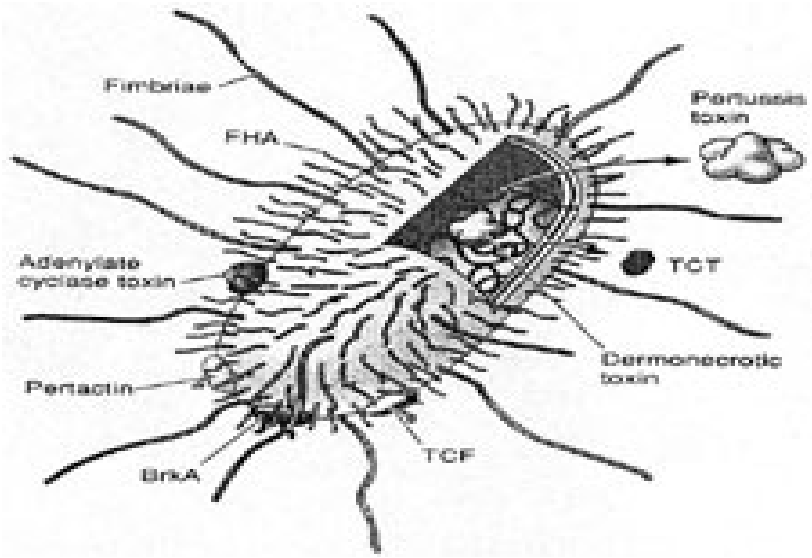

Figure 4. Virulence factors of *B. pertussis* (www.rivm.nlinfectieziektenbulletinbul1602kinkhoest.jpg).

1.3.1. Adhesins

Of various virulence factors *B. pertussis* produces under the control of the BvgA/S system, a number of adhesins are found at the surface of the organism, several of which have been studied in some detail. These include filamentous hemagglutinin, fimbriae, also called agglutinogens, pertactin, the serum-resistance protein Brk and the attachment factor Tcf.

1.3.1.1. Filamentous hemagglutinin (FHA)

Filamentous hemagglutinin (FHA), a primary component of acellular pertussis

vaccines, is a large, β-helical, highly immunogenic protein that is both surface associated and secreted. *In vitro* studies suggested that FHA functions for adhesion, and several binding domains have been identified (Inatsuka et al., 2005). FHA is synthesized as a 367 kDa precursor 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 full-length FHA contains three distinct binding sites that are thought to contribute to its adhesin property: an N-terminal glycosaminoglycan-binding site, two arg–gly-asp (RGD) sequences and a carbohydrate recognition domain (CRD). The protein is highly immunogenic, with two main immunodominant regions. Sera from convalescent patients and vaccinated infants specifically recognize these two immunodominant domains of FHA, identified as type I and type II domains, respectively (Piatti et al., 1999).

The type I domain at the carboxy terminus appears to be more immunodominant than the N-terminal type II domain, containing the most reactive epitopes. Vaccination with purified antigen alone is usually insufficient to induce a protective immune response; adjuvants are usually required (Knight et al., 2006).

1.3.1.2. Fimbriae (FIM)

Like many gram-negative pathogenic bacteria, *bordetellae* express filamentous, polymeric protein cell surface structures called fimbriae (FIM). *B. pertussis* fimbriae are composed of a major subunit (either Fim2 or Fim3) and a minor subunit (FimD). Fim2, Fim3, and FimD have molecular weights of 22.5 kDa, 22 kDa, and 40 kDa, respectively. *B. pertussis* has three major (*fim2*, *fim3*, *fimX*) and one minor (*fimY*) fimbrial subunit genes; however, *fimX* and *fimY* do not appear to be expressed. The gene cluster *fim23XY* is

found scattered around the chromosome. An additional gene cluster consisting of *fimBCD* encodes the fimbrial chaperone, the anchorage protein and a minor fimbrial subunit, respectively. This *fim* cluster is located between the genes *fhaB* and *fhaC*, which encode the filamentous hemagglutinin (FHA) respectively (Crowcroft et al., 2002).

Fimbriae of *B. pertussis* are required for colonization of mouse trachea, in that Fim2 and Fim3 deletion mutants were significantly disadvantaged in this function. FimD is located at the fimbrial tip and has been shown to bind the integrin very late antigen (VLA)-5 and heparin, both of which are ubiquitous in the respiratory tract. Fim2 also binds heparin; the binding regions show sequence similarity with the heparin-binding regions of fibronectin and are immunodominant (Kerr and Matthews 2000). Recent studies suggest that FIM-mediated interactions with epithelial cells and/or monocytes/macrophages may play important roles not only in adherence but also in the nature and magnitude of the host immune response to *Bordetella* infection (Mattoo et al., 2005).

1.3.1.3. Pertactin (PRN)

Pertactin is an outer-membrane protein with an apparent molecular weight of 69 kDa. The structural gene, *prn*, encodes a protein of 94 kDa which is transported into the periplasm by a classical signal-peptide dependant secretion mechanism. It then undergoes proteolytic processing resulting in the removal of an approximately 30 kDa C-terminal region. This C-terminal region remains anchored in the outer membrane and is essential for the secretion of pertactin. It resembles the C-terminal domains of the precursors of several secreted proteins from gram negative bacteria that are known to be autotransporters (Locht, 1999).

Pertactin has been proposed to play a role in attachment since all three PRN proteins contain an Arg-Gly-Asp (RGD) tripeptide motif as well as several proline-rich regions and leucine-rich repeats, motifs commonly present in molecules that form protein-protein interactions involved in eukaryotic cell binding (Mattoo et al., 2005). Several studies have shown that P.69 pertactin is important for immunity against disease. Antibody levels to P.69 pertactin were shown to correlate with clinical protection (Hijnen et al., 2005).

1.3.1.4. Serum-resistance protein (BrkA)

Serum resistance or resistance to killing by antibody dependent pathway of complement 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 complementation and is involved in adherence and invasion (Fernandez et al., 1998). *BrkA* is an autotransporter, 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. Although cleaved, the 73 kDa BrkA passenger domain remains tightly associated with the bacterial surface and is not detected in *B. pertussis* culture supernatants (Lambert et al., 1993).

1.3.1.5. Tracheal colonization factor (TCF)

A recently identified virulence factor in *B. pertussis* is tracheal colonization factor (TCF) encoded by the *tcfA* gene. When a strain of *B. pertussis* lacking this protein was used to infect mice by aerosol challenge, the number of bacteria isolated from the trachea decreased tenfold when compared with the parent strain (Kerr and Mathews, 2000). A derived amino acid sequence after

removal of a prokaryotic signal sequence predicts a 64 kDa proline-rich protein that contains the RGD motif. The amino terminal shows 50% identity with the pertactin precursor (Kerr et al., 2000).

1.3.2. Toxins

Most *B. pertussis* toxins are protein toxins, except for lipopolysaccharide, also called endotoxin, and the tracheal cytotoxin, which is a fragment of the *Bordetella* peptidoglycan. The three major protein toxins are the pertussis toxin, the adenylate cyclase toxin and the dermonecrotic toxin.

1.3.2.1. Pertussis toxin (PT)

Pertussis toxin (PT) is one of the major components of acellular pertussis vaccine with FHA. It is the main toxin of *B. pertussis* and one of the most important virulence factors. PT is a globular protein with a molecular weight of 117 000 Da. This ADP-ribosylating protein (PT) has several biological activities and is composed of five subunits, S1 to S5, which assemble into two functionally distinct moieties (Özcengiz et al., 2004). The enzymatically active A component consists of the S1 subunit which ADP ribosylates a family of GTP-binding regulatory proteins involved in signal transduction in eukaryotic cells. The B oligomer binds a eukaryotic cell and mediates translocation of the S1 subunit into the cell. Structurally, the subunits comprising the B oligomer (one copy each of subunits S2, S3, and S5 and two copies of S4) form a pentameric ring that is penetrated by the carboxy terminus of the S1 subunit. Residues of the S1 subunit between positions 219 and 235 are involved in an interaction with the B oligomer (Farizo et al., 2002).

The action of PT 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, PT 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 PT is the major protective antigen in all the new acellular vaccines available today (Locht, 1999).

1.3.2.2. Adenylate cyclase toxin (AC)

Bordetella species produce and secrete a bifunctional adenylate cyclase/haemolysin, termed cyclolysin, which is a 45 kDa protein encoded by *cyaA* gene. 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 cAMP concentration leads to paralysis of the killing functions in phagocytic and immune effector cells (Kerr et al., 2000).

The AC catalytic domain is located in the first 400 amino acid residues. It exhibits a high catalytic activity upon activation by calmodulin which binds with a high affinity to the enzyme and stimulates its activity more than 1000 fold. The carboxy-terminal part of CyaA (residues 400–1706) mediates the binding and the internalization of the toxin into eukaryotic cells. AC toxin is endowed with a unique mechanism of penetration into eukaryotic cells: its catalytic domain is directly translocated across the plasma membrane of the target cells from the cell surface into the cytosol. Although CyaA can invade a variety of cell types, recent studies have shown that it interacts specifically with the $\alpha_M\beta_2$ integrin, suggesting that cells expressing this integrin might be the primary target of AC toxin (Dautin et al., 2002).

1.3.2.3. Dermonecrotic toxin (DNT)

Dermonecrotic toxin was one of the first virulence factors to be described for *B. pertussis* by Bordet and Gengou in 1909, although they misidentified it as endotoxin (Walker et al., 1994). The heat-labile dermonecrotic toxin (DNT) of *B. pertussis* causes necrotic lesions on subcutaneous injection into mice at low doses and is lethal at high doses. It has a molecular weight of 102 kDa, with subunits of 30 and 24 kDa. Although it has been reported to be localised in the cytoplasm of the organism, a part of the toxin is probably exposed at the cell surface (Kerr, 2000).

1.3.2.4. Tracheal cytotoxin (TCT)

Tracheal cytotoxin destroys ciliated epithelial cells of the respiratory tract, causes an increase in the number of cells with sparse ciliation and the extrusion of cells from the epithelial surface. *In vitro*, TCT inhibits DNA synthesis in tracheal epithelial cells and induces the production of intracellular interleukin-1 and of nitric oxide, the most likely triggers of TCT-mediated cytopathy (Locht, 1999). TCT alone is necessary and sufficient to reproduce the specific ciliated-cell cytopathology characteristic of *B. pertussis* infection in explanted tracheal tissue (Mattoo et al., 2005)

1.3.2.5. Lipopolysaccharide

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 is therefore more similar to rough-type

LPS (Mattoo et al., 2005). It is lethal in galactosamine-sensitized mice, and is pyrogenic and mitogenic in spleen cell cultures. It activates macrophages and induces the production of tumor necrosis factor. *B. pertussis* contains two LPS classes, LPSI and LPSII, which differ in their polysaccharide moieties. The ratio of LPSI to LPSII may differ between variants of the same strain. However, the precise role in pathogenesis of these structural differences is not clear (Locht, 1999).

1.4. *B. pertussis* BVG system

In *B. pertussis*, the *vir/bvg* locus coordinately regulates the expression of many of the known virulence-associated determinants of the organism (Finn et al., 1995).

Expression of many of the proteins involved in *Bordetella* pathogenesis is regulated in response to environmental stimuli by a two-component regulatory system called BvgA/S. Environmental stimuli cause the inner membrane sensor histidine kinase BvgA protein. Phosphorylated BvgA activates the transcription of a number of virulence-activated genes (vags) by binding to their sites in their promoters. One such gene encodes the regulator BvgR that represses the transcription of a number of other genes called virulence-repressed genes (vrgs). Thus, activation of Bvg markedly changes the gene expression profile of the bacterium. Many genes implicated in pathogenesis are upregulated when the Bvg system is active, suggesting that this phase (Bvg-plus) is adopted by bacteria on entering the host during the course of infection. Conversely, the Bvg-minus phase is thought to be expressed when the bacteria are in the environment. A third distinct phenotypic phase, Bvg-intermediate (Bvg_i), is characterized by the lack of expression of vrgs and by the expression of only a few vags (Parkhill et al., 2003). The signal to which Bvg responds *in vivo* are unknown, but *in vitro*,

the system is activated at 37 ^{o} C and silenced by adding sulfate ions or nicotinic acid to the growth medium, regardless of growth temperature (Kinnear et al., 2001, Merkel et al., 2003).

1.5. Resurgence of whooping cough (pertussis)

Pertussis is one of the ten most common causes of death from infectious disease worldwide, accounting for 300,000–400,000 deaths each year. The manifestations of *B. pertussis* vary with the host, symptoms ranging from the most typical cough outbursts with inspiratory whoop, periods of apnoea, and post-tussive vomiting, to symptoms so mild as to be confounded with those of a common cold. *B pertussis* is increasingly recognized as a principal cause of persistent cough in adults.

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%. Estimates for individual efficacy of pertussis vaccines are around 70–80% and induced protection is of limited duration (König et al., 2002). Despite high vaccination coverage, *B pertussis* remains endemic and reports of increasing incidence in the USA, Canada, the Netherlands, 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, raising concerns about household transmission to vulnerable infants and calling for a reappraisal of

clinical management in adolescents and adults and a reinforcement of vaccination strategies (Águas et al., 2006).

1.6. Whooping cough in Turkey

In Turkey, whole cell pertussis vaccine is being administered in the 2nd, 3rd, and 4th months of life, in combination with a booster dose administered between the 18th and 24th months. If the booster is delayed, it can be administered to children younger than six years old. After participation in the Expanded Programme on Immunisation of the The World Health Organization (WHO), immunization was accelerated with Turkey's "National Vaccination Campaign" in 1985. As a result of this campaign, DPwT vaccination increased 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 (http://www.saglik.gov.tr/extras/istatistikler/temel2004/tablo-39.htm). 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, balancing need with an assessment of local circumstances (Vatansever et al., 2005).

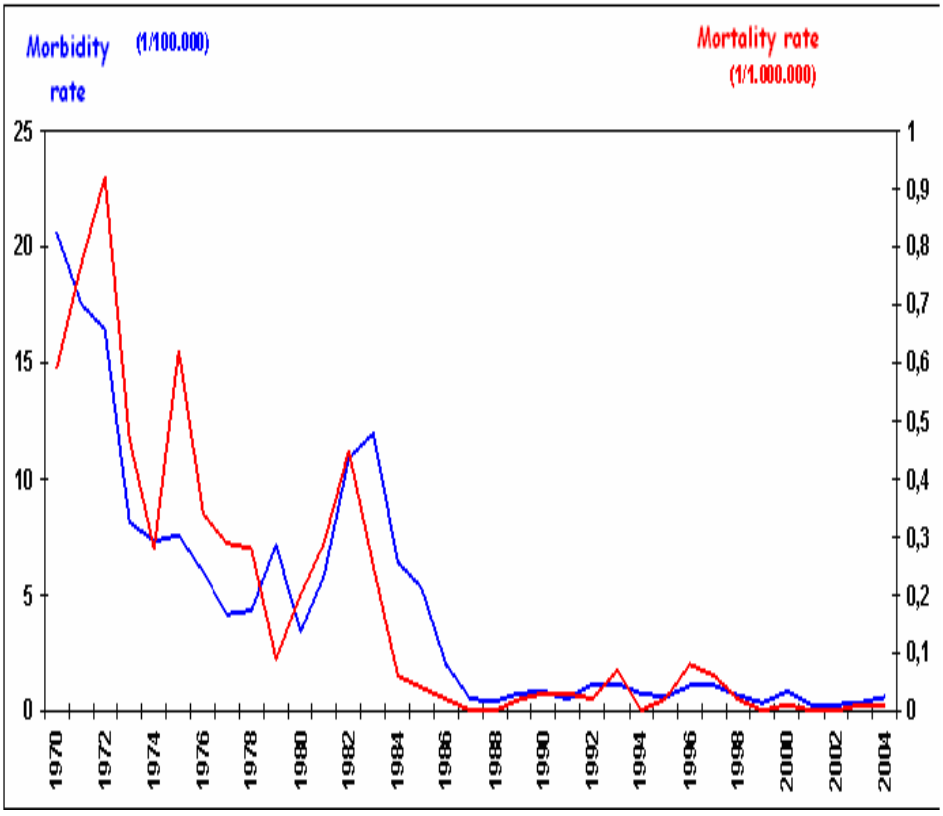

Figure 5. Pertussis morbidity and mortality rates in Turkey, 1970-2004 (http://www.saglik.gov.tr/extras/istatistikler/temel2004/tablo-39.htm).

1.7. Proteomics

The term proteome, in analogy to the term genome, was coined to describe the complete set of proteins that an organism has produced under a defined set of conditions (Wasinger et al., 1995). The genome is static because it represents the blueprint for all cellular properties that a cell is able to develop. In contrast, the proteome is highly dynamic and much more complex than the genome. It is critical for survival that the protein composition of a cell is constantly adjusted to meet the challenges of changing environmental conditions. Already in 1975, the powerful method of

two-dimensional-polyacrylamide gel electrophoresis (2D PAGE) was introduced that allowed one to separate highly complex cellular protein extracts into individual proteins on a single gel based on two properties of the proteins the isoelectric point (pI) and the molecular weight (MW), (O'Farrell, 1975). Based on a well-annotated genomic sequence, it became possible to introduce large-scale mass spectrometry (MS) techniques to identify virtually every protein detected on a 2D gel. The increase in throughput, the partial automation, and the higher reproducibility of 2D-PAGE analysis recently made it a very attractive tool to study cellular functions on a molecular level.

Proteins are the functional output of the cell and therefore might be expected to provide the most relevant information, particularly when interpretation of their expression takes into account their dynamics in specific biological contexts. The expression or function of proteins is modulated at many points from transcription to post-translation, which generally cannot be predicted from analysis of nucleic acids alone. There is poor correlation between the abundance of mRNA transcribed from the DNA and the respective proteins translated from that mRNA and the transcript can be spliced in various ways to yield different protein forms. Extensive changes can also be introduced during or after translation for example, the addition of specific carbohydrate side-chains or phosphorylation leading to multiple protein products from a single gene. Preliminary studies suggest an average number of protein forms per gene of one to two in bacteria, three in yeast, and three to more than six in human beings. Thus, the human body may contain more than half a million modified proteins. Understanding of their interplay is a formidable undertaking, even at a cellular level, where only 5000–10 000 genes (although many more proteins) may be expressed, particularly with their functional state varying with post-translational modifications such as phosphorylation and dephosphorylation, glycosylation, cleavage, complex formation and translocation within the cell (Banks et al, 2000).

Globally, proteomic approaches have three major advantages over more traditional approaches. First, these technologies allow for the analysis of up to thousands of proteins simultaneously, in any tissue or organelle, under any given physiological condition. Second, prior to proteomic applications, no limitations are set for the proteins analyzed on cellular functions or role in specific signal transduction pathways. Therefore, these studies enable the investigation of proteins and protein populations that are not a priori expected to be linked to any physiological conditions, allowing for the discovery of novel molecular mechanisms, opening novel research avenues. In addition, proteomic studies are not limited to proteins that have already been characterized. They allow for the study of links between physiological conditions and novel proteins, of which thus far only hypothetical amino acid sequences exist, deduced from the nucleic acid sequence of corresponding genes (Arckens et al., 2006). Third, the high sensitivity of proteomic technologies allows for these large-scale screening studies utilizing only a minimal amount of protein. For example, while for a typical Western blotting experiment, 40 µg of protein sample is loaded on a gel for the analysis of one or a few proteins, proteomic expression analysis of more than a thousand proteins in a single sample can be performed using as little as 50 µg of protein. The requirement of such a little amount of protein sample for these high-throughput studies is particularly advantageous when the availability of tissues is limited, for example when samples are obtained from transgenic animals, small brain regions or human tissues. Because of these unique properties, an overview of the expression profiles of thousands of proteins can be established for each sample investigated (Kislinger et al, 2006). Comparative proteomic studies in which protein expression in diseased and control tissue is quantitatively compared are particularly suitable for the discovery of novel biomarkers of diseases (Fu and Van Eyk, 2006).

Moreover, time point proteomic studies permit follow-up studies of changes in expression levels of hundreds of proteins at different time points before and after drug treatment, or after various physical conditions. Data obtained from proteomic analyses can subsequently be combined with more traditional approaches such as Western blotting and confocal microscopy for biological validation and functional studies.

1.7.1. Steps in proteome work

1.7.1.1. Isoelectric focusing and immobilized pH gradient

Isoelectric focusing (IEF) is an electrophoretic method which separates proteins according to their isoelectric points (pI) (O' Farrell, 1975). In nature, proteins are amphoteric molecules. They carry either negative, positive or zero net charge, depending on their surrounding pH level. The net charge of a protein is the sum of all the positive and negative charges of R groups (amino acid side chains) and amino- and carboxyl-termini. Isoelectric point (pI) is the specific pH where the net charge of a protein is zero. Proteins are positively-charged with pH values below their pI and negatively-charged with pH values above their pI. If the net charge of a protein is plotted versus the pH of its environment, the resulting curve intersects the x-axis at its pI.

The presence of the pH gradient is essential to the IEF method. In the pH gradient, under the influence of an electrical field, a protein will migrate to a location in the gradient where its net charge is zero. 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 pI. On the contrary, proteins with negative net charges will migrate toward anode, becoming less negatively charged as it moves through the pH gradient until it also reaches a zero net charge. When a protein diffuses away

from its pI, it suddenly gains charge and moves back to its pI position. This is called "focusing" effect of IEF, which concentrates proteins at their pIs and permits proteins to be separated on the basis of very small charge differences. EF is performed under denaturing conditions. Complete solubilization and denaturation is achieved by using a mixture of detergent, urea and ampholines. Employing this mixture ensures that each protein is present in only one configuration, aggregation and intramolecular interaction. After the first dimension is completed the second dimension is achieved on the base of protein size using sodium dodecyl sulfate polyacrylamide gel electrophoresis.

1.7.1.2. Visualisation of protein spots and image analysis

There are a number of visualizing methods for the protein spots resolved by the 2-DE. The highest sensitivity is achieved with radioactivity/fluorography detection. A number of non-radioactive methods (Silver staining, Coomassie brilliant blue staining) are applied, with big difference in sensitivity, linearity and dynamic range. Sensitive protein staining, like the ammoniacal silver staining, permits the detection of proteins at or below nanogram quantities.

1.7.1.3. Mass spectrometry analysis

An array of approaches has been developed for addressing proteome analysis and protein identification. Methods of protein identification have included immunoblotting, peptide sequencing, amino acid composition and more recently the use of mass spectrometry (Celis et al., 2000). One of the earliest methods used for protein identification was microsequencing by Edman chemistry to obtain N-terminal amino acid sequences.

Mass spectrometry (MS) enables protein structural information (i. e., peptide masses or amino acid sequences) to be obtained. Hence, this information can be employed to identify the protein by searching nucleotide and protein databases. Identification of proteins by mass spectrometry requires three major stages: (i) sample preparation, (ii) sample ionization and (iii) mass analysis. Extraction of a whole protein or its constituent peptide from the gel is commonly very difficult and mostly inefficient. On the other hand, if a protein is "in gel" digested with proteases (e.g. with trypsin) many of the peptides can be extracted from the gel (Andersen and Mann, 2000). Therefore, *in situ* digestion is more efficient at sample recovery than other common methods such as electroblotting. Additionally, the conversion of a protein into its parts provides more information than that can be obtained from a whole protein itself.

First requisite for biological sample to be analyzed by MS is that the molecule must be charged and dry (Graves and Hystead, 2002). Conversion of the samples into desolvated ions is the most commonly used application. Although there are different techniques available, the most preferable methods are Electrospray Ionization (ESI) and Matrix-assisted Laser Desorption/Ionization (MALDI). 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 analysis follows the conversion of protein or peptides into molecular ions. This is performed by the mass analyzer in a mass spectrometer which resolves the molecular ions on the bases of their mass and charge in a vacuum. Although there are few available techniques, a time-of-flight (TOF) is one of the simplest mass analyzer. It measures the m/z (mass to charge)

ratio of an ion by determining the time required for it to transverse the length of a flight tube. From the MS scan, after subtracting the backgrounds, amino acid sequence information for the peptide is obtained with the aid of software. The information obtained is then used to search DNA and protein databases (SWISS-PROT, FASTA; MOWSE, ProFound, PepFrag, PepSea and so on).

1.7.2. The benefits of pathogen proteomics and immunoproteomics

Compared to eukaryotic cells, bacteria are great model organisms to study regulatory networks, protein function, and even cell differentiation, because their genomes are relatively small and adaptation processes are less complex and involve smaller numbers of protein components. Some bacteria are easily genetically manipulated and are thus excellent models to study protein function. Bacteria also have an even more direct impact on human life in that a variety of species are indispensable for aspects as immune system maturation, nutrition digestion, and vitamin production. On the other hand, interactions harmful to the human host occur when bacteria override the defense barriers and cause infections.

So far, 554 complete genomes of pathogenic and nonpathogenic bacteria are available and the genome of approximately 1000 bacteria is being sequenced [Genomes OnLine database (GOLD) at http://wit.integratedgenomics.com/GOLD/]. Fueled by growing DNA sequence information, the analysis of the proteome (the entire set of proteins expressed by a genome) becomes a valuable and useful tool for antigen discovery and thus vaccine design (Grandi, 2001; Rappuoli et al., 2006).

Infections by microorganisms cause some 17 million deaths each year according to WHO statistics (www.who.org). Although most of the deaths

occur in the less-developed countries, death due to infectious diseases is back to rank number three even in the most developed countries. One important reason for that unpleasant development is the fact that bacteria that were previously susceptible to the large armory of antibiotics have now developed resistance against them. Another reason is ironically provided by the progress in medicine in general, because we are becoming older and more often subject to aggressive treatment regimens; for example, in surgery, transplantation, and cancer chemotherapy. All of those manipulations lead to a suppression of our immunological defense capabilities, and, thereby, to more serious and more difficult to treat infections. Thus, novel treatment options are urgently required, and the need for novel antibacterial agents without cross-resistance to existing antibiotics as well as the development of alternative treatment regimens should have high priority on any meaningful public health agenda. In that environment, it is not astonishing that proteome analysis of the consequences of antimicrobial treatment for bacteria has recently gained increasing interest (Brötz et al, 2005). It can provide a deeper insight into how a bacterium responds to a certain antimicrobial treatment. In addition, benefits are expected in many other aspects of modern drug development approaches such as the identification of novel target areas and the elucidation of the molecular mechanisms of action of novel drug candidates (Rappuoli et al, 2005).

Proteomics approaches to pathogens may have different targets: (i) Characterization of submicrobial proteomes (for example, secreted proteins, surface proteins and immunogenic proteins), (ii) comparative analysis of different strains and physiological states, (iii) identification of proteins related to pathogenicity, (iv) identification of proteins involved in host–pathogen interactions and (v) evaluation of mechanisms of action of antimicrobials are the most important ones (Hanash, 2003).

Much of information about immunogenic components can be derived from proteomics coupled to Western blotting, namely immunoproteomics (Rappuoli et al, 2005; Peng et al, 2004). Proteomics has been used to identify novel bacterial vaccine candidates against several human pathogens. Integrated proteomic strategies have also been successfully applied in the discovery of antigens from *Helicobacter pylori* (Nilsson et al, 2000; Krah et al., 2004; Lin et al., 2006; Santucci et al., 2006), *Shigella flexneri* (Peng et al., 2004, Ying et al., 2005a, Ying et al., 2005b), *Staphylococcus aureus* (Vytvytska et al, 2002), *Streptococcus agalactiae* (Hughes et al, 2002) *Plasmodium falciparum* (Doolan et al, 2003), *Bacillus anthracis* (Ariel et al, 2003), *Haemophilus influenzae* (Langen et al, 2000, Thoren et al, 2002), *Francisella tularensis* (Havlasová et al., 2005), *Aeromonas hydrophila* (Peng et al., 2004), *Anaplasma marginale* (Brown et al., 2005) *Brucella abortus* (Neubauer et al, 2006), *Corynebacterium diphtheria* (Hansmeier et al., 2006), *Neisseria meningitides* (Vipond et al., 2006), *Streptococcus iniae* (Shin et al., 2006), *Lactococcus garvieae* (Jung et al., 2007), *Leishmania donovani* (Gupta S. K et al, 2007), *Mycoplasma hyopneumoniae* (Pinto P, 2007), *Bacillus anthracis* (Shafferman et al., 2007) and *Streptococcus pyogenes* (Severin et al., 2007). Some of these studies are briefly decribed in next section.

1.7.3. Immunoproteomics approaches to pathogenic bacteria

H. pylori has been one of the most popular pathogen for immunproteomics. 29 proteins from *H. pylori* G27 proteome strongly reacted with sera derived from *H. pylori* infected patients suffering from different gastroduodenal pathologies (Kimmel et al., 2000). Recently, by using a proteomics approach, protein composition of two *H. pylori* fraction, soluble proteins and structure-bound proteins (including membrane proteins) were investigated. Both fractions differed markedly in the overall protein composition as determined by 2-DE. Numerous outer membrane proteins, the vacuolating cytotoxin

VacA, other potential virulence factors and few ribosomal proteins were detected (Backert et al., 2005).

Shigellosis is a major public health problem in infants and young children in poor countries and is the major etiologic agent of traveler's diarrhea. In the first immunoproteomics approach to *S. flexneri*, 13 immunogens were identified which included seven outer membrane and six soluble proteins. Of these 13 proteins, 12 were shown to be novel immunogens (Peng et al., 2004). In a more recent study with the same pathogen, 5 known antigens and 8 novel antigens were successfully identified. A hypothetical protein (YaeT) was detected which might be a candidate vaccine (Ying et al., 2005a). The same group also identified 22 OMPs and 12 extracellular proteins as immunogenic. Eight of these proteins were novel antigens with potential as vaccine candidates (Ying et al., 2005b).

In a proteomic analysis of meningococcal outer membrane vesicles of *N. meningitidis*, a major pathogen of meningitis and septicaemia, 74 spots from the proteome map were identified including the outer membrane protein antigens PorA, PorB, RmpM and OpcA. In addition to outer membrane proteins, outer membrane vesicles contained periplasmic, membrane-associated and cytoplasmic proteins (Vipond et al., 2006). *F. tularensis* live vaccine strain infection of mice has been established as an experimental model of tularemia that is suitable for studies of immune mechanisms against the intracellular pathogen. Globally, 36 different proteins were identified which strongly reacted with sera from experimentally infected mice, including several putative virulence markers of intracellular pathogens as nucleoside diphosphate kinase, isocitrate dehydrogenase, RNA-binding protein Hfq and molecular chaperone ClpB. Of them, 27 proteins were described for the first time as immunorelevant (Havlasová, 2005).

Hansmeier et al. (2006) presented the first systematic reference map of the extracellular and cell surface proteome fractions of the type strain *C diphtheriae* which is the etiological agent of the acute, communicable disease diphtheria. The data suggested that the extracellular and cell surface proteome of *C. diphtheriae* consists of two major groups of proteins. The first class of proteins generally executes functions in the cytoplasm and is not known to be actively secreted. This class includes, for instance, proteins participating in translation and protein folding as well as in molecular mechanisms of detoxification. The second class of proteins was apparently secreted actively, including solute-binding components of siderophore-type iron ABC transporters and proteins that are encoded by pathogenicity islands.

B. abortus is the etiologic agent of bovine brucellosis and causes a chronic disease in humans known as undulant fever. For the development of a safer and equally efficacious vaccine, immunoproteomics was utilized to identify novel candidate proteins from the cell envelope. A total of 163 proteins were identified using 2-DE with MALDI-TOF MS and Liquid Chromatography-MS/ MS. Some of the major protein components included outer membrane protein 25, OMP31, Omp2b porin, and 60 kDa chaperonin GroEL. 2-DE Western blot analyses probed with antiserum from bovine and a human patient infected with *Brucella* identified several new immunogenic proteins such as fumarate reductase flavoprotein subunit, F0F1-type ATP synthase a subunit and cysteine synthase A (Connolly et al, 2006).

S. aureus, an opportunistic pathogen and the most pathogenic staphylococcal species, causes illnesses ranging from minor infections to life-threatening diseases. Vytvytska et al. (2002) focussed on the analysis of two particular subproteomes, surface preparations of *S. aureus* COL grown under standard or iron-stress conditions The COL strain was used as its genome was nearly completely sequenced and it is a methicillin-resistant clinical isolate. Their

approach led to the discovery of several new antigens with potential for vaccine development. These included membrane proteins Aux1 (a putative transmembrane protein phosphatase involved in resistance to methicillin) and LP309 (hypothetical lipoprotein with close homology to *S. epidermidis* iron-regulated ABC-type transporter), the latter only detected under stress conditions (iron depletion) consistent with a role in the physiologically important and difficult iron uptake during infection. Both antigens were proposed by the authors as not only vaccine candidates, but also potential drug targets, as they appeared to be involved in important virulence related functions (drug resistance, iron uptake).

Four novel highly antigenic proteins of *M. hyopneumoniae* were identified from its soluble proteome, a heat shock protein 70, an elongation factor Tu, a pyruvate dehydrogenase E1-beta subunit and the P76 membrane protein (Marcos et al., 2007).

K. pneumoniae is an opportunistic pathogen which causes pneumoniae, urinary tract infections and septicemia in immunocompromised hosts. To identify candidate antigens of *K. pneumoniae* for vaccine development, sera from patients with acute *K. pneumoniae* infections and a control group of sera from healthy individuals were analyzed for reactivity by Western blot against *K. pneumoniae* outer membrane proteins separated by 2-DE. Twenty highly immunogenic protein spots were identified and the ones that were most frequently recognized by positive *K. pneumoniae* sera included OmpA, OmpK36, FepA, OmpK17, OmpW, colicin I receptor protein and three novel proteins (Kurupati et al., 2006).

1.8. Aim of the present study

The identification of antigenic proteins as putative whole protein subunit vaccines is a key goal of immunovaccinology. Recent sequencing of *Bordetella pertussis* genome (Parkhill et al, 2003) provided an essential database for applying proteomics approaches to this organism, recalling an obvious need for reinforcement of vaccination strategies against whooping cough. In the present study, we took the advantage of the availability of complete *B. pertussis* genome sequence and attempted to construct the first systematic immunoproteome map of *B. pertussis* with the aim of providing further insight into the biology and immunogenicity of this pathogen. Our approach involved the use of total soluble proteome, secretome and outer membrane subproteome, respectively, for the identification of the predominant immunogenic proteins via 2-DE followed by immunoblotting and MALDI-TOF MS analyses.

CHAPTER 2

MATERIALS AND METHODS

2.1. Bacterial strains

The strains of *B. pertussis* used in this study were Tohama and Saadet. While Tohama is a standard strain of *B. pertussis* used worldwide for both vaccine manufacture and research, Saadet is a local *B. pertussis* strain which was isolated in 1948 from a baby girl named Saadet and then used as the primary vaccine strain in Refik Saydam Central Institute of Hygiene till 1996, the year at which the manufacture of vaccines was completely terminated in Turkey. Both strains were kindly provided by Dr. Erkan Özcengiz (VBR Vaccine Res. Co., Ankara).

2.2. Culture media, growth and maintenance

B. pertussis Tohama and Saadet strains were grown on Cohen-Wheeler agar medium (Sato et al., 1972) for 48 h, stored at 4^{O} C and subcultured at 2 week intervals. For long term maintenance, the bacteria were collected from the agar surface, resuspended in phosphate buffered saline (PBS) to an OD_{600} of 0.6 (corresponding to ca. 10^9 cells/ml) and stored at – 80^{O} C after covering with 20% glycerol. When necessary, the strains were also grown on modified Morse and Bray liquid medium (Sato et al., 1974) for 48 h at 37^{0} C (Appendix C).

2.3. Buffers and Solutions

The composition and preparation of buffers and solutions are presented in Appendix A.

2.4. Chemicals and Enzymes

The chemicals and enzymes used and their suppliers are listed in Appendix B.

2.5. Sample preparation for 2-DE

2.5.1. Total soluble proteome

B. pertussis Tohama and Saadet strains were grown on Cohen-Wheeler agar medium for 48 h at 37^0 C. For the preparation of whole cell lysate, the cells were collected and suspended in cold TE buffer, centrifuged, and the pellet was resuspended in 1 ml of lysis buffer composed of 0.04 M lysosyme in TE buffer and incubated at 37^o for 30 min. The pellet was solubilized in rehydration buffer (7 M urea, 2M thiourea, %4 CHAPS, pH 3-10 ampholyte, DTT), vortexed at 4^oC for 30 min, and recentrifuged. The protein samples were stored at -20 ^{0} C.

2.5.2. Extracellular proteome (secretome)

B. pertussis Tohama secretome was prepared as in Tianyi et al., 2005. The organism was cultured in 100 ml of modified Morse and Bray liquid medium by shaking at 200 rpm for 48 h at 37^0 C. Supernatant was collected by centrifugation (Sigma Laboratory Centrifuges, 3K30) for 15 min at 20 000 *g* at 4^0 C and then filtered through 0.22 µm membrane. To protect protein sample against proteases, phenylmethylsulfonyl fluoride (PMSF) was added to a final concentration of 0.1 mM. The supernatant was treated with 15% TCA in ice for 30 min to precipitate protein. The precipitate was collected by centrifugation at 10 000 *g* for 20 min at 4^0C, washed three times with ice cold acetone containing 0.1% DTT to remove traces of TCA and finally acetone was removed by speed vacuum treatment (Heto spin vacuum system, VR-1).

Extracellular proteins were solubilized with 7 M urea, 2 M thiourea, 4% CHAPS, and 50 mM DTT.

2.5.3. Outer membrane subproteome

Two different methods were used for outer membrane protein (OMP) extraction; Sarkosyl and Urea extraction methods, respectively.

2.5.3.1. Sarkosyl method

The Sarkosyl method described by Leyt and Griffth (1992) was used. The cells were thawed and PMSF was added to a final concentration of 0.1 mM. The cells were disrupted by a sonicator (Ultrasonic processor CP 70T, Cole-Parmer) for 10 $^{1\text{-min}}$ bursts (50% cycle), while being cooled in an ice-water bath. Whole cells and large debris were removed by centrifugation at 5,000 x *g* for 20 min, and the total membrane fraction was harvested from the supernatant by centrifugation at 100,000 x *g* for 1 h at 4°C. The membrane fraction was suspended in 1% (w/v) Sarkosyl (sodium lauryl sarcosine) in 10 mM HEPES [*4*-(2-hydroxyethyl)-1-piperazineethanesulfonic acid] buffer, pH 7.4 containing 0.1 mM PMSF for 30 min at room temperature. The detergent-insoluble material containing the OMP-enriched fraction was harvested by centrifugation at 100,000 x *g* for 60 min at 4°C. The final insoluble pellet was suspended in rehydration buffer (7 M urea, 2M thiourea, %4 CHAPS, pH 3-10 ampholyte, DTT). The protein samples were stored at -20 ^{0} C.

2.5.3.2. Urea extraction method

For the preparation of OMP-enriched fraction, culture was suspended in 8 M urea, 50 mM Tris-Cl pH 8.0, 1 mM PMSF and incubated at room temperature for 30 min (Wright et al., 2005). The sample was then centrifuged, and the

pellet was resuspended in 1 ml of rehydration buffer (7 M urea, 2M thiourea, %4 CHAPS, pH 3-10 ampholyte, DTT), vortexed at 4^{o}C for 30 min, and recentrifuged. The protein sample was stored at -20 ^{0} C.

2.6. Protein Estimation

To determine total protein concentration, the modified Bradford assay described by Ramagli and Rodrigez (1985) was used. 5 X Bradford reagent (containing 500 mg Comassie 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, 0.3 % DTT, 1% (w/v) CHAPS, 0,3 % (w/v) DTT, 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 reagents 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.

2.7. Preparation of antisera against *Bordetella pertussis*

For antisera preparation against *B. pertussis*, BALB/c mice were subjected to three different immunizations; intranasal challenge, intraperitonal challenge and subcutaneous immunization, respectively.

2.7.1. Subcutaneous immunization

B. pertussis Tohama and Saadet strains were grown on Cohen-Wheeler agar media for 48 h. For both strains, the cells were suspended in 0.85 % saline solution to contain ca. $4x10^{10}$ bacteria/ml. The suspension was inactivated at 56^{o} C for 30 min. Inactivated bacterial cells were used as the antigen and anti-*B. pertussis* polyclonal antibodies were raised by immunizing mice. For each strain, ten 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. Serum antibody levels were measured by the micro-agglutination test which was performed on a 96 well-plate. The sera were serially diluted two fold (for 12 times) and the antibody titer was determined as 1/512 for both Saadet and Tohama immunization antibodies control mice (three as a total) received no treatment.

2.7.2. Intraperitonal challenge

B. pertussis Tohama and Saadet strains were grown on Cohen-Wheeler agar media for 48 h. The live cells were suspended in 0.85 % saline solution to contain approximately $4x10^{10}$ cells/ml. Anti-*B.pertussis* polyclonal antibodies were raised by intraperitonally challenging mice with this suspension. For each strain, ten mice received one intraperitonal injection of 50 µl per animal. Their sera were collected and pooled 14 days after the injection. The antibody titer was measured using micro-agglutination test which was performed on a 96 well-plate. The sera were serially diluted two fold (for 12 times) and the antibody titer was determined as 1/256 for both Saadet and Tohama immunization antibodies. Control mice (three as a total) received no treatment.

2.7.3. Intranasal challenge

B. pertussis Tohama and Saadet strains were grown on Cohen-Wheeler agar media for 48 h. The cells were suspended in 0.85 % saline solution to contain $4x10^{10}$ cells/ml. Anti-*B.pertussis* polyclonal antibodies were raised by intranasally challenging mice by using whole live cells. For each strain, ten mice received one intranasal injection of 25 µl per animal. Their sera were collected and pooled 14 days after the injection. The antibody titer was measured using micro-agglutination test which was performed on a 96 well-plate. The sera were serially diluted two fold (for 12 times) and the antibody titer was determined as 1/512 for Saadet and 1/256 for Tohama immunization antibodies. Control mice (three as a total) received no treatment.

2.8. 1-D gel electrophoresis and Western blotting

SDS-PAGE of total cell extract (40 µg) was carried out by using a 4% stacking gel (pH 6.8) and a 12% separating gel (pH 8.8). The electrophoresis was performed with a constant voltage of 120 V. The gels were stained with Comassie G-250 (Neuhoff et al, 1988). Proteins from the identical, but non-stained gels were transferred to a nitrocellulose membrane for 18 h at 30 mA in transfer buffer (48 mM Tris, 39 mM glycine and 20% methanol). After the transfer, the membrane was blocked for 3 h with 5% skim milk in TBS at 37°C. After rinsing three times for 5 min with TBS-Tween, the membrane was incubated with primary antibody, mouse anti-*B. pertussis*, at a dilution of 1:300 in 0.05% Tween-20 in TBS (TTBS) containing 5% skim milk for 1 h at room temperature on a gentle shaker. The membrane was rinsed three times for 10 min and incubated with anti-mouse IgG (whole molecule)-alkaline phosphatase at a dilution of 1:1000 in TTBS containing 5% skim milk for 1 h.

The membrane was then washed with TBS and developed with substrate (AP Conjugate Substrate Kit, Bio-Rad, USA) until optimum color was developed.

2.9. 2D-PAGE and 2-DE Western blotting

IPG strips were passively rehydrated by applying 400 µl of rehydration buffer (8 M urea, 2 M thiourea, 1% w/v CHAPS, 20 mm DTT and 0,5% v/v ampholyte 3-10) containing 500 µg protein sample for 16 h**.** IEF was performed with commercially available IPG strips (17 cm, pH 3–10, Bio-Rad) and the Protean IEF Cell (Bio-Rad USA). Rehydrated strips were taken to the IEF process. The following voltage profile was used for IEF: 5 h 50 V; 5 h 100 V; 1 h 300 V; 1h 600 V; 1 h 1000 V; 3 h 3000 V; 2 h 5000 V followed by a linear increase to 8000 V. The final phase of 8000 V was terminated after 75 000 Vh. The IPG strips were equilibrated for 30 min each in 5mL of solution 1 (6 M urea, 50mM Tris-HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 50mg DTT) and then in 5mL of solution 2 (6 M urea, 50mM Tris-HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 225mg iodacetamide) (Gorg et al., 2004). The isolated proteins were separated in 12 % acrylamide/bis-acrylamide gels with a Bio-Rad Cell system (Bio-Rad, USA), applying approximately 30 mA per gel. To visualize the separated proteins, each gel was stained with colloidal Coomassie blue (Neuhoff et al, 1988). The procedure used for Western blotting of 2 DE gels was the same as that used for 1-DE Western blotting.

2.10. Delta2D image analysis

Coomassie stained gels were digitized by using a scanner (HP Scanjet 4070 Photosmart scanner, USA). Spot pattern analyses were accomplished by using the 2D image analysis software Delta2D version 3.3 (Decodon, Germany). The software was also used to compare the proteomes of *B. pertussis* Tohama and Saadet strains.

2.11. Protein identification

The identifications were accomplished by mass spectrometry according to established protocols. 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^{O}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.

2.12. Database searches

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

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. Animal experiments: Preperation of antisera against *B. pertussis*

B. pertussis pathogenesis has been studied mainly by using the mouse model of respiratory tract infection. Intranasal and aerosol challenge experiments using *B. pertussis* and *B. parapertussis* in mice have yielded important insights into the roles of specific virulence factors in determining colonization. Mouse respiratory as well as intracerebral challenge experiments have been used to determine immunity generated in response to *B. pertussis* infection. Nevertheless, since *B. pertussis* is restricted to humans, often large infectious doses are required to colonize the animals (Mattoo et al., 2005).

As explained in Section 2.7, three different routes were used for sera preparation against *B. pertussis*. Mice weights were regularly controlled after intranasal and intraperitonal challenge with live whole cells until collecting sera in order to see the effects of *B. pertussis* infection on mice, i.e. to prove the pathogenicity of our strains in mice. Since inactivated whole cells were used for subcutaneous immunization, mice weight gain was not monitored in this set.

Normally, a healthy mouse gains 1 gram/day during growth. The effects on mice of intranasal and intraperitonal challenges were quite different. In spite of an apparent weight loss, there was no death as a result of intranasal challenge. On the other hand, only 4 out of 10 mice and 5 out of 10 mice intraperitonally challenged with Saadet and Tohama strains, respectively could survive (Fig 8 and Fig 9). This demonstrated that intraperitonal

challenge with *B. pertussis* is more lethal for mice. Furthermore, these results proved well the pathogenicity of both strains in mice. The weight gain by the mice challenged with live whole cells of *B. pertussis* Tohama intranasally and intraperitonally is presented in Figure 6 and Figure 7, respectively.

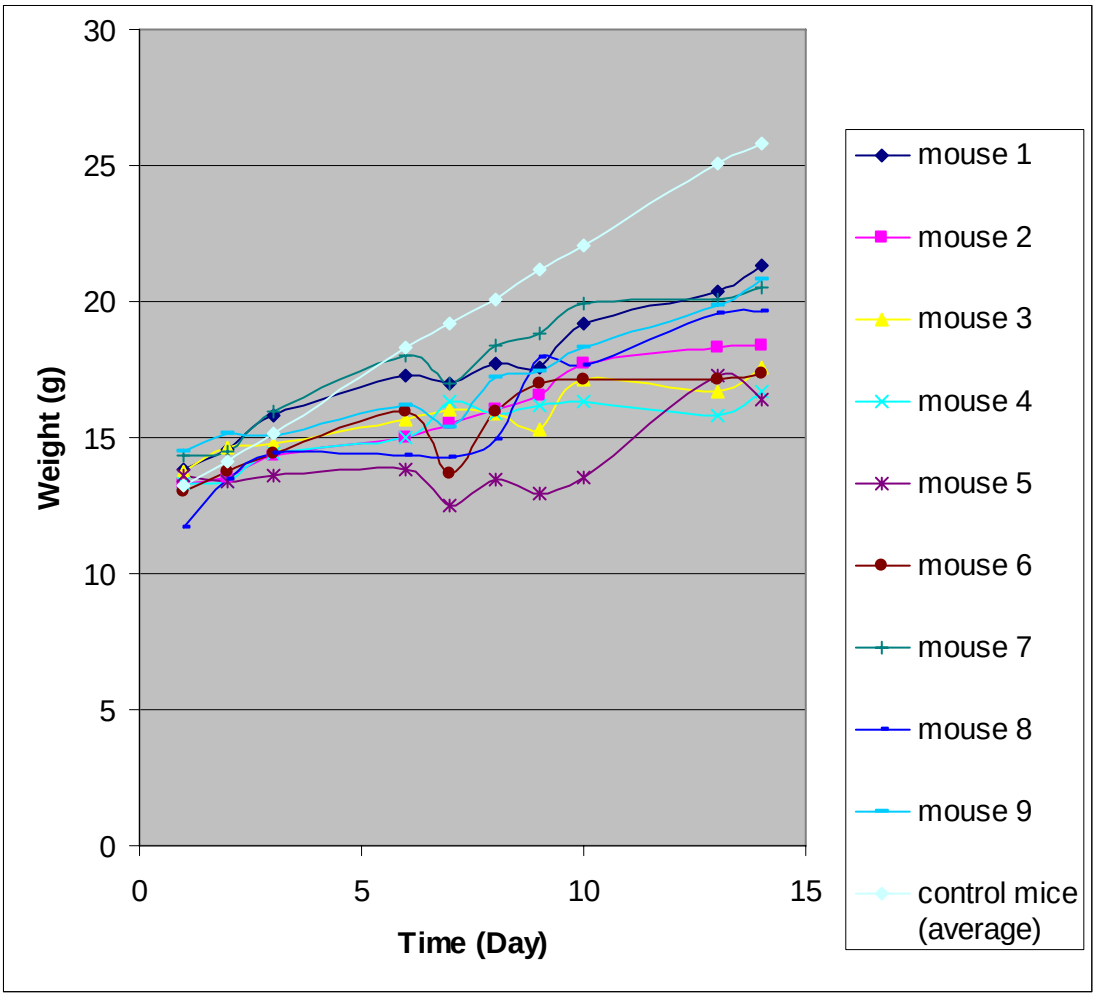

Figure 6. Weight gain as a function of time in mice intranasally challenged with live whole cells of *B. pertussis* Tohama.

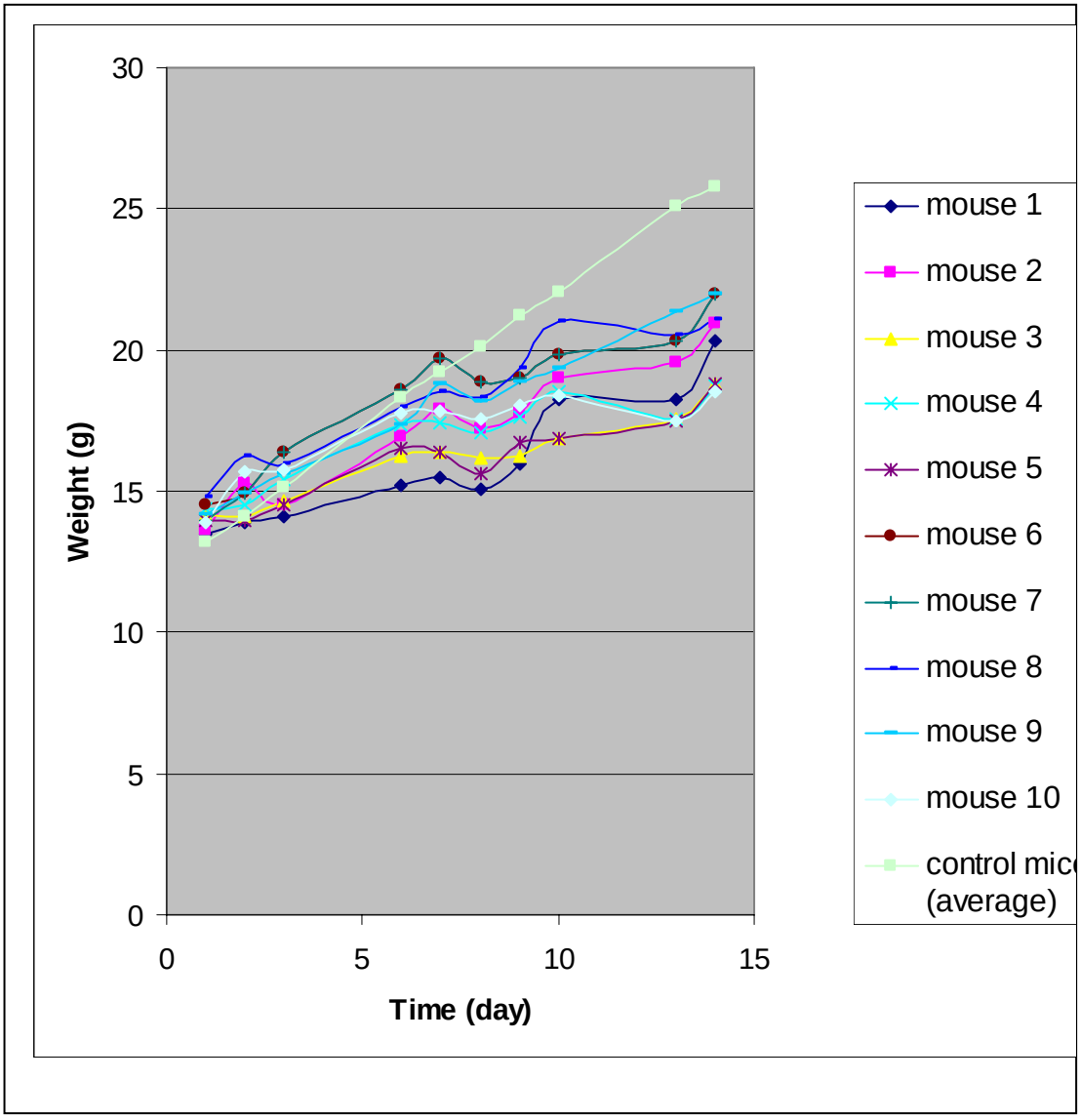

Figure 7. Weight gain as a function of time in mice intranasally challenged with live whole cells of *B. pertussis* Saadet.

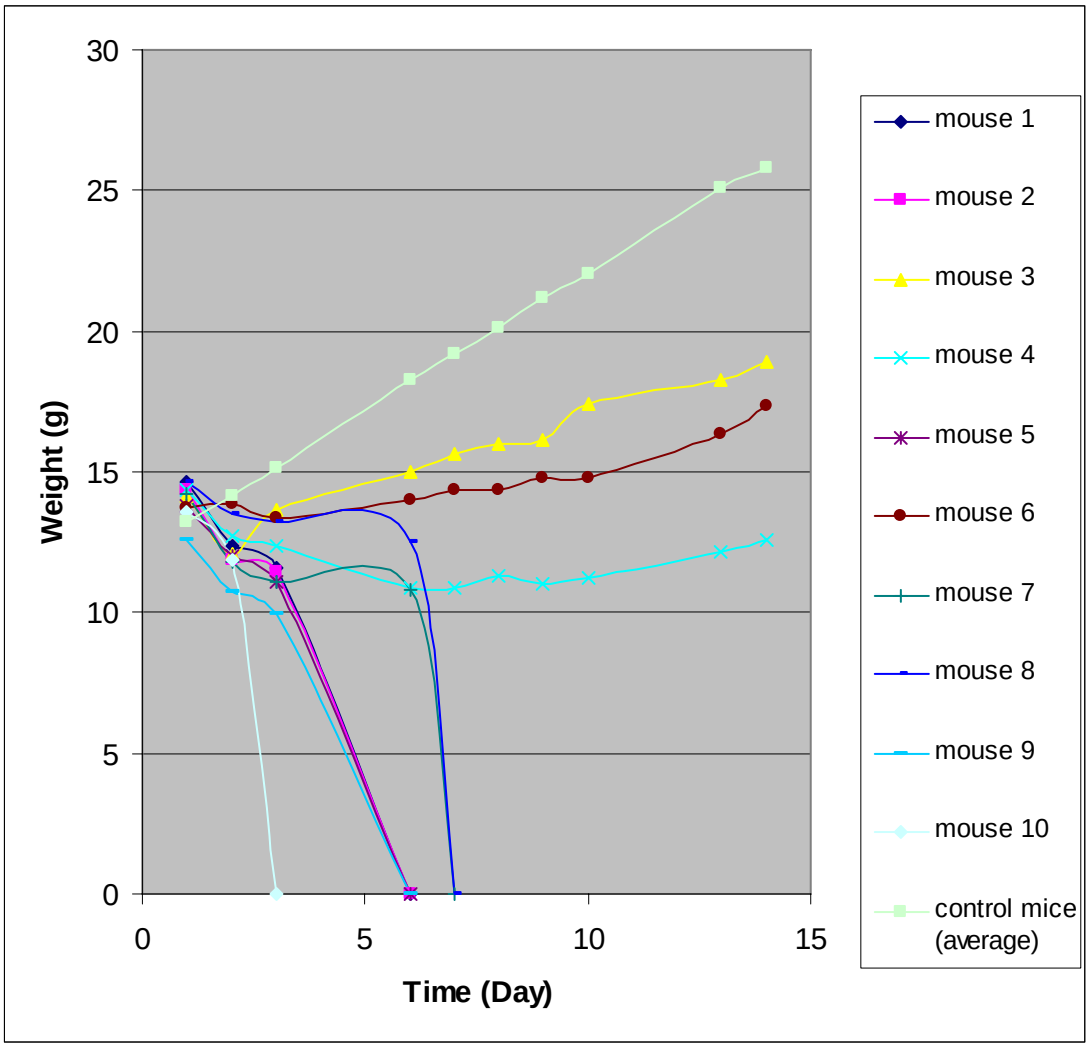

Figure 8. Weight gain as a function of time in mice intraperitonally challenged with live whole cells of *B. pertussis* Saadet.

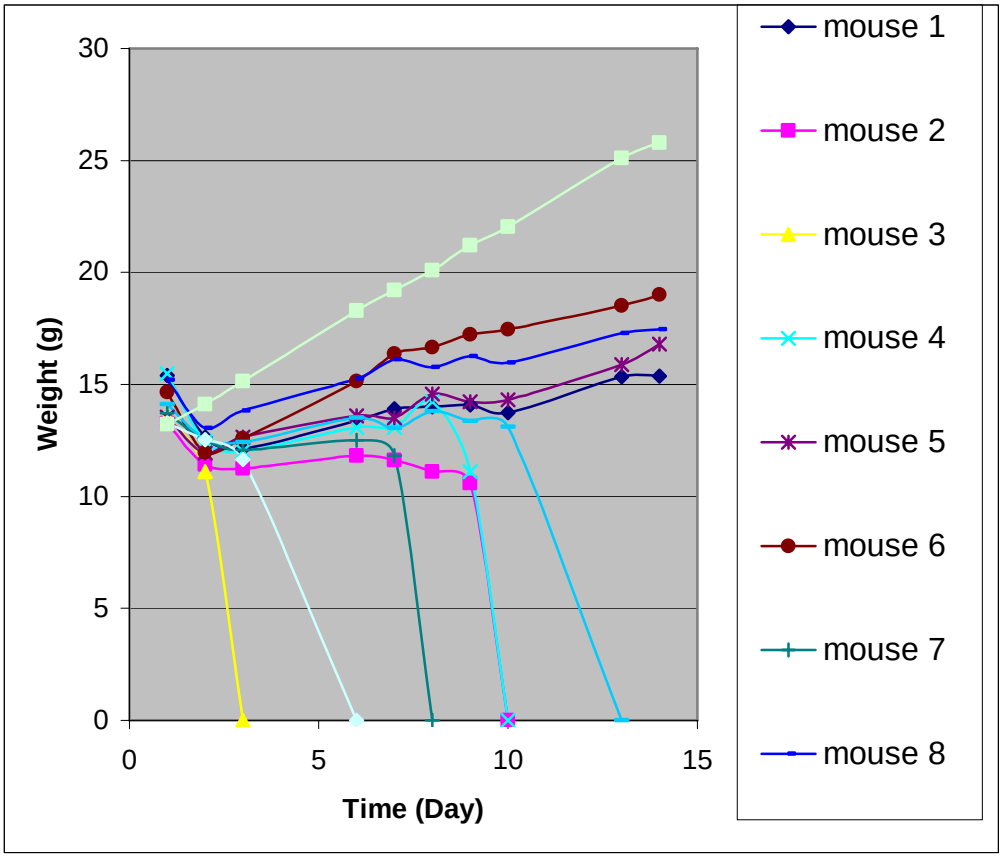

Figure 9. Weight gain as a function of time in mice challenged with live whole cells of *B. pertusis* Tohama intraperitonally.

To summarize, we used four different sera for immunoblot analyses:

Serum A: The serum was obtained from mice subcutaneously immunized with whole cell*s* of *B. pertussis* Tohama.

Serum B: The serum was obtained from mice subcutaneously immunized with inactivated whole cell*s* of *B. pertussis* Saadet.

Serum C: The serum was obtained from mice intraperitonally challenged with whole live cell*s* of *B. pertussis* Tohama.

Serum D: The serum was obtained from mice intraperitonally challenged with whole live cell*s* of *B. pertussis* Saadet.

3.2. Soluble protein profile in 1-DE and 1-DE immunoblots

Before 2-DE analyses, 1-DE analysis was made to see the efficiency of the protein extraction, the challenge/immunization time tables as well as the cross-reactions between two strains, Saadet and Tohama.

The protein profile of Tohama and Saadet strains were almost identical (Figure 10, 11 and 12). One of the most prominent and common band at the top of the gel was very likely FHA, 220 kDa. The second prominent band of about 60 kDa was thought to be chaperonine 60kDa. The thick band on 14 kDa at the bottom of the gel corresponded to lysozyme used for protein extraction.

Figure 10 shows the result of Western blotting by using *B. pertussis* Tohama immunization serum (Serum A). As expected, the serum recognized more proteins of Tohama strain than that of Saadet and gave stronger reactions. A total of 18 proteins from Tohama and 12 proteins from Saadet reacted with this serum and the strongest reactions were given with the two most prominent proteins of both strains (220 and 60 kDa, respectively).

Except for the two most prominent proteins of both strains (220 and 60 kDa, respectively), the Saadet immunization serum (Serum B) gave weaker reactions than the Tohama serum, recognized less antigens of the strains and did not identify specific antigens of Saadet. As a total, 13 proteins of Tohama and 7 proteins of Saadet were found to react with this serum (Fig. 11). The Western blot analysis by using Saadet intraperitonal challenge serum (Serum D) identified only two antigenic proteins for both strains (Figure 12), indicating that intraperitonal challenge is not convenient for raising a good antibody response against *B. pertussis* most probably because of acute and strong nature of infection (Figure 8).

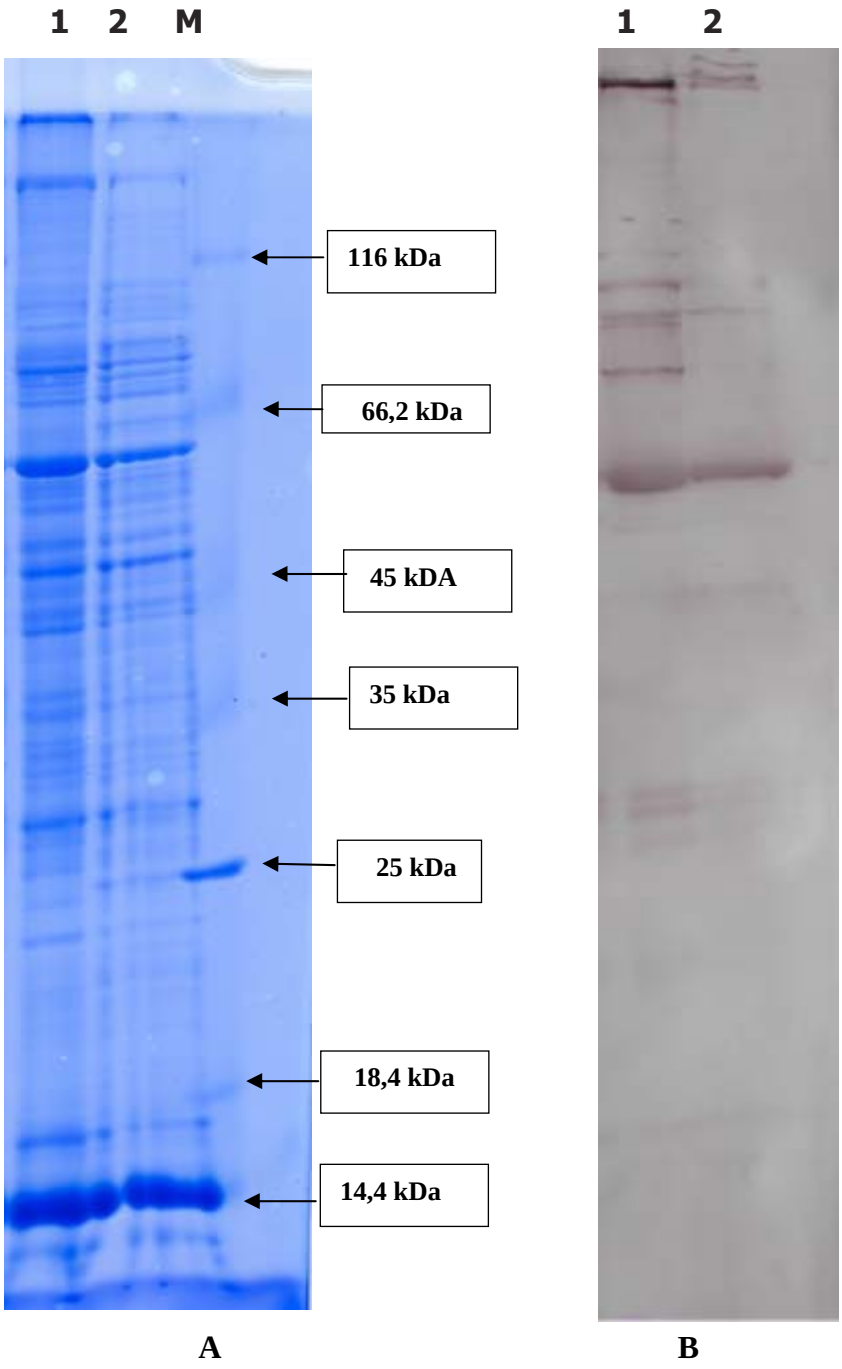

Figure 10. A: Commassie-stained SDS-PAGE gel of whole cell extracts of B. pertussis Tohama (1) and *B. pertussis* Saadet (2) strains. B: Western blot analysis with the serum obtained from mice subcutaneously immunized with inactivated whole cells of *B. pertussis* Tohama (Serum A).

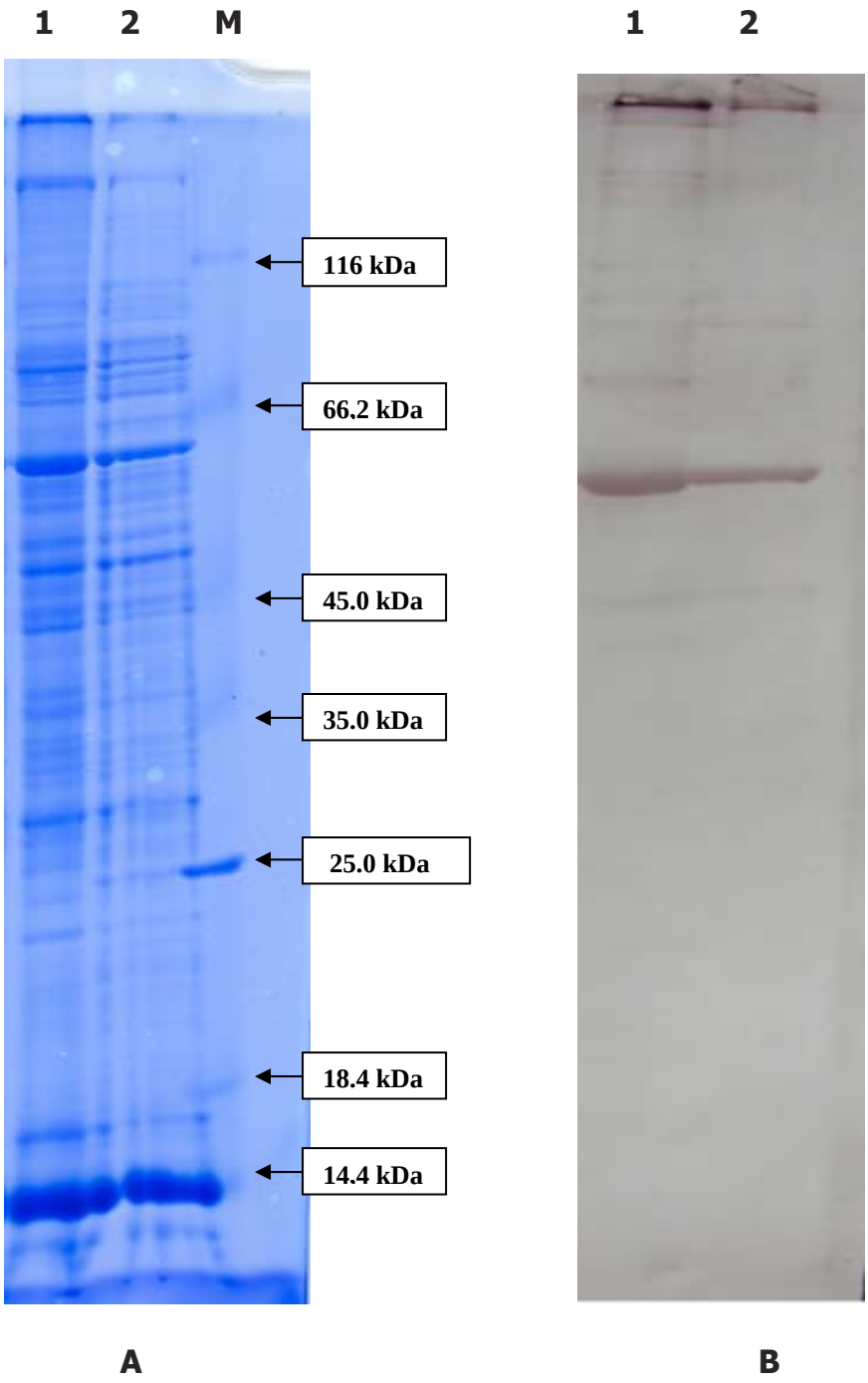

Figure 11. A: Commassie-stained SDS-PAGE gel of whole cell extracts of *B. pertussis* Tohama (1) and *B. pertussis* Saadet (2) strains. B: Western blot analysis with the serum obtained from mice subcutaneously immunized with inactivated whole cells of *B. pertussis* Saadet (Serum B).

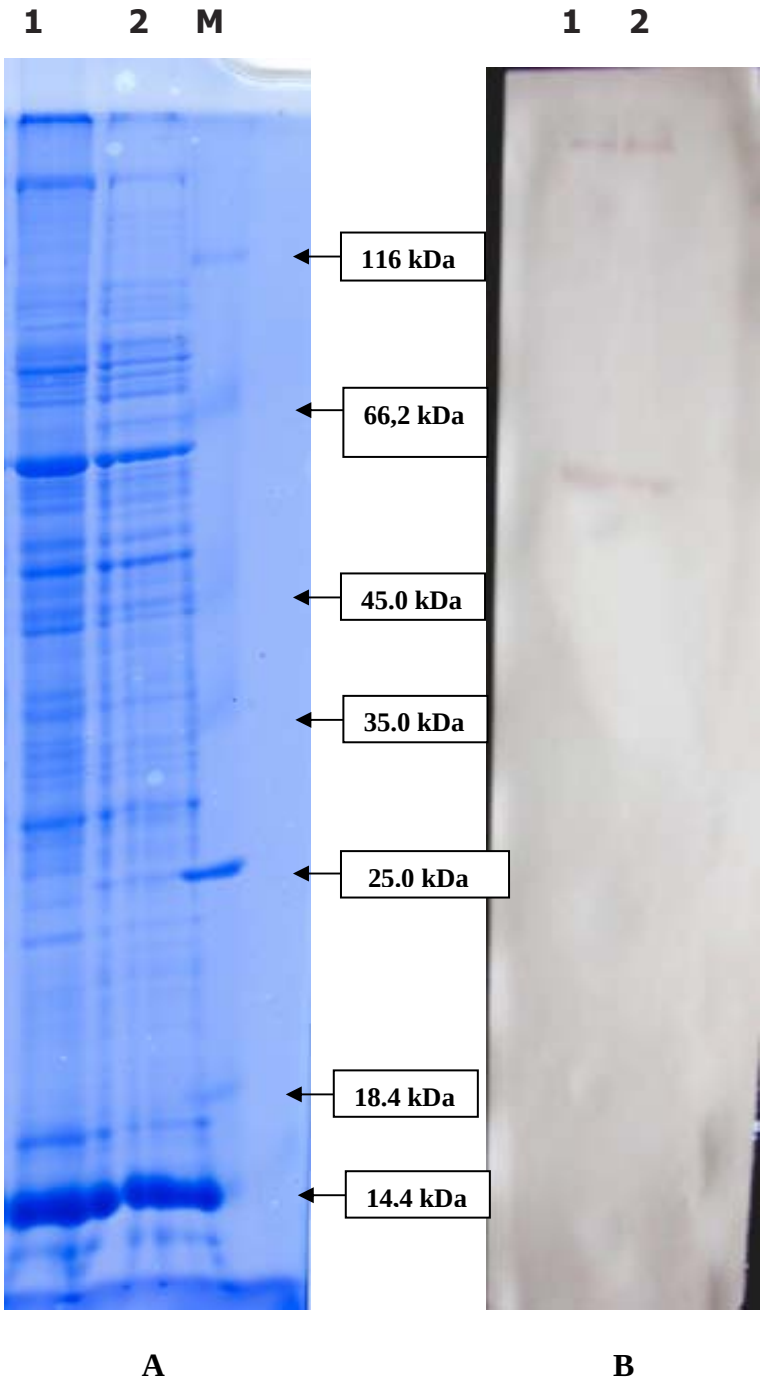

Figure 12. A: Commassie-stained SDS-PAGE gel of whole cell extracts of *B. pertussis* Tohama (1) and *B. pertussis* Saadet (2) strains. B: Western blot analysis with the serum obtained from mice intraperitonally challenged with live whole cells of *B. pertussis* Saadet (Serum D).

3.3 Theoretical proteome map

To determine the optimal range for standard 2-D gel-based proteome analysis of *B. pertussis*, the theoretical gel was obtained from the gelbank database (http://gelbank.anl.gov/proteomes/insilico/257313.asp). According to the theoretical map (Figure 13), a p*I* range between 3 and 10 was chosen as the analytical window to cover most of the proteins.

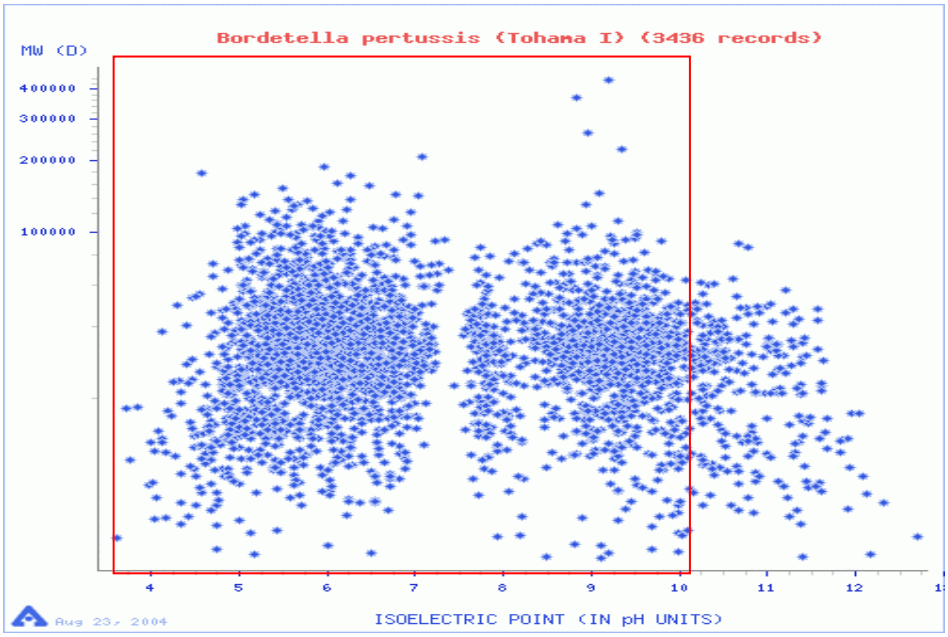

Figure 13. Predicted 2-D gel separation of the *B. pertussis* proteome. The red rectangle indicates the analytical pI 3-10 range.

3.4. Total soluble proteomes and immunoproteomes of *B. pertussis* Tohama and Saadet

The profile of total soluble proteins of *B. pertussis* Tohama separated by 2-DE gel and stained by Comassie G-250 is shown in Figure 14. A total of 630 protein spots could be detected on the 2-DE gel and 40 of them reacted with polyclonal antibodies (Serum A) against inactivated whole cells of *B. pertussis* Tohama (Figure 15). Among these 40 proteins, 28 (56 spots in total from the

master gel duplicates) could be cut and analyzed by MALDI-TOF MS. Of these 28 proteins, 17 corresponded to 10 Bordetellae protein entries which comprised of 4 known and 6 novel antigens, as listed in Table 7 and shown in Figure 16. On the other hand, only 8 proteins reacted with polyclonal antibodies (Serum C) against live whole cells of *B. pertussis* Tohama (Figure 17). Among these 8 proteins, 6 (12 spots in total from the master gel duplicates) could be cut and analyzed by MALDI-TOF MS. Of these 8 spots, 3 corresponded to 2 different Bordetellae protein entries, comprised of 1 known and 1 novel antigen, as listed in Table 8 and shown in Figure 18.

The profile of soluble proteins of *B. pertussis* Saadet separated by 2-DE gel and stained by Comassie G-250 is shown in Figure 19. 600 protein spots could be detected on the 2-DE gel and 17 of them reacted with polyclonal antibodies (Serum B) against inactivated whole cells of *B. pertussis* Saadet (Figure 20). Among these 17 proteins, all could be cut (34 spots in total from the master gel duplicates) and analyzed by MALDI-TOF MS. These 17 proteins corresponded to 9 *Bordetellae* protein entries, comprised of 1 known and 8 novel antigens, as listed in Table 9 and shown in Figure 21. On the other hand, 14 of the soluble proteins reacted with polyclonal antibodies (serum D) against live whole cells of *B. pertussis* Saadet (Figure 22). Among these 12 spots, 6 could be cut (12 spots in total from the master gel duplicates) and analyzed by MALDI-TOF MS. These 6 proteins corresponded to 4 different Bordetellae protein entries which are known to be immunogenic proteins, as listed in Table 10 and shown in Figure 23.

Well-separated master gels presented in Figure 14 and 19 constituted first experimental proteome maps of two different strains of *B. pertussis* although the identification of every spot has not yet been accomplished. The studies are under way to define the whole proteome which will form a basis for physiological proteomics of the pathogen planned as future research.

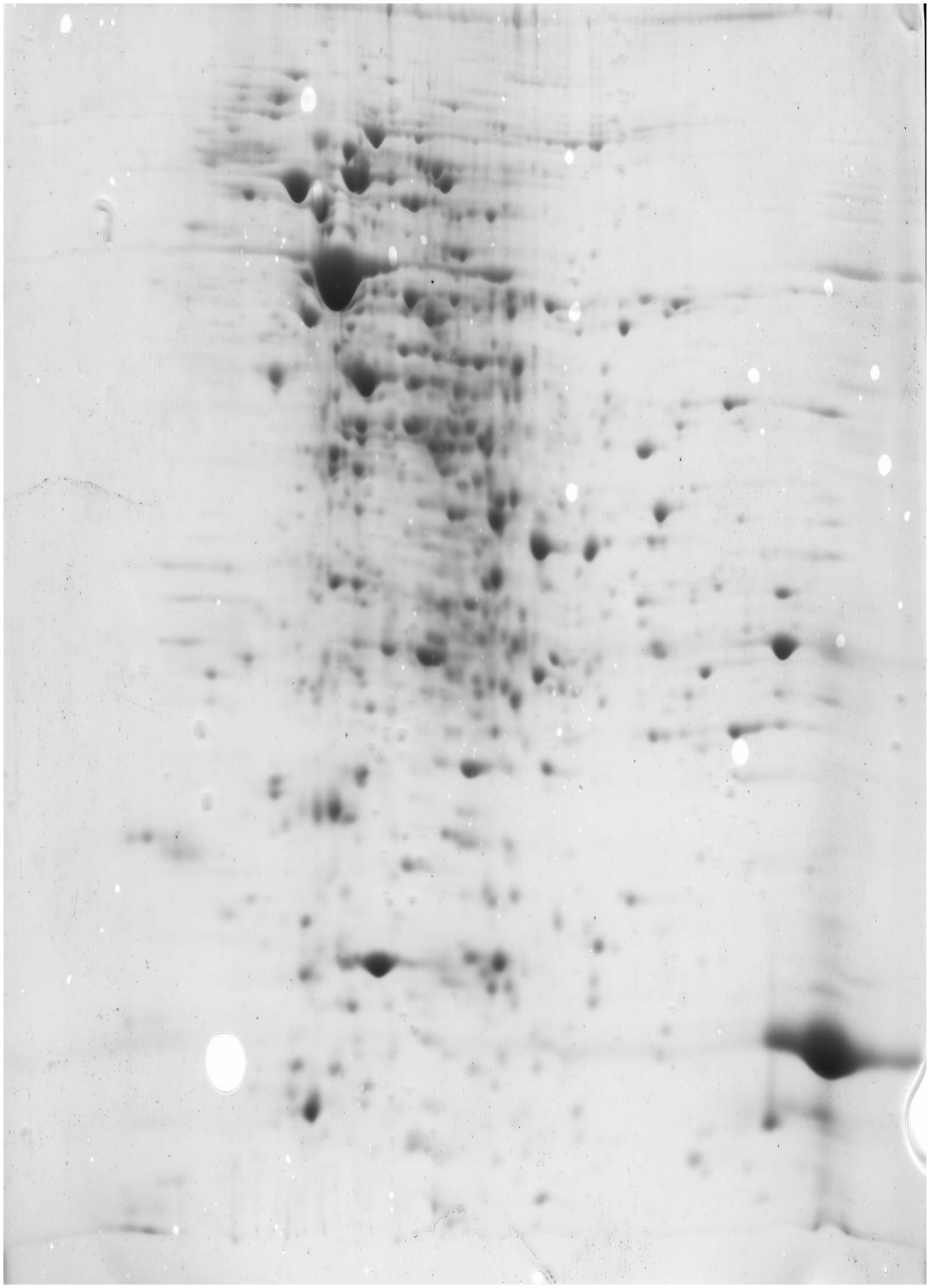

Figure 14. A reference 2-DE map (master gel) of total soluble proteins of *B. pertussis* Tohama (pI 3-10).

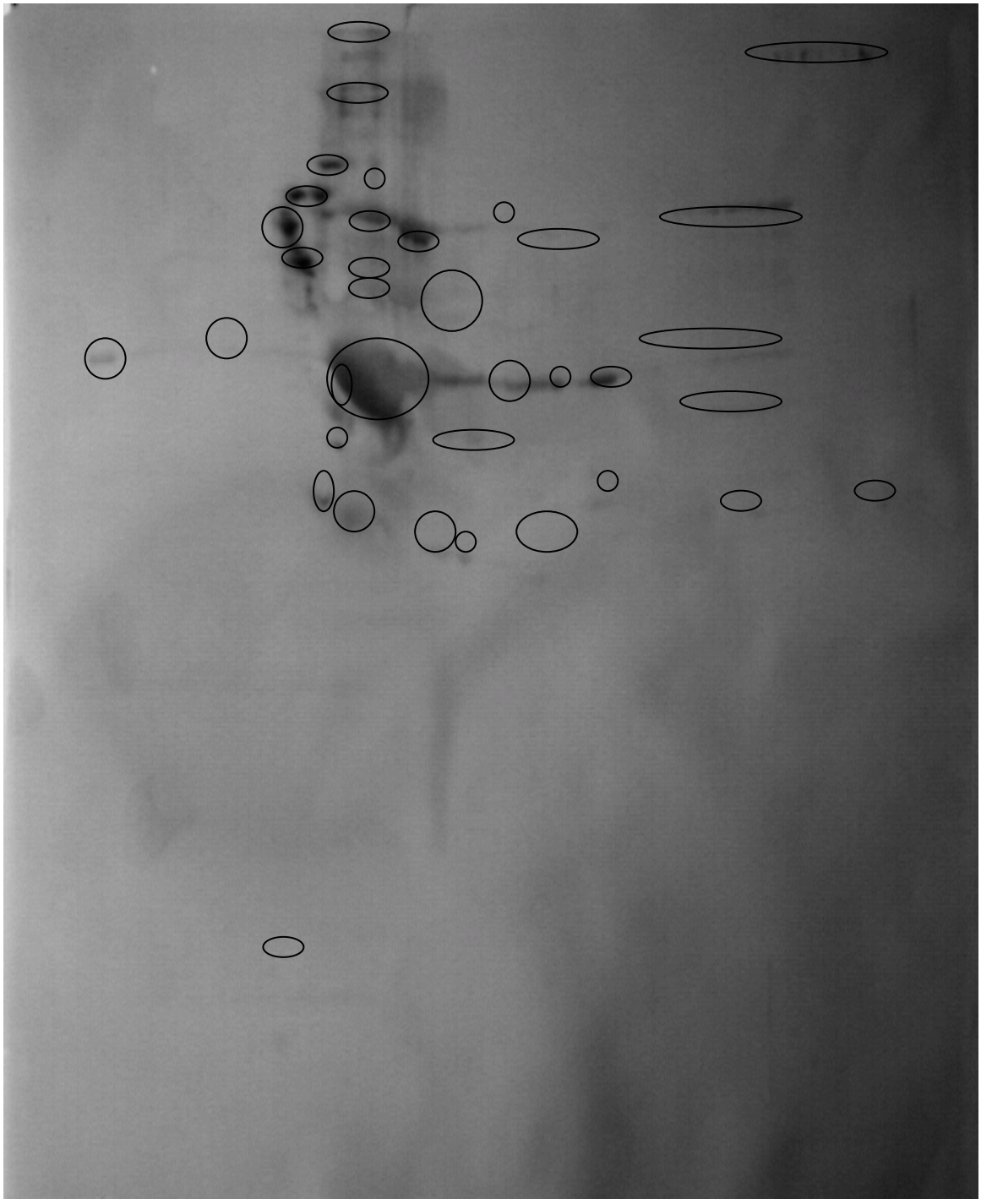

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Figure 15. Western blot analysis of the soluble proteome of *B. pertussis* Tohama. The serum was obtained from mice immunized with inactivated whole cell*s* of *B. pertussis* Tohama (Serum A).

Table 2. List of identified immunogenic soluble proteins of *B. pertussis* Tohama (Serum A)

	Protein Name	Species	Accession Number	Protein MW	Protein pI	Peptide Count	Protein Score	Multiple spots
1	60 kDa chaperonin	*Bordetella pertussis* Tohama I	gi|33564445	57446,45	5,13	18	586	5
2	Pertactin	*Bordetella pertussis*	gi|72539793	68644,38	6,92	12	351	2
3	Heat shock protein	*Bordetella pertussis* Tohama I	gi|33570977	71110,52	5,04	11	310	3
4	Lysyl-tRNA synthetase	*Bordetella pertussis*	gi|33571884	56593,81	5,27	20	288	1
5	Putative chromosome partition protein	*Bordetella pertussis* Tohama I	gi|33564502	130596	5,02	24	355	1
6	Serum resistance protein	*Bordetella pertussis* Tohama I	gi|33564444	103314,7	6,62	13	308	1
7	ATP-dependent protease, ATPase subunit	*Bordetella pertussis* Tohama I	gi|33571973	96274,57	5,37	30	507	1
8	Preprotein translocase secA subunit	*Bordetella bronchiseptica* RB50	gi|33568795	103237,4	5,47	11	88	1
9	RNA polymerase alpha subunit	*Bordetella pertussis* Tohama I	gi|406284	36136,08	5,6	14	216	1
10	S-adenosylmethionine synthetase	*Bordetella pertussis* Tohama I	gi|33564072	41976,16	5,12	10	174	1

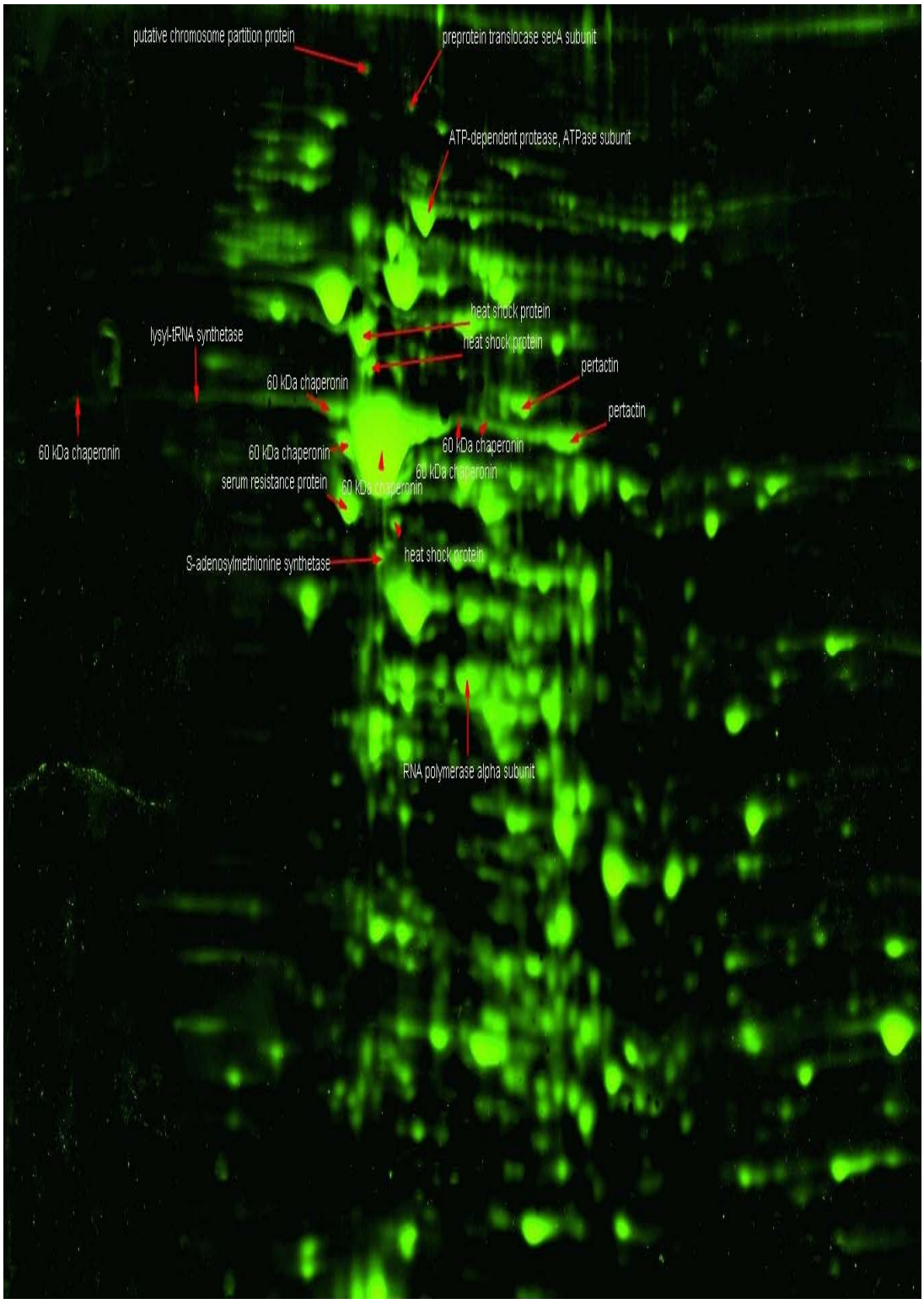


Figure 16. Representation of identified soluble immunogenic proteins of B. pertussis Tohama on 2-DE gel (Serum A).

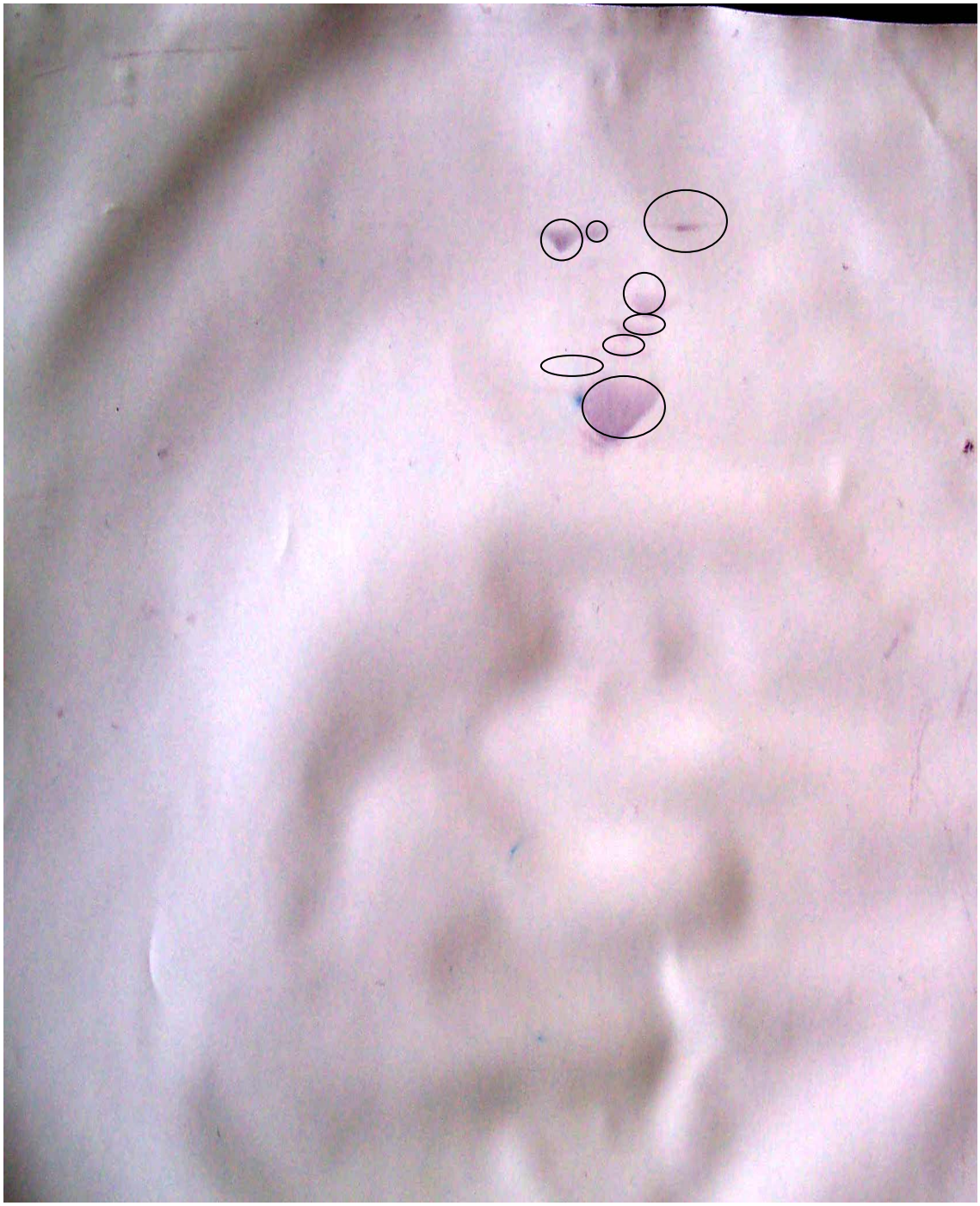


Figure 17. Western blot analysis of the soluble proteome of *B. pertussis* Tohama by using intraperitonal challenge serum. The serum was obtained from mice intraperitonally challenged with whole cells of *B. pertussis* Tohama (Serum C).

Table 3. List of identified immunogenic soluble proteins of *B. pertussis* Tohama (Serum C).

	Protein Name	Species	Accession Number	Protein MW	Protein pI	Peptide Count	Protein Score	Multiple spots
1	60 kDa chaperonin	*Bordetella pertussis* Tohama I	gi|33564445	57446	5,13	20	632	1
2	30S ribosomal protein S1	*Bordetella pertussis* Tohama I	gi|33571751	62979	5,1	9	146	1

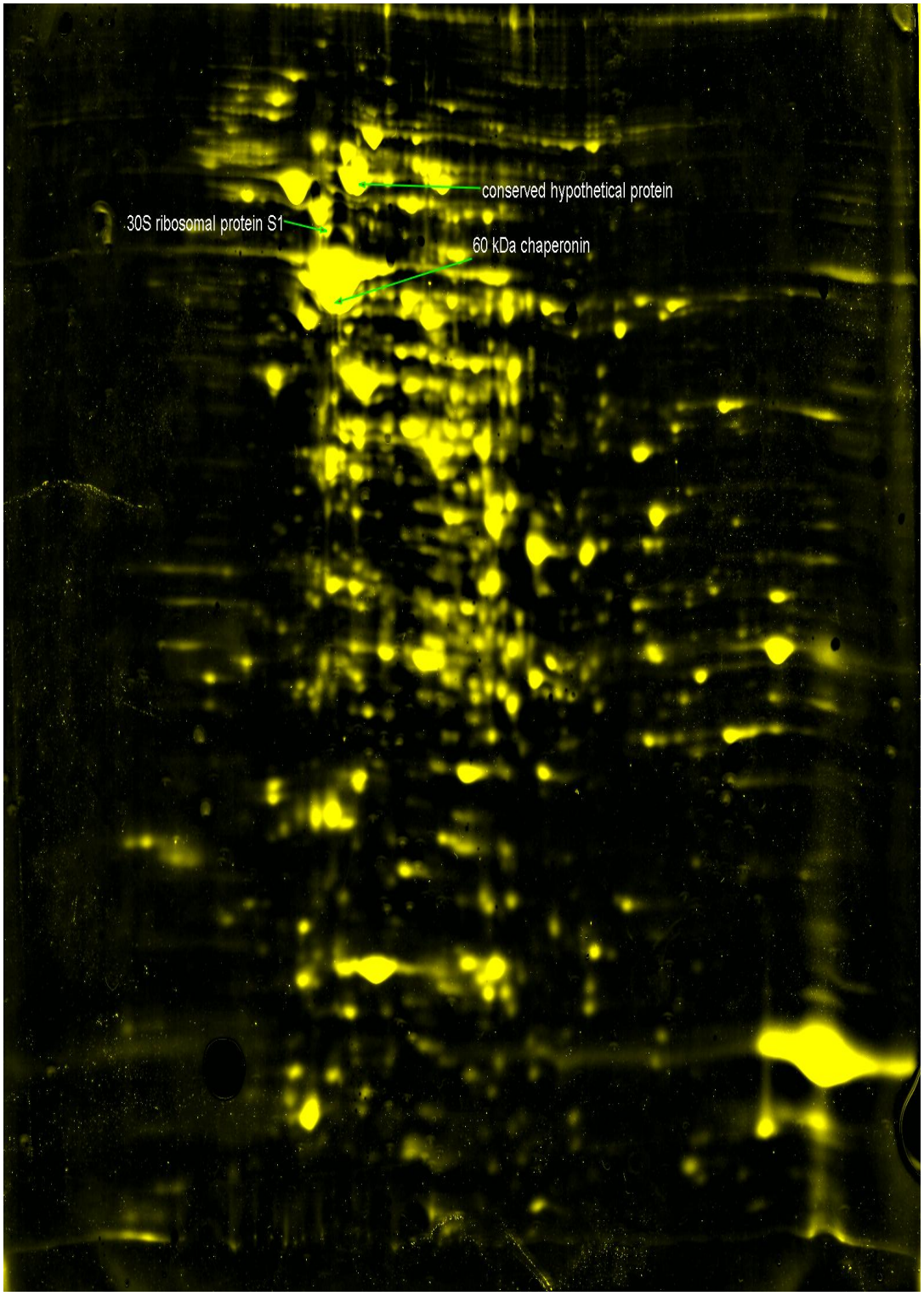


Figure 18. Representation of identified soluble immunogenic proteins *of B. pertussis* Tohama on 2-DE gel (Serum C)

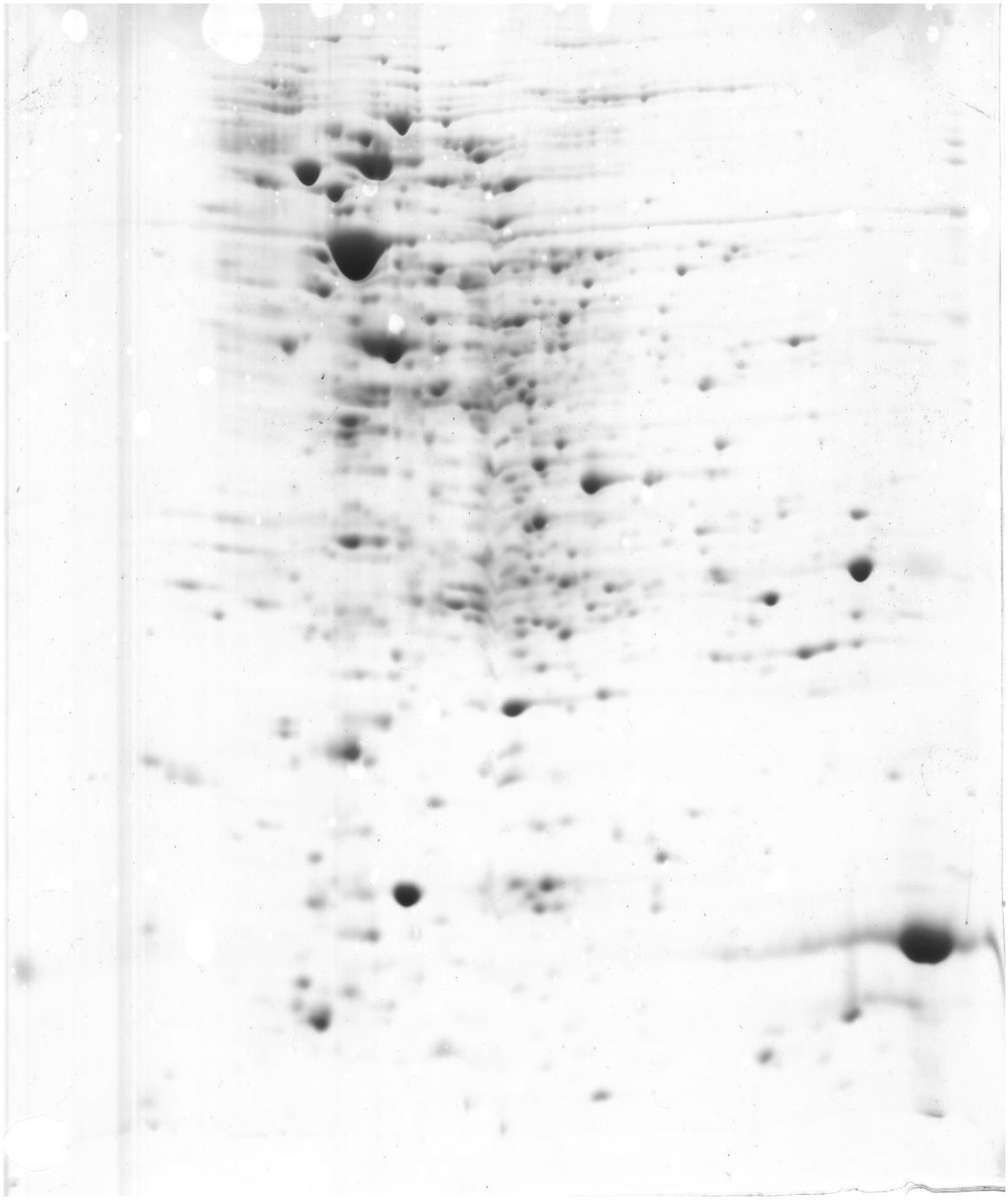


Figure 19. A reference 2-DE map (master gel) of total soluble proteins of *B. pertussis* Saadet strain (pI 3-10).

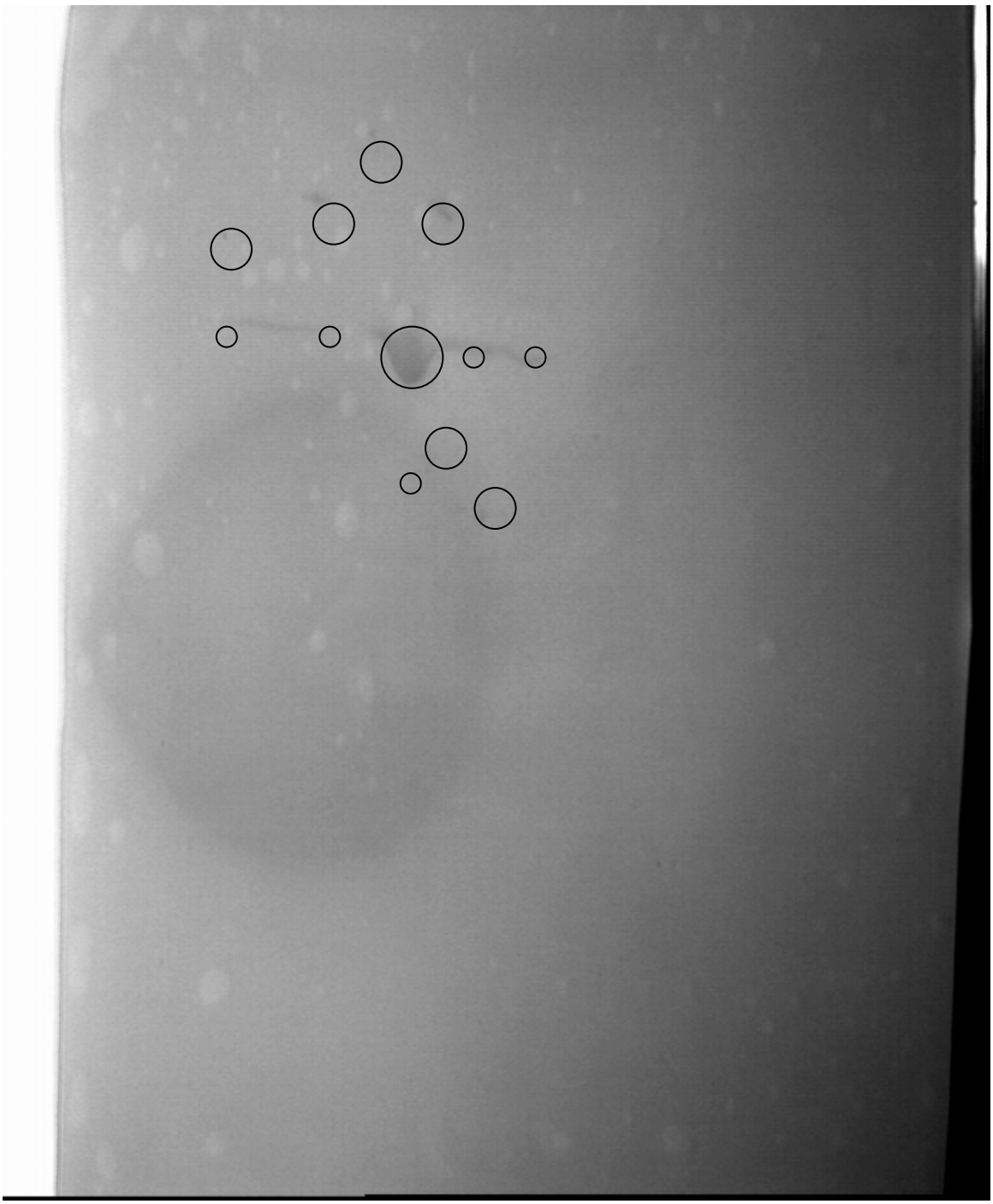


Figure 20. Western blot analysis of the soluble proteome of *B. pertussis* Saadet by using the serum obtained from mice subcutaneously immunized with inactivated whole cells of *B. pertussis* Saadet (Serum B).

Table 4. Identified immunogenic soluble proteins of *B. pertussis* Saadet (Serum B)

	Protein Name	Species	Accession Number	Protein MW	Protein pI	Peptide Count	Protein Score	Multiple Spots
1	60 kDa chaperonin	*Bordetella pertussis*	gi|33564445	57446,4492	5,13	18	586	5
2	Putative substrate-CoA ligase	*Bordetella bronchiseptica*	gi|33577798	54538,0508	5,62	9	255	1
3	30S ribosomal protein S1	*Bordetella pertussis*	gi|33571751	62978,7383	5,1	11	309	2
4	ATP-dependent protease, ATPase subunit	*Bordetella pertussis*	gi|33571973	96274,5703	5,37	30	507	1
5	Carbamoyl-phosphate synthase large chain	*Bordetella pertussis*	gi|33572197	118066,523	5,25	27	596	1
6	Sadenosylmethionine synthetase	*Bordetella pertussis*	gi|33564072	41976,1602	5,12	10	174	1
7	Elongation factor Tu	*Bordetella bronchiseptica*	gi|33575066	42889,1289	5,34	9	268	1
8	RNA polymerase alpha subunit	*Bordetella pertussis*	gi|406284	36136,0781	5,6	14	216	1
9	Ketol-acid reductoisomerase	*Bordetella bronchiseptica*	gi|33577302	36211,3906	5,76	13	609	1

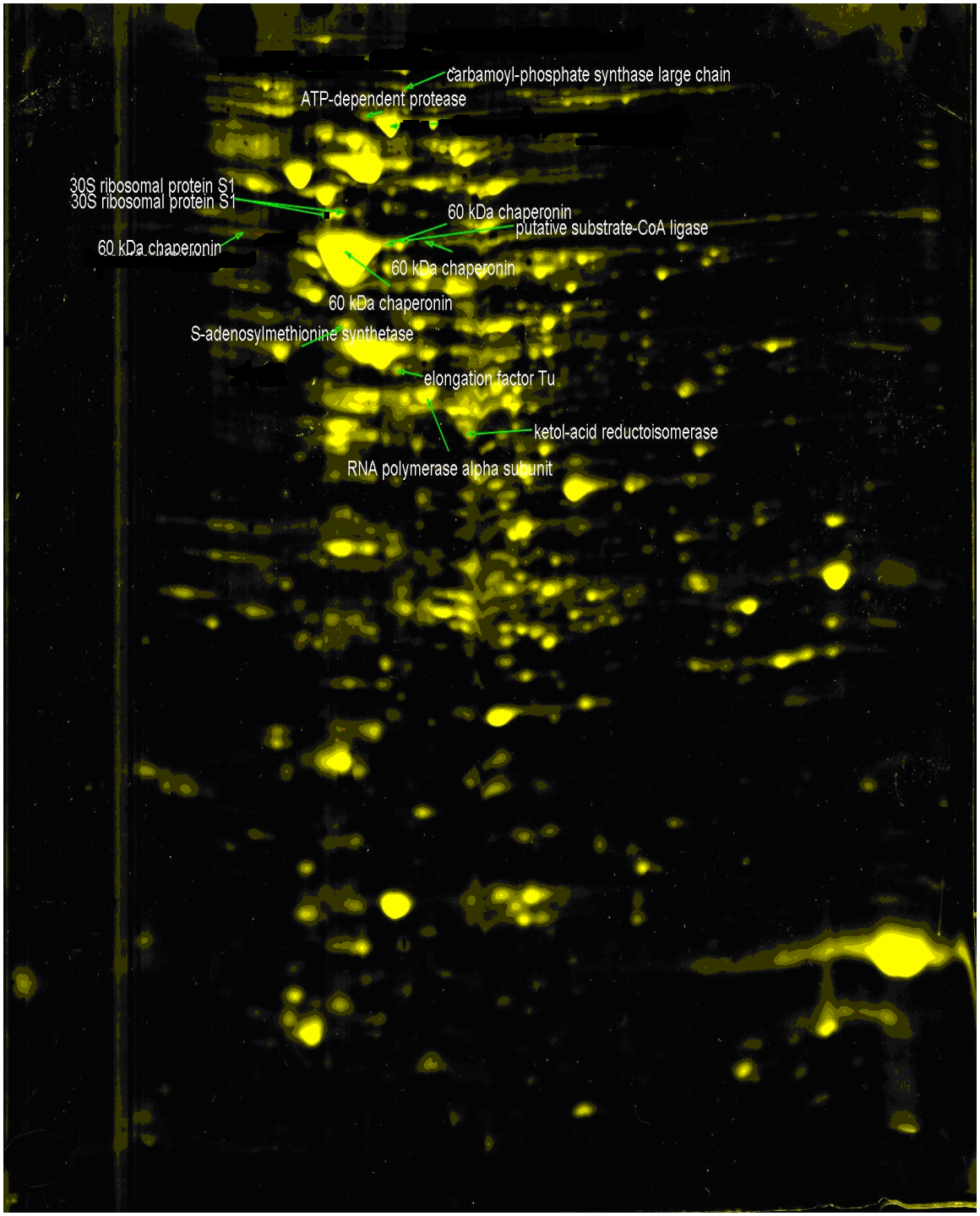


Figure 21. Representation of identified soluble immunogenic proteins *of B. pertussis* Saadet on 2-DE gel (Serum B)

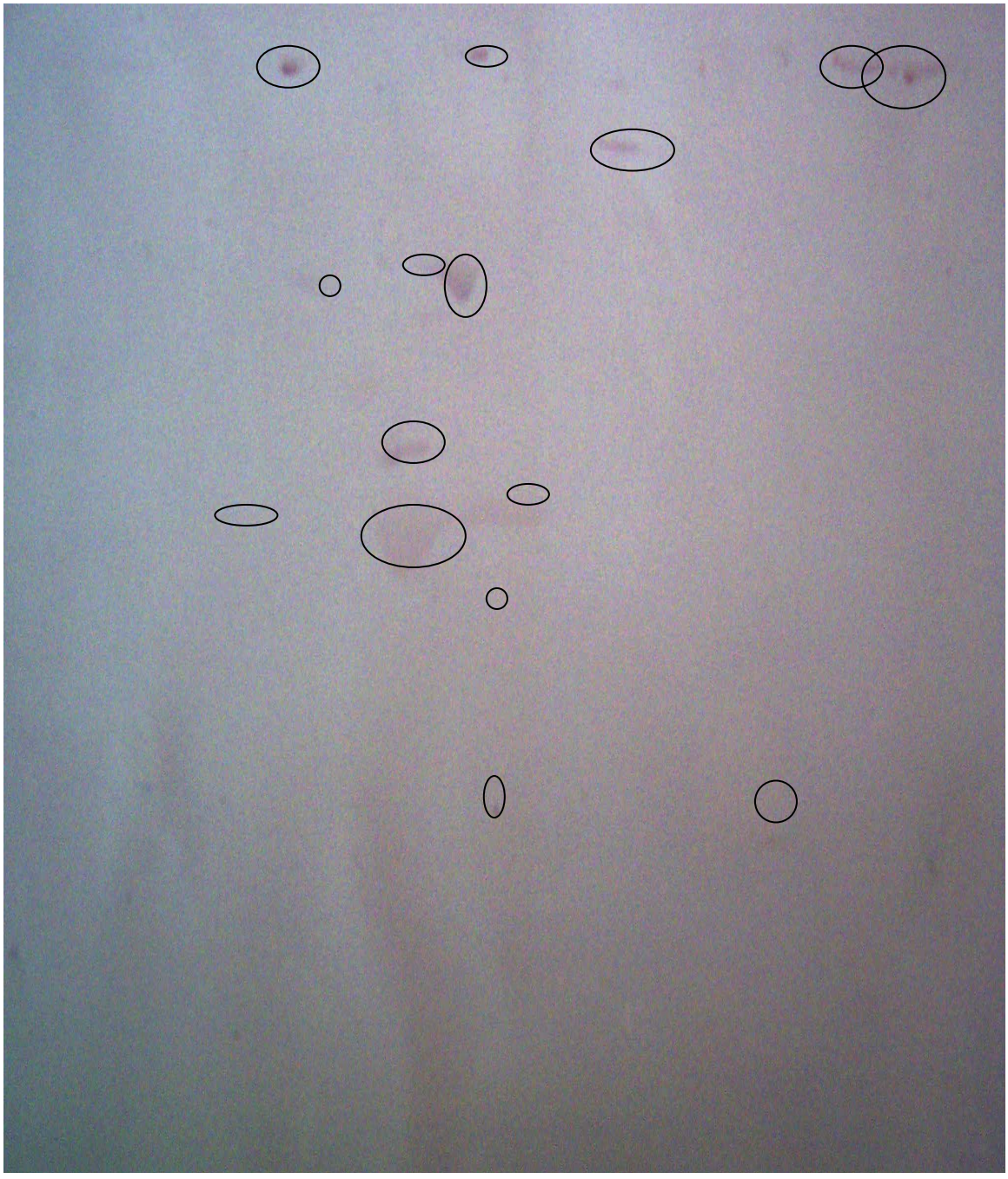


Figure 22. Western blot analysis of the soluble proteome of *B. pertussis* Saadet by using the serum obtained from mice intraperitonally challenged by live cells of *B. pertussis* Saadet (Serum D).

Table 5. Identified immunogenic soluble proteins of *B. pertussis* Saadet (Serum D)

	Protein Name	Species	Accession Number	Protein MW	Protein pI	Peptide Count	Protein Score	Multiple Spots
1	60 kDa chaperonin	*Bordetella pertussis*	gi|33564445	57446	5,13	15	572	5
2	Serum resistance protein	*Bordetella pertussis*	gi|33564444	1E+05	6,62	13	308	1
3	Pertactin	*Bordetella pertussis*	gi|72539793	68644	6,92	12	351	3
4	Heat shock protein	*Bordetella pertussis*	gi|33570977	71111	5,04	11	310	2

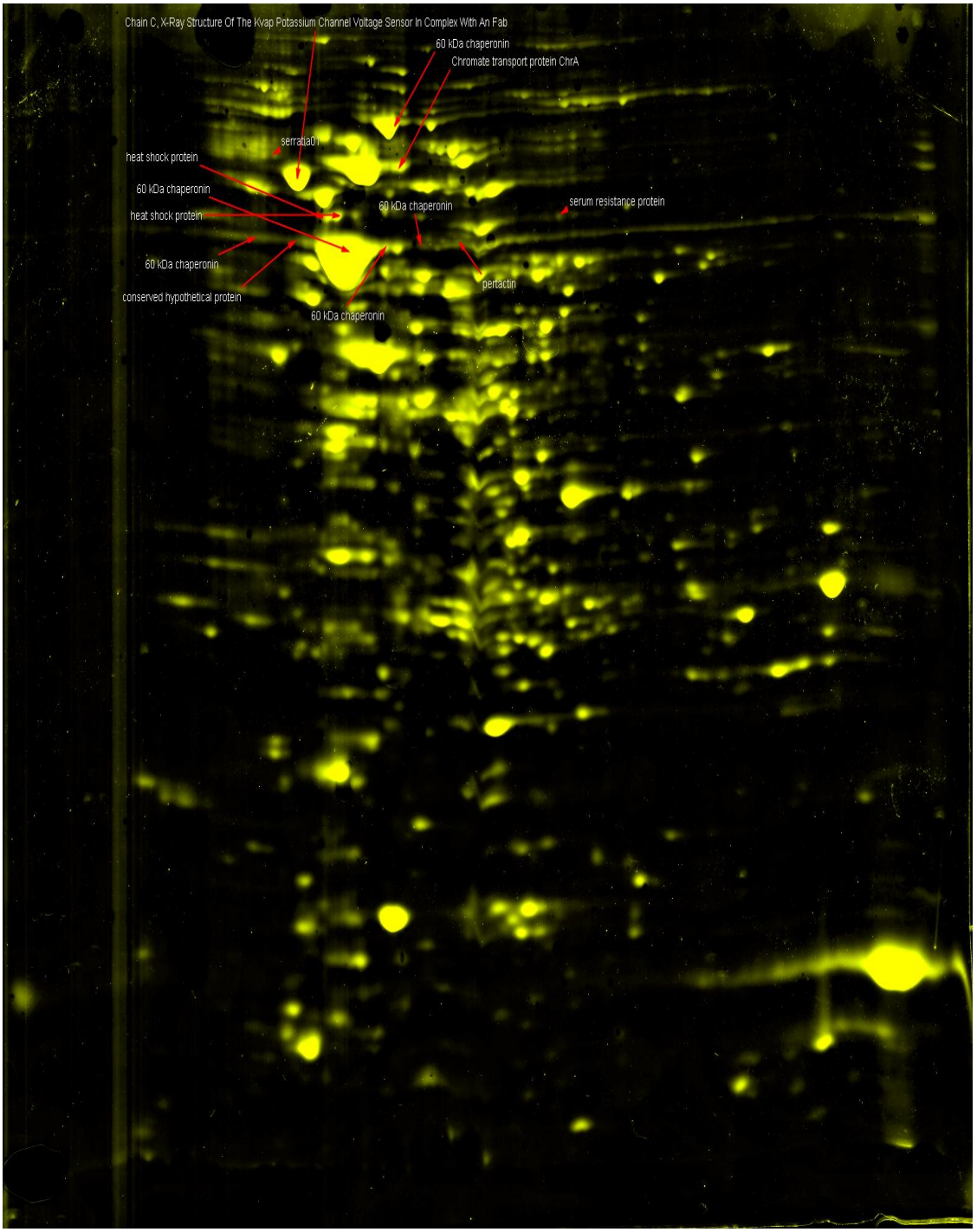


Figure 23. Representation of identified soluble immunogenic proteins *of B. pertussis* Saadet on 2-DE gel (Serum D).

An epitope is the part of a macromolecule that is recognized by immune system, specifically by antibodies, B cells or cytotoxic T cells. Peptide-based vaccines in which epitopes are used to provoke an immune reaction have attracted considerable attention recently as a potential means both of treating infectious diseases and promoting the destruction of cancerous cells by a patient's own immune system. With the availability of large sequence databases and computers fast enough for rapid processing of large numbers of peptides, computer-aided design of peptide-based vaccines has emerged as a promising approach to screening among billions of possible immune active peptides to find those likely to provoke an immune response to a particular cell type (Florea et al., 2003). In the present study, the artificial network-based B-cell epitope prediction server ABCpred (Saha et al., 2006) was used to predict the epitopes of the identified proteins of *B. pertussis* (Table 11). Since most of the proteins identified in this work are known to be cytoplasmic or membrane bound, epitope prediction is thought to be particularly useful for design of respective peptide-based vaccines in future.

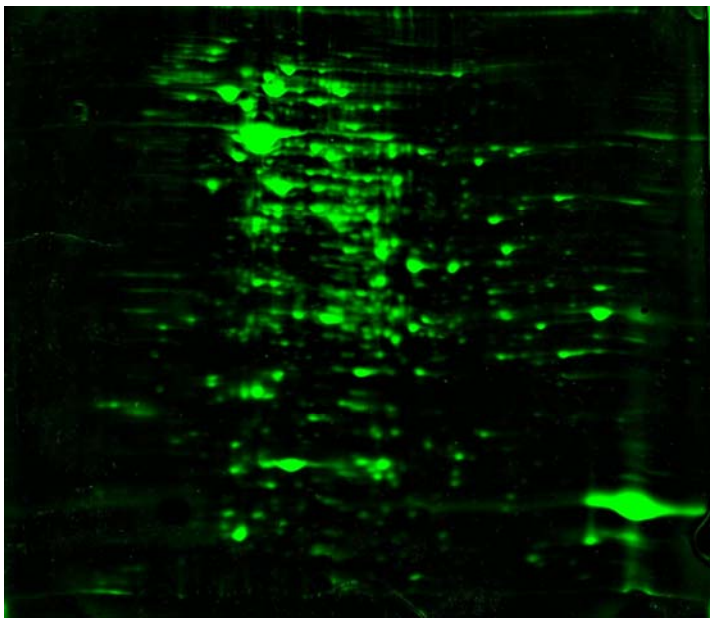
Table 6. Artificial network-based B-cell epitope prediction for *B. pertussis* immunogenic proteins

	Protein Name	Rank	Sequence	Start position	Score
1	60 kDa chaperonin	1	TGLKGDTADQNAGIKL	462	0.90
2	Pertactin	1	TLTGGADAQGDIVATE	419	0.95
3	Heat shock protein	1	KIYYVTADTFTAAANS	466	0.93
4	Lysyl-tRNA synthetase	1	IGDIIAIEGPVFKTNK	155	0.92
5	Putative chromosome partition protein	1	RELAEMPDEWRKASWL	979	0.93
6	Serum resistance protein BrkA	1	YSLAEDPKTHVWSLQR	721	0.95
7	Preprotein translocase secA subunit	1	TRSIETAQRKVEGRNF	681	0.96
8	S-adenosylmethionine synthetase	1	TSESVSEGHPDKVADQ	50	0.90
9	30S ribosomal protein S1	1	KQLGEDPWVGLARRYP	306	0.94
10	Putative substrate-CoA ligase	1	CVALLAPPTPDAMVCL	89	0.95
11	Carbamoyl-phosphate synthase large chain	1	VEKIIEREKPDALLPT	123	0.96
12	Elongation factor Tu	1	PGSINPHTDFTAEVYI	326	0.97
13	Ketol-acid reductoisomerase	1	ARDMWMKVFYDKDCDL	30	0.91
14	ATP-dependent protease, ATPase subunit	1	RMEIDSKPEVMDRLDR	5	0.94
15	RNA polymerase alpha subunit	1	NELLKTPNLGRKSLNE	331	0.93

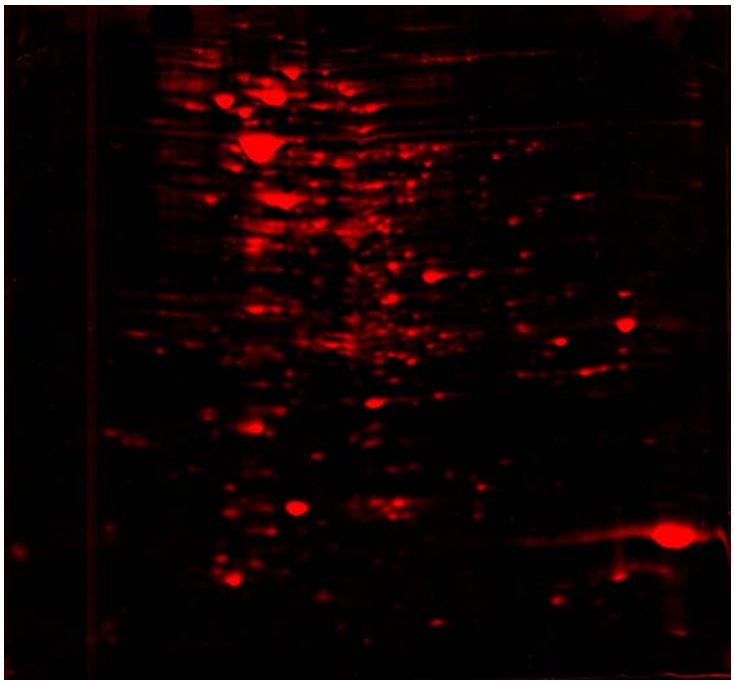
3.4. Comparison of 2-DE gel images and immunogenic proteins of Tohama and Saadet strains

2-DE gel images of *B. pertussis* Tohama and Saadet strains were compared by using Delta 2D image analysis software (Figure 24). We detected 64 proteins

which were specific to Saadet strain and not found in Tohama. Likewise 74 proteins which were specific to Tohama could not be detected in Saadet.



Tohama



Saadet

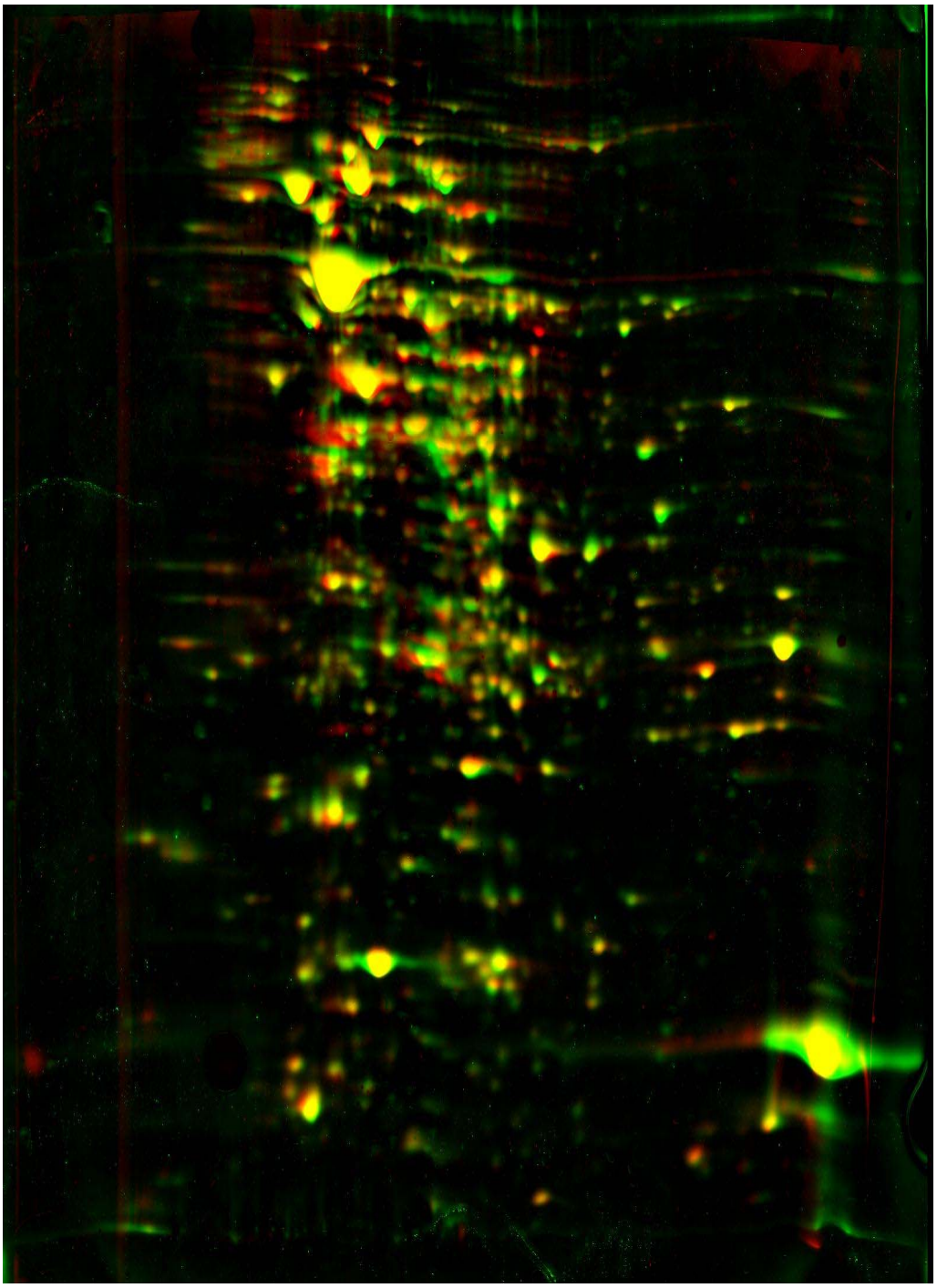
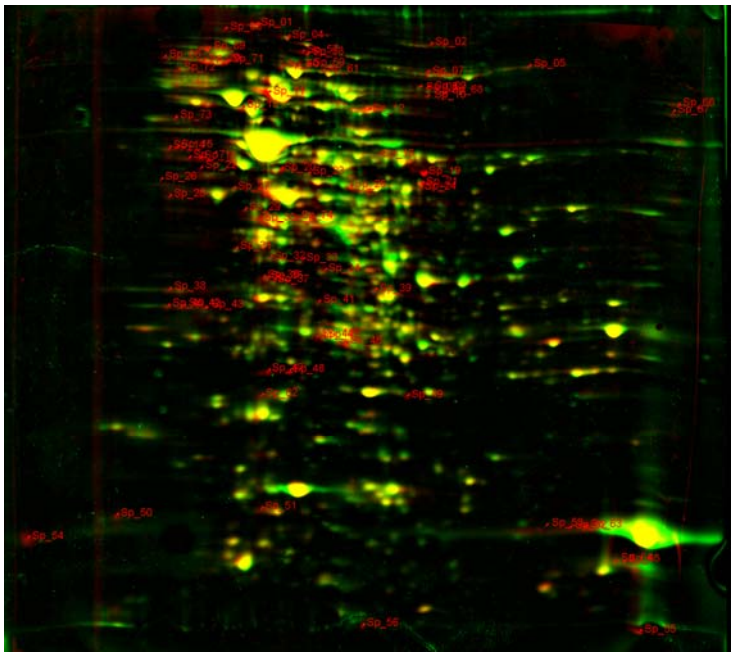
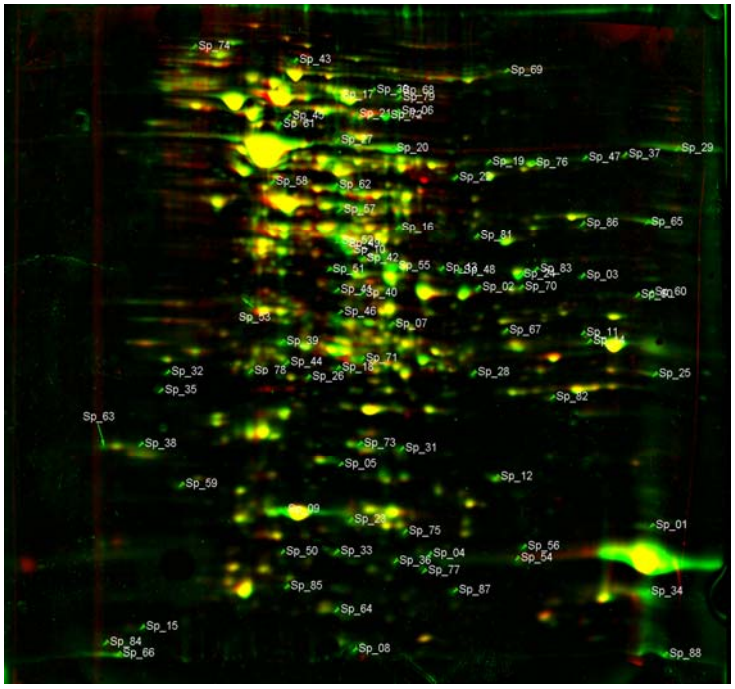


Figure 24. Comparison 2D images of *B. pertussis* Tohama (green) and Saadet (red) strains by Delta 2D image analysis software.



A



B

Figure 25. A: The proteins which are specific to *B. pertussis* Saadet. B: The proteins which are specific to *B. pertussis* Tohama.

Immunogenic proteins were next assigned to their source strain(s). 60 kDa chaperonin, pertactin, heat shock protein, ATP-dependent protease-ATPase subunit, RNA polymerase alpha subunit, S-adenosylmethionine synthetase and 30S ribosomal protein S1 were the common ones that could be identified from both of the strains. Putative chromosome partition protein, preprotein translocase secA subunit could be found only in Tohama whereas putative substrate-CoA ligase, carbamoyl-phosphate synthase large chain, elongation factor Tu and ketol-acid reductoisomerase could be shown only in Saadet.

Table 7. Assignment of immunogenic proteins to the source strain(s) used for immunization or infection as well as the sera used for their identification.

No.	Protein Name	Serum A Tohama	Serum C Tohama	Serum B Saadet	Serum D Saadet
1	60 kDa chaperonin	+	+	+	+
2	Pertactin	+			+
3	Heat shock protein	+			+
4	Lysyl-tRNA synthetase	+			
5	Putative chromosome partition protein	+			
6	Serum resistance protein	+			+
7	ATP-dependent protease, ATPase subunit	+		+	
8	Preprotein translocase secA subunit	+			
9	RNA polymerase alpha subunit	+		+	
10	S-adenosylmethionine synthetase	+		+	
11	30S ribosomal protein S1		+	+	
12	Putative substrate-CoA ligase			+	
13	Carbamoyl-phosphate synthase large chain			+	
14	Elongation factor Tu			+	
15	Ketol-acid reductoisomerase			+	

The cellular functions, involvement in pathogenesis and the potential as a vaccine candidate of the immunogenic proteins of *Bordetella* identified in this work are discussed in next section.

3.5. Evaluation of immunogenic proteins

Heat shock proteins Hsp 60 (chaperonine, 60kDa) and Hsp 70:

When entering the host from the environment, a microbial pathogen is confronted by several changes, some of which are highly stressful. To protect itself against the host, the pathogen activates various evasion mechanisms including Hsp synthesis (Kaufmann et al, 1999). It has been reported in an increasing number of studies that chaperonin 60 is either found on the surface of bacteria or actively secreted by them (Coates et al, 2000; Ranford et al., 2002). The importance of Hsp for survival in the host holds true for a variety of intracellular pathogens. A GroEL-like protein, which represents approximately 2% of the total cytoplasmic protein of *B. pertussis*, was first purified in 1991 (Arciniega, et al). In 1992, Burns et al. produced the three monoclonal Abs specific to *B. pertussis* Cpn60 and showed its immunogenicity (Burns et al, 1992). Very recently, Hsp 60 was used as an adjuvant in pertussis vaccine and the resulting vaccine showed very good performance in terms of protection as compared to the traditional vaccine (Vera et al., 2006)

Other members of the Hsp family have also been described as dominant antigens in several infectious diseases. Increased antibody levels to Hsp70, for example, have been identified in sera of patients suffering from malaria, leishmaniasis, schistosomiasis, filiariasis and candidiasis. In contrast to Hsp60, responses to

pathogen-derived Hsp70 seem to be more restricted, sometimes exclusively species-specific (Shinnick et al, 1991). The immune response against Hsp 70 induced by whole cell *B. pertussis* vaccine was reported first in 1993 (Lambert et al., 1993). As verified by our results, not only Hsp 60, but also Hsp 70 might be a good vaccine candidate for *B. pertussis*.

Autotransporters (pertactin and serum resistance protein BrkA):

Bordetella strains express a number of surface-associated proteins belonging to the autotransporter secretion system that are positively regulated by BvgAS. The autotransporter family includes functionally diverse proteins such as proteases, adhesins, toxins, invasins, and lipases that appear to direct their own export to the outer membrane (Mattoo et al., 2005). Serum resistance protein BrkA is a virulence factor of *B. pertussis* that confers serum resistance to killing by the classical pathway of complementation and is involved in adherence and invasion. *BrkA* is an autotransporter, outer membrane protein that mediate its own export across 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. Although cleaved, the 73 kDa BrkA passenger domain remains tightly associated with the bacterial surface and is not detected in *B. pertussis* culture supernatants (Lambert et al., 1993). Another autotransporter protein detected in the present study was 69kDa pertactin, one of the most commonly shown autotransporter known to be involved in adhesion (Emsley et al., 1996; Walker et al., 2001).

Lysyl-tRNA synthetase:

Aminoacyl tRNA synthetases (aminoacylRS) are essential proteins that are

extremely conserved throughout the evolution from prokaryotes to eukaryotes. These proteins have various additional functions extending to several critical cellular activities, such as tRNA processing, RNA splicing, RNA trafficking and transcriptional and translational regulation (Razin et al., 2005). *Borrelia burgdorferi,* causative agent of Lyme disease contains a functional class I-type lysRS, in contrast to its mammalian hosts, which contain class II-type enzymes. This fundamental difference between pathogen and host in an essential enzyme suggests that LysRS may be exploitable as a target for the development of antibiotics (Dieter et al., 1997). Kaniga et al. (1998) reported the importance of lysRS in *Salmonella enterica* infection and showed the presence of the encoding gene (poxA) in other pathogens like *S. flexneri*, *Y. enterocolitica*, *K. pneumoniae*, and *P. multocida*. In *S. enterica*, a poxA mutation resulted in an attenuated strain, thus the authors concluded that poxA disruption could be useful in designing live vaccines from *Salmonella* and other pathogens (Kaniga et. al., 1998). In addition to its involvement in infection process, our study demonstrated that it is an immunogenic protein.

Putative chromosome partition protein:

P1*par* family members promote the active segregation of a variety of plasmids and plasmid prophages in gram-negative bacteria. The P1*par* (partition) region consists of an operon containing *parA* and *parB* genes and a centromere analog site, *parS*, that lies downstream (Sergueev et al., 2005). Like similar systems found in other low-copy number plasmids, it assures the faithful distribution of the plasmids between dividing cells by an active process akin to mitosis. The *parA* gene encodes a Walker type ATPase essential for plasmid movement during partition. The *parB* gene encodes a protein that can bind tightly to the partition site, *parS.* It is required for capturing the plasmid at the cell center prior to

partition in *S. flexneri, Yersinia pestis* and many other pathogens have shown to possess these genes (Youngren et al., 2000). Our study was the first implication of the existence of this protein in the immunoproteome of a bacterial pathogen.

ATP-dependent protease, ATPase subunit:

Proteins are usually degraded when they become damaged, misfolded, mislocalized, or simply when they are no longer needed. Recently, it has become apparent that a number of pathogens require energy-dependent proteases to cause disease. A variety of ATP-dependent proteases have been identified in bacteria and many of them have homologues in eukaryotes. HslVU, presently the mechanistically best-characterised ATP-dependent protease, is the most recent addition to the set of known ATP-dependent proteases in bacteria. This protease has been detected in the pathogens like *Yersinia* sp., *L. monocytogenes*, *S. aureus* (Darwin et al., 2006) *S. enterica* with a disrupted ATP-dependent protease can be useful in developing live vaccine strains (Yamamoto et al., 2003). As the putative chromosome partition protein, ATP-dependent protease of *B. pertussis* acted as an immunogen during infection *in vivo*.

Preprotein translocase secA subunit:

A number of protein secretion mechanisms have been identified in gram-negative pathogens. Many of these secretion systems are dependent on the Sec translocase for protein export from the cytoplasm into the periplasm and then utilize other mechanisms for transport from the periplasm through the outer membrane (Newman et al., 2004). Proteins to be exported to the periplasm or outer membrane of bacteria are synthesized with a signal sequence and bind to the polar, SecA subunit of preprotein translocase. Translocase is a complex

enzyme, consisting of a dimer of SecA bound to membrane-embedded heterotrimeric SecYEG or heterohexameric SecYEGDFyajC domains. SecA has binding sites for ATP and for preprotein. Soluble SecA, which can spontaneously bind to lipid, might associate with both the outer and inner surfaces of the plasma membrane after cell lysis (Economou, 1998; Wickner et al, 1998). The main virulence factors of *B. pertussis*, autotransporter proteins BrkA and pertactin, main adhesion protein FHA and pertussis toxin are known to be Sec-dependent for protein export (Weiss et al., 2004). To our knowledge, the present study shows as the first time the immunogenicity of preprotein translocase secA subunit in a host.

RNA polymerase alpha subunit:

Alpha subunit of RNA polymerase has been initially known to play a role in the assembly of multisubunit RNA polymerase complex, providing a scaffold for the assembly of β and β4 (Roe et al., 1996). As mentioned earlier, in *B. pertussis*, expression of virulence factors is regulated by the Bvg two-component signal transduction system, comprising the sensor BvgS and the transcriptional activator BvgA. Carbonetti et al. (2000) reported that overexpression of the RNA polymerase α-subunit in *B. pertussis* reduces expression of the virulence factor PT. The alpha subunit is a common site of interaction of RNA polymerase with transcription activator proteins . The researchers therefore hypothesized that the observed effect on virulence factor expression was due to interaction of the excess alpha with BvgA, effectively reducing the level of BvgA present in cells for functional interactions with RNA polymerase.

Boucher et al. (2003) more recently found that the response regulator BvgA and RNA polymerase subunit C-terminal domain bind simultaneously to different faces

of the same segment of promoter DNA. These results reveal a previously undescribed mode of interaction between RNA polymerase subunit C-terminal domain and a transcriptional activator (Boucher et al., 2003).

Immunogenicity of RNA polymerase β subunit was demonstrated earlier in *Chlamydia trachomatis* (Campillo et al., 1999), *H. pylori* (Bumann D. et al., 2002). and *K. pneumoniae* (Kurubati et al., 2006), and it is shown to be immunogenic in *B. pertussis*, too, by the present study.

S-Adenosylmethionine synthatase:

Bacteria can attain a high cell density before virulence determinants are expressed, and in doing so, they are able to make a concerted attack, produce virulence factors, and be present in sufficient numbers to overwhelm the host defences (Kievit, 2000). It has been shown that many pathogens use quorum sensing to regulate virulence. It was shown that the LuxS protein is the AI-2 synthase and AI-2 is produced from S-adenosylmethionine by three enzymaticreactions. Evidence was provided that the biosynthetic pathway and biochemical intermediates in AI-2 biosynthesis are identical in *E. coli, S. typhimurium, V. harveyi, V. cholerae,* and *E. faecalis*. Thus, AI-2 is a unique, 'universal' signal that can be used by a variety of bacteria for communication among and between species (Griffiths et al., 2003). The immunogenicity of *S*-adenosylmethionine synthatase was previously shown in a *H. pylori* immunoproteomics study (Lin et al., 2006). Herein, we demonstrated that this enzyme is also an immunogenic protein of *B. pertussis.*

Putative substrate-CoA ligase:

The initial step necessary for synthesis of lipids is activation of endogenous and exogenous fatty acids to their corresponding acyl-CoA esters. This is achieved by the action of ATP dependent acid: CoA ligases (AMP forming) also called acyl CoA synthetases. Formation of an energy rich thioester bond between the fatty acid anion and CoA is achieved by hydrolysis of ATP which undergoes pyrophosphate cleavage yielding AMP and inorganic pyrophosphate (Knights et al., 2000). Moreover, substrate CoA-ligase has also a function in fatty acid and phospholipid metabolism (Deborah et al., 2004). The present study reports the immunogenicity of this enzyme for the first time.

Elongation factor Tu (EF-Tu) :

Elongation factor Tu (EF-Tu) is one of the most abundant proteins in prokaryotes, representing about 5% of the total cellular protein of *Escherichia coli* . During protein biosynthesis, that is, the elongation process, EF-Tu catalyzes the binding of each aminoacyl-tRNA to the ribosome. As a multifunctional protein, it interacts with several macromolecules and guanine nucleotides, including EF-Ts, GDP, GTP and some ribosomal proteins. The precence of protein in *B. bronchiseptica* was shown in 1995 (Weber S. et al., 1995).

In gram-positive bacteria, elongation factor Tu appears to be cell surface associated, but its location in gram-negative bacteria is unclear. EF-Tu associates with the cell surface of *Escherichia coli* and that of *Lactobacillus johnsonii*. Furthermore, in *L. johnsonii*, EF-Tu had properties of an adhesion factor, mediating attachment of bacteria to human intestinal cells. In *Mycobacterium leprae* and *Mycobacterium pneumoniae*, EF-Tu was identified as a cell wall-associated protein (Lopez et al., 2005).

Recently immunogenicity of elongation factor was shown by using immuproteomics technology for *Staphylococcus epidermidis* (Sellman et al., 2005)*, Anaplasma marginale* (Brown et al., 2005) and *Mycoplasma hyopneumoniae* (Marcos et al., 2006), *Lactococcus garvieae* (Shin et al., 2007). Our study constitutes the first report on the immunogenicity of this protein in *B. pertussis*.

Ketol-acid reductoisomerase:

The biosynthetic pathway for the three branched amino acids begins with two enzymes that catalyze reactions leading to all three products. The second enzyme of the pathway, ketol acid reductoisomerase (KARI) catalyzes the reaction. The two-step reaction involves an alkyl migration and a ketone reduction that occurs at a single active site on the enzyme without dissociation of any reaction intermediates (Rane et al., 1997).

A recent study showed that ketol-acid reductoisomerase is a cold inducible factor of *B. bronchiseptica* (Stu et al., 2005). Cold inducible factors are also heat shock proteins and usually have a relation with virulence. The immunogenicity of ketol-acid reductoisomerase was showed by Fernández-Arenas et al. (2004) by an immunoproteomics approach in *Candida albicans*. The present article constitutes the first report on the immunogenicity of ketol-acid reductoisomerase for *B. pertussis*.

30S ribosomal protein S1:

In gram-negative bacteria, a protein that differed from the bulk of ribosomal proteins both by its larger length and its general biochemical properties (it is an acidic protein, pI 4.9, and it is only loosely bound to ribosomes) was found to be present in ribosomes (Danchin et al., 1997). The shape of ribosomal protein S1 is unusual in that it is an elongated protein of about 23 nm containing two domains. The N-terminal portion binds to the 30S ribosomal subunit, while the C-terminal portion contains three repeated segments that may be involved in binding mRNA. Ribosomal protein S1 appears to function as a relatively nonspecific mRNA binding protein that brings the mRNA into the vicinity of the ribosomal region at which translation is initiated. Its relatively low specificity allows the translation of mRNAs that lack or have a poorly defined ribosome binding site (Clark et al., 1996).

To our knowledge, the present study shows as the first time the immunogenicity of 30S ribosomal protein S1 subunit in a bacterium.

Carbamoyl phosphate synthase large chain:

Carbamoyl phosphate synthetase plays a critical role in both arginine and pyrimidine biosynthesis by providing an essential precursor, namely carbamoyl phosphate (Thodent et al., 1998). The enzyme is composed of two polypeptide chains, referred to as the large and small subunits. The small subunit catalyzes the hydrolysis of glutamine, while the large subunit is responsible for the two phosphorylation events. In addition, the large subunit provides the regions of the polypeptide chain that are responsible for binding physiologically important monovalent cations and effector molecules, such as ornithine, an activator, and UMP, an inhibitor (Holden et al., 1999).

The presence of this protein in the supernatant of *S. aureus* (Burlak et al., 2007) and total proteome of *N. meningitidis* serogroup A (Bernardini et al. 2004) was shown by proteomics approaches. The present study constitutes the first report showing the immunogenic function of carbamoyl phosphate synthase in a bacterial pathogen.

It is to be noted that further work must be performed with above-mentioned antigenic proteins in order to determine if they generate a protective immune response either by alone or in different combinations. Antigen-specific T cell responses remain to be determined as well evaluate total cellular immune response to evaluate antigens during infection or vaccination.

3.7. Extracellular proteome of *B. pertussis*

Extracellular proteome of *B. pertussis* Tohama was obtained by TCA-Acetone extraction method. A total of 46 protein spots could be detected on the 2-DE gel of the secretome (Fig. 26) and 8 of them reacted with polyclonal antibody (Serum A) against inactivated whole cells of *B. pertussis* Tohama (Figure 27).

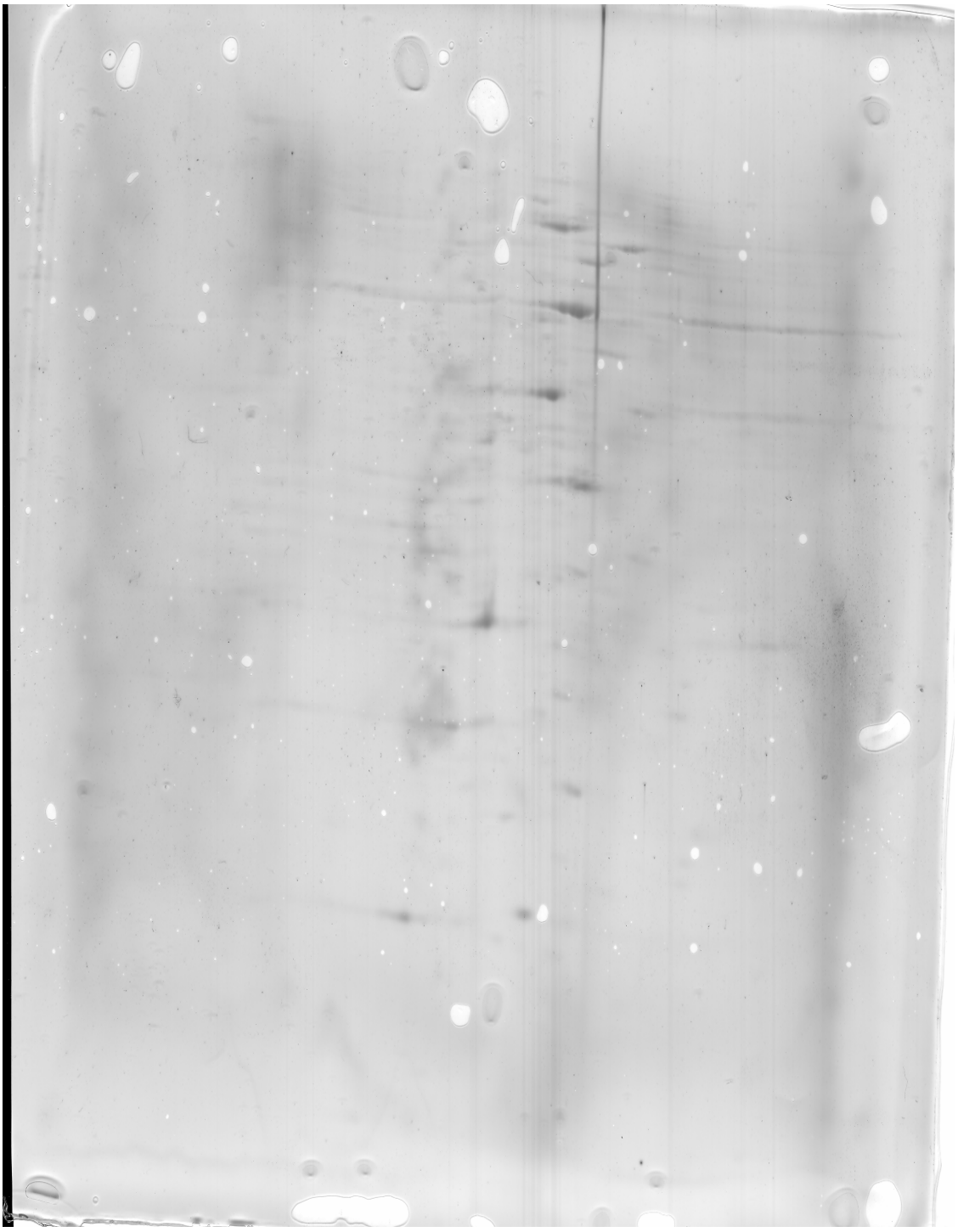


Figure 26. Extracellular soluble proteome of *B. pertussis* Tohama.

Figure 27. Western blot analysis of the extracellular soluble proteome of *B. pertussis* Tohama (Serum A).

3.8. Outer membrane proteins of *B. pertussis*

Lauryl sarcosine method has been used in numerous studies because of its ability to efficiently and selectively solubilizing the inner membrane of ruptured gram-negative bacteria. Inner membrane proteins (IMPs) are released in a soluble form, but neither the outer membrane (OM) peptidoglycan complex nor the OMPs are affected, thus the method enables their recovery by ultracentrifugation after detergent exposure (Rhomberg et al, 2004). Moreover, this method was a previously used one for preparing *Bordetella* OMP samples (Leyht, 1992; Passerini de Rossi et al., 1999). Urea extraction is also an important method for OMPs, and like Sarkosyl method, was used earlier for extracting OMPs of *B. pertussis* (Hewlett et al., 1989, 1994*).* In this method, the protein samples must be freshly prepared and no warmer than 30 ^{o}C to avoid carbomylation reaction which causes incorrect results in MALDI-TOF analysis (Westermeier et al., 2002). In the present study, a modified version of this procedure ((Wright et al., 2005) was employed and over 60 soluble proteins were obtained on the 2D gel (Figure 28) while the sarcosyl method could resolve only about 30 spots (data not shown). Western blot analysis of urea-extracted protein remains to be undertaken.

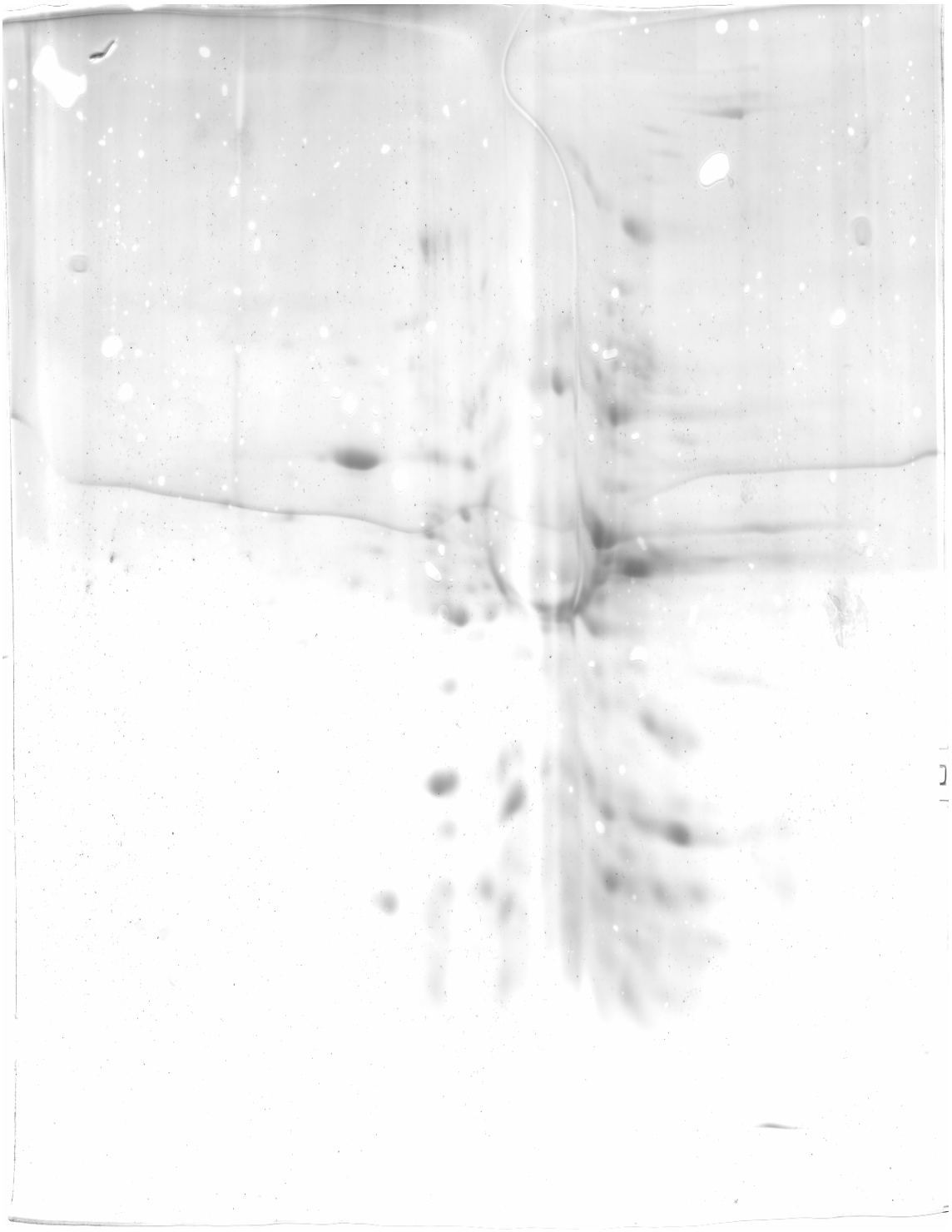


Figure 28. *B. pertussis* Tohama outer membrane proteins obtained by using urea extraction method.

CHAPTER 4

CONCLUSION

- The results indicate that the humoral response of mice to intraperitonal challenge with *B. pertussis* is rather weak, most probably because it creates an acute and fatal infection. Subcutaneous immunization, on the other hand, yields immune sera that can be more efficiently used for identification of immunogenic proteins.

- In order to fractionate *B. pertussis* proteome into subproteomes, the protocol of Tianyi et al. (2005) for extracting extracellular proteins works well and the urea extraction method is better suited than the Sarkosyl method for our organism in terms of the total number of outer membrane proteins obtained on the gel.

- The most prominent proteins expressed during infection *in vivo* with either Tohama and Saadet strains of *B. pertussis* are known now. Among a total of 15 such proteins, 5 proteins (RNA polymerase alpha subunit, S-adenosylmethionine synthatase, putative substrate-CoA ligase, elongation factor Tu and ketol-acid reductoisomerase) are immunogenic for *B. pertussis* and 6 proteins (ATP-dependent protease, carbamoyl-phosphate synthase large chain, lysyl-tRNA synthetase, putative chromosome partition protein, preprotein translocase secA subunit and 30S ribosomal protein S1 subunit) have an immunogenic effect in a pathogenic bacterium as shown for the first time in the present study. Further work must be performed with these antigenic proteins in order to determine if they generate a protective immune response either by alone or in different combinations. Antigen-specific T cell responses also remain to be determined.

- Our findings are expected to facilitate future proteomics analyses with *B. pertussis* including physiological proteome and aid in development of new generation vaccines against the pathogen.

REFERENCES

1. **Águas, P. G., Guilherme, G. M., Gabriela, M. (2006).** Pertussis: increasing disease as a consequence of reducing transmission. *Lancet Infect Dis* 6: 112–17.

2. **Andersen, J. S. and Mann, M. (2000).** Functional genomics by mass spectrometry. *FEBS Lett* 480: 25-31.

2. **Arckens, L. Vercauteren, F. G., Quirion, R. (2007).** Applications and current challenges of proteomic approaches, focusing on two dimensional electrophoresis. *Amino Acids* In press.

3. **Ariel, N., Zvi, A., Makarava, K. S.; Chitlaru, T.; Elhanany, E.; Velan, B.; Cohen, S. ; Friedlander, A. M. ; Shafferman, A. (2003).** Genome-based bioinformatic selection of chromosomal *Bacillus anthracis* putative vaccine candidates coupled with proteomic identification of surface-associated antigens. *Infect. Immun* 71: 4563-4579.

4. **Backert, S., Kwok, T., Schmid, M., Selbach, M., Moese, S., Peek, R., König, W., Meyer, T. and Jungblut, P. R. (2005).** Subproteomes of soluble and structure-bound *Helicobacter pylori* proteins analyzed by two dimensional gel electrophoresis and mass spectrometry. *Proteomics* 5: 1331–1345.

5. **Banks, R. E., Dunn, M. J, Hochstrasser, D. F., Sanchez, J., Walter, B., Pappin, D., Selby, P. J., (2000).** Proteomics: new perspectives, new biomedical opportunities. *Lancet* 356: 1749–56.

6. **Bernardini, G., Giovanni, R., Maurizio, C., Roberta, M., Simona, A., Chiara, D., Stefania, B., Lorenza, T., Guido, G., Paola, M., Mark, A.,**

Andrea, S., Giulio, R. and Annalisa S. (2004). Proteome analysis of *Neisseria meningitidis* serogroup A. *Proteomics* 4: 2893–2926.

7. **Bjellqvist, B., Hughes, G. J., Pasquali, C., Paquet, N., Ravier, F., Sanchez, J. C., Frutiger, S., Hochstrasser, D. (1993).** The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14: 1023-31.

8. **Gueirard, P., Guiso, N. and Chaby, R. (1997).** Antigenic polymorphism of the lipopolysaccharides from human and animal isolates of Bordetella bronchiseptica. *Microbiology* 143: 1433-1441.

9. **Boucher, P. E., Maris, A. E., Yang, M. S., Stibitz, S. (2003).** The response regulator BvgA and RNA polymerase alpha subunit C-terminal domain bind simultaneously to different faces of the same segment of promoter DNA. *Mol Cell* 11: 163-73.

10. **Brown, W. C., William, F. S., Guy, H. P., Kelly, A. B., McGuire, T. C., Norimine, J., and Job, E. L. (2005).** Identification of novel antigenic proteins in a complex *Anaplasma Marginale* outer membrane immunogen by mass spectrometry and genomic mapping. *Infect Immun* 73: 8109–8118.

11. **Brötz H. O., Julia E. B. and Harald L. (2005).** Bacterial proteomics and its role in antibacterial drug discovery. *Mass Spectrom Rev* 24: 549– 565.

12. **Bumann D., Petra H., Frank S., Jan K., Nicolas S., Stefanie L., Ursula Z., Peter R. J., and Thomas F. M. (2002).** A comparison of murine and human immunoproteomes of *Helicobacter pylori* validates the preclinical murine infection model for antigen screening. *Infect Immun* 70: 6494–6498.

13. **Burlak, C., Carl, H. H., Mary, R., Adeline, R. W., Martin, J. M., Barry, N. K. and Frank, R. D. (2007).** Global analysis of community-associated methicillin-resistant *Staphylococcus aureus* exoproteins reveals molecules produced in vitro and during infection. *Cell Microbiol* In press.

14. **Burns, D. L., Gould-Kostka, J. L., Kessel, M., and Arciniega, J. L. (1991).** Purification and immunological characterization of a GroEL-like protein from *Bordetella pertussis*. *Infect Immun* 59: 1417–1422.

15. **Burns, D. L., Kessel, M., Arciniega, J. L., Karpas, A., Gould-KostkA, J., (1992).** Immunochemical localization of a region of chaperonin-60 important for productive interaction with chaperonin-10. *J Biol Chem* 267: 25632-25635.

16. **Campillo-Sanchez, M., Bini, L., Comanducci, M., Raggiaschi, R., Marzocchi, B., Pallini, V., Ratti, G. (1999).** Identification of immunoreactive proteins of *Chlamydia trachomatis* by Western blot analysis of a two dimensional electrophoresis map with patient sera. *Electrophoresis* 20: 2269-2279.

17. **Carbonetti, N.H., Romashko, A., Irish, T.J. (2000).** Overexpression of the RNA polymerase alpha subunit reduces transcription of Bvg-activated virulence genes in *Bordetella pertussis*. *J Bacteriol* 182: 529–531.

18. **Celis, J. E., Kruhoffer, M., Gromova, I., Frederiksen, C., Ostergaard, M., Thykjaer, T., Gromov, P., Yu, J., Palsdottir, H., Magnusson, N. and Orntoft, T.F. (2000).** Gene expression profiling: monitoring transcription and translation products using DNA microarrays and proteomics. *FEBS Lett* 25: 2-16.

19. **Clark, M. A., Baumann, L., Baumann, P., Rouhbakhsh, D. (1996).** Ribosomal protein S1 (RpsA) of *Buchnera aphidicola*, the endosymbiont of aphids: characterization of the gene and detection of the product. *Curr Microbiol* 32: 89-94.

20. **Coates, A. R., Henderson, B. (2000).** Chaperonins are cell-signalling proteins: the unfolding biology of molecular chaperones. *Expert Rev Mol Med* 15: 1-17.

21. **Connolly, J., Diego, C., Timothy, G. A., Alexander, W., Marian, Q., Ryan, C., Paul, G., Cesar, Mujer., Rodolfo, A. U. and DelVecchio, V. G. (2006).** Proteomic analysis of *Brucella abortus* cell envelope and identification of immunogenic candidate proteins for vaccine development. *Proteomics* 6: 3767–3780.

22. **Crowcroft, N. S., Britto, J. (2002).** Whooping cough--a continuing problem. *BMJ 4:1537*–8.

23. **Danchin, A. (1997).** Comparison between the *Escherichia coli* and *Bacillus subtilis* genomes suggests that a major function of polynucleotide phosphorylase is to synthesize cdp. *DNA Res.* 72: 6355–6363.

24. **Dautin, N., Karimova, G., Ladant, D. (2002).** *Bordetella pertussis* adenylate cyclase toxin: a versatile screening tool. *Toxicon* 40: 1383–1387.

25. **Darwin, H., Butler, S. M., Festa, R. A. and Pearce, M. J. (2006).** Self-compartmentalized bacterial proteases and pathogenesis. *Mol Microbiol* 60: 553–562.

26. **Deborah, E. G., Deepak, K., Chiew, K., Sandeep, T., Yukari, C. M., Benjamin, G. S., Robert, D. F., Norman, E. M., Paul, J. C., Ping, C.**

and William, R. B. (2004). Attenuation of late-stage disease in mice infected by the *Mycobacterium tuberculosis* mutant lacking the sigf alternate sigma factor and identification of sigf-dependent genes by microarray analysis. *Infect Immun* 72: 1733–1745.

27. **Delvecchio, V. G., Connolly, J.P., Alefantis, T. G., Walz, A., Quan, M. A., Patra, G., Ashton, J.M., Whittington, J. T., Chafin, R. D., Liang, X., Grewal, P., Khan, A. S., Mujer, C.V. (2006).** Proteomic profiling and identification of immunodominant spore antigens of *Bacillus anthracis*, *Bacillus cereus*, and *Bacillus thuringiensis. Proc. Nati. Acad Sci* 86: 8673-8677.

28. **Diavatopoulos, D. A., Cummings, C. A., Schouls, L. M., Brinig, M. M., Relman, D. A., Mooi, F. R., (2005).** *Bordetella pertussis*, the causative agent of whooping cough, evolved from a distinct, human-associated lLineage of *B. bronchiseptica*, *PLoS Pathog* 1: 373-383.

29. **Dieter S., Ibba M., Bono J. and Patricia A. Rosa (1997).** Archaeal-type lysyl-tRNA synthetase in the Lyme disease spirochete *Borrelia burgdorferi. Proc. Natl. Acad. Sci* 94: 4383–14388.

30. **Donnes, P. and Hoglund, A. (2004).** Predicting protein subcellular localization: past, present, and future. *Genomics Proteomics Bioinformatics* 2: 209-15.

31. **Doolan, D. L., Southwood, S., Freilich, D. A., Sidney, J., Graber, N. L., Shatney, L., Bebris, L., Florens, L., Dobano, C., Witney, A. A., Appella, E., Hoffman, S. L., Yates, J. R., Carucci, D. J., Sette, A. (2003).** Identification of *Plasmodium falciparum* antigens by antigenic analysis of genomic and proteomic data. *Proc. Natl. Acad. Sci.* 100: 9952–9957.

32. **Dowsey, A. W., Dunn, M. J. and Yang, G. Z. (2003).** The role of bioinformatics in two-dimensional gel electrophoresis. *Proteomics* 3:1567- 96.

33. **Economou, A. (1998).** Bacterial preprotein translocase: mechanism and conformational dynamics of a processive enzyme. *Mol Microbiol* 27: 511-518.

34. **Emsley, P.*,* Ian, G. C., Neil, F. F., Neil, W. I. (1996).** Structure of *Bordetella pertussis* virulence factor P.69 pertactin. *Nature* 381: 90 – 92.

35. **Ewanowich, C. A., Melton, A. R., Weiss, A. A., Sherburne, R. K., and Peppler, M. S. (1989).** Invasion of HeLa 229 cells by virulent *Bordetella pertussis*. *Infect Immun* 57: 2698–2704.

36. **Farizo, M. K., Fiddner, S., Cheung, A. M., and Burns, D. L. (2002).** Membrane localization of the S1 subunit of pertussis toxin in *Bordetella pertussis* and implications for pertussis toxin secretion. *Infect Immun* 70: 1193–1201.

37. **Fernández-Arenas E., Gloria M., César N., Rosalía D. O. and Concha G. (2004).** Low virulent strains of *Candida albicans*: Unravelling the antigens for a future vaccine. *Proteomics* 4: 3007–3020.

38. **Fernandez, R., Weiss, A. (1998).** Serum resistance in bvg-regulated mutants of Bordetella pertussis. *FEMS Mic. Let.* 163: 57-63.

39. **Finn, T., Li, Z. and Kocsis, E. (1995).** Identification of a *Bordetella pertussis bvg*-regulated porin-like protein, *J Bacteriol* 177: 805–809.

40. **Florea, L., Halldorsson, B., Kohlbacher, O., Schwartz, R., Hoffman, S., Istrail, S. (2003).** Epitope prediction algorithms for peptide-based vaccine design. *Proc IEEE Comput Soc Bioinform Conf* 2: 17-26.

41. **Frutiger, S. and Hochstrasser, D. (1993).** The focusing positions of polypeptides in immobilized pH gradients can be predicted from their amino acid sequences. *Electrophoresis* 14: 1023-31.

42. **Fu, Q., Van-Eyk, J. E. (2006).** Proteomics and heart disease: identifying biomarkers of clinical utility. *Exp Rev Proteomics* 3: 237–249.

43. **General Directorate of Primary Health Care of Ministry of Health, Turkey.** The Annual of Statistics 2001. Table 40.
http://www.saglik.gov.tr/extras/istatistikler/temel2004/tablo-39.htm

44. **Goo, A. Y., Eugene, C. Y., Weiguo, A. T., Min, P., Ruedi, A., David, R. G., Leroy, H., Wailap, V. N. (2003).** Proteomic Analysis of an Extreme Halophilic Archaeon, *Halobacterium sp.* NRC-1. *Mol Cel Proteomics* 2: 506–524.

45. **Gorg, A., Weiss, W., Dunn, M. J. (2004).** Current two-dimensional electrophoresis technology for proteomics. *Proteomics* 4: 3665-3685.

46. **Grandi, G., (2001).** Antibacterial vaccine design using genomics and proteomics. *Trends Biotechnol* 19: 181-188.

47. **Graves, P. R., Haystead, T. A. (2002).** Molecular biologist's guide to proteomics. *Microbiol. Mol. Biol. Rev*. 66: 39-63.

48. **Griffiths, M. W., Anand, SK. (2003).** Quorum sensing and expression of virulence in *Escherichia coli* O157:H7. *Int J Food Microbiol* 85: 1 – 9.

49. **Gupta, S. K., Sisodia, B, Sudhir, S., Hajela, K.,Naik, S., Shasany, K. and Dube, A. (2007)** Proteomic approach for identification and characterization of novel immunostimulatory proteins from soluble antigens of *Leishmania donovani* promastigotes. *Proteomics* 7: 816–823.

50. **Hanash, S. (2003).** Disease proteomics*. Nature*, 422: 226-232.

51. **Hansmeier, N., Chao, T., Kalinowski, J., Pühler, A. and Tauch, A. (2006).** Mapping and comprehensive analysis of the extracellular and cell surface proteome of the human pathogen *Corynebacterium diphtheriae*. *Proteomics* 6: 2465–2476.

52. **Havlasová, J., Hernychová, L., Brycht, M., Hubálek, M., Lenco, J., Larsson, P., Lundqvist, Ma., Forsman, M., Krocová, Z., Macela, A. (2005).** Proteomic analysis of anti-*Francisella tularensis* LVS antibody response in murine model of tularemia. *Proteomics* 5*:* 2090–2103.

53. **Hélène, H., (2006).** Secretion signal of the filamentous haemagglutinin, a model two-partner secretion substrate, *Mol Microbiol* 61: 368–382.

54. **Heveker, N., Bonnaffe, D., Ullmann, A. (1994).** Chemical fatty acylation confers hemolytic and toxic activities to adenylate cyclase protoxin of *Bordetella pertussis*. *J Biol Chem* 269: 32844-7.

55. **Hewlett, L. E., (1989).** Adenylate cyclase toxin from *Bordetella pertussis. J Biol Chem* 264: 19379-19384.

56. **Hijnen, M., Van, G., Pieter, G. M., Berbers, G. A. M., Van W. T. (2005).** The *Bordetella pertussis* virulence factor P.69 pertactin retains its immunological properties after overproduction in *Escherichia coli*. *Protein Expr Purif* 41: 106–112.

57. **Holden, H. M., Thoden, J. B., Raushel, F. M., Wesenberg, G. (1999).** The binding of inosine monophosphate to *Escherichia coli* carbamoyl phosphate synthetase. *J Biol Chem* 274: 22502-7

58. **Holden, H. M., Thoden, J. B., Raushel, F. M., Benning, M. M., Rayment, I. (1999).** The structure of carbamoyl phosphate synthetase determined to 2.1 A resolution. *Acta Crystallogr D Biol Crystallogr* 55: 8-24.

59. **Hughes, M. J., Joanne, C. M., Jonathan, D. L., Rebecca, W., Philippa, K. P., Zabin, N. Y., Richard, J. D., Paul, E., Andrew, J. R., Joanne, M. R., Fiona, M. G., Thanai, P., Maria, P., Howard, R. M., Robert, G. F.and Joseph, D. S. (2002).** Identification of major outer surface proteins of *Streptococcus agalactiae*. *Infect Immun* 70:1254–1259.

60. **Inatsuka, C. S. (2005).** *Bordetella* filamentous hemagglutinin plays a critical role in immunomodulation, suggesting a mechanism for host specificity, *PNAS* 102: 18578–18583.

61. **Jung, T., Shin, Y. S., Lee, E. G., Shin, G. W., Kim, Y. R, Lee, E. Y., Kim, J. H., Jang, H., Gershwin, L. J., Kim, D. Y., Kim, Y. H., Kim, G. S., Suh, M. D. (2004).** Identification of antigenic proteins from *Neospora caninum* recognized by bovine immunoglobulins M, E, A and G using immunoproteomics. *Proteomics* 4: 3600–3609.

62. **Jung, T. S., Shin, G. W., Palaksha, K. J, Kim, Y. R., Nho, S. W, Cho, J. H., Heo, N. E., Heo, G. J., Park, S. C. (2007).** Immunoproteomic analysis of capsulate and non-capsulate strains *of Lactococcus garvieae*. *Vet Microbiol* 31: 205-12.

63. **Kaniga K., Compton M. S., Roy, C., and Preeti S. (1998).** Molecular and functional characterization of *Salmonella enterica*, serovar typhimurium poxA gene: effect on attenuation of virulence and protection, *Infect Immun* 66: 5599–5606.

64. **Kaufmann, S. H., Zugel, U. (1999).** Role of heat shock proteins in protection from and pathogenesis of infectious diseases. *Clin Microbial Rev* 12: 19–39.

65. **Kerr, R. C., Matthews, J. R. (2000).** *Bordetella pertussis* infection: pathogenesis, fiagnosis, management, and the role of protective immunity. *J Clin Microbiol Infect Dis* 19: 77–88.

66. **Khan, A. S., Mujer, C. V., Alefantis, T. G., Connolly, J. P., Mayr, U. B., Walcher, P., Lubitz, W., Delvecchio, V. G. (2006).** Proteomics and bioinformatics strategies to design countermeasures against infectious threat agents. *J Chem Inf Model.* 46: 111-5.

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

68. **Kievit, T. R., Iglewski, B. H. (2000).** Bacterial quorum sensing in pathogenic relationship. *Infect. Immun* 68: 4839– 4849.

69. **Kimmel, B., Armin, B., Rainer, F., Roy, G., Wernerr, G., and Dagmar B. (2000).** Identification of immunodominant antigens from *Helicobacter pylori* and evaluation of their reactivities with sera from patients with different gastroduodenal pathologies. *Infect. Immun* 68: 915-920.

70. **Kinnear, S. M., Marques, R. R., Carbonetti, N. H. (2001).** Differential regulation of Bvg-activated virulence factors plays a role in *Bordetella pertussis* pathogenicity. *Infect Immun* 69: 1983–1993.

71. **Kislinger, T., Cox, B., Kannan, A., Chung, C., Hu, P., Ignatchenko, A., Scott, M. S., Gramolini, A. O., Morris, Q., Hallett, M. T., Rossant, J., Hughes, T. R., Frey, B., Emili, A. (2006).** Global survey of organ and organelle protein expression in mouse: combined proteomic and transcriptomic profiling. *Cell* 125: 173–186.

72. **Knight JB, Huang YY, Halperin SA, Anderson R, Morris A, Macmillan A, Jones T, Burt DS, Van Nest G, Lee SF. (2006).** Immunogenicity and protective efficacy of a recombinant filamentous haemagglutinin from *Bordetella pertussis*, *Clin and Exp Immunol* 144: 543–551.

73. **Knights, K. M. and Drogemuller, C. J. (2000).** Xenobiotic-CoA ligases: kinetic and molecular characterization. *Cur Drug Metab* 1: 49-66.

74. **König, C. H. W. (2002).** Pertussis of adults and infants. *Lancet Infect Dis*; 2: 744–50.

75. **Krah. A., Miehlke. S., Pleissner. K., Zimny-Arndt, U., Kirsch, C., Lehn, Z., Meyer, T., Jungblut, P. and Aebischer, T. (2004).** Identification of candidate antigens for serologic detection of *Helicobacter pylori*-infected patients with gastric carcinoma. *Int. J. Cancer* 108: 456–463

76. **Kurupati, P., The, B. K., Kumarasinghe, G., Poh, C. L. (2006).** Identification of vaccine candidate antigens of an ESBL producing *Klebsiella pneumoniae* clinical strain by immunoproteome analysis. *Proteomics* 6: 836-844.

77. **Lambert, H. P., Giudice, G., Gervaix, A., Costantino, P., Wyler, C.A., Tougne, C., Graff-Meeder, E. R., Rappuoli, R. (1993).** Priming to heat shock proteins in infants vaccinated against pertussis, *J Immunol* 150: 2025-2032.

78. **Langen, H., Fountoulakis M., Takacs, B., (2000).** Two-dimensional map of the proteome of *Haemophilus influenzae*. *Electrophoresis* 21: 411–429.

79. **Leyht, R. and Griffith, R. W. (1992).** Characterization of the outer membrane proteins of *Bordetella avium*. *Infect. and Immun*. 60: 958-964.

80. **Lin, Y. F., Wu, M., Lin, J. T., Sun, Y., Chen, D., Chow L. (2006).** Comparative immunoproteomics of identification and characterization of virulence factors from *Helicobacter pylori* related to gastric cancer. *Mol Cell Proteomics*. 5: 1484-1496.

81. **Locht, C., (1999).** Molecular aspects of *Bordetella pertussis* pathogenesis. *Internatl Microbiol* 2:137–144.

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

83. **Madhavi, R. J. and Calvo, K. C. (1997).** Reversal of the nucleotide specificity of ketol acid reductoisomerase by site-directed mutagenesis identifies the NADPH binding site. *Arch Biochem Biophys* 338: 83-9.

84. **Marcos, P. P., Chemale, G., Luiza, C., Costa, A., Kich, J., Vainstein, M., Zaha A., Ferreira, H. (2007).** Proteomic survey of the pathogenic *Mycoplasma hyopneumoniae* strain 7448 and identification of novel post-translationally modified and antigenic proteins. *Vet Microbiol* 121: 83-93.

85. **Maskell, D. J., Preston, A. (2002).** A new era of research into *Bordetella pertussis* pathogenesis, *J Infection* 44: 13-16.

86. **Kerr, J. R., Matthews, R. C. (2000)**. *Bordetella pertussis* infection: pathogenesis, diagnosis, management, and the role of protective immunity. *Eur J Clin Microbiol Infect Dis* 19: 77–88.

87. **Mattoo, S. and Cherry, J.D. (2005).** Molecular pathogenesis, epidemiology, and clinical manifestations of respiratory infections due to *Bordetella pertussis* and other *Bordetella s*ubspecies*. Clin Microbiol Rev* 18: 326-82.

88. **Merkel, T. J., Boucher, P. E., Stibitz, S., Grippe, V. K., Merkel, T. J., (2003).** Analysis of *bvgR* expression in *Bordetella pertussis*. *J Bacteriol* 18: 6902–6912.

89. **Neubauer, H., Dahouk, S., Nöckler, K.; Scholtz, H., Tomaso, H., Bogumil, R. (2006).** Immunoproteomic characterization of *Brucella abortus* 1119-3 preparations used for the serodiagnosis of *Brucella* infection. *J Immunol Methods* 309: 34– 47.

90. **Neuhoff, V., Arold, N., Taube, D., Ehrhardt, W. (1988).** Improved staining of proteins in polyacrylamide gels including isoelectric focusing gels with clear background at nanogram sensitivity using Coomassie Brilliant Blue G-250 and R-250. *Electrophoresis* 9: 255–262.

91. **Newman, C. L., Stathopoulos, C. (2004).** Autotransporter and two-partner secretion: Delivery of large-size virulence factors by gram-negative bacterial pathogens. *Crit Rev Microbiol* 30: 275–286.

92. **Nilsson, C. L., Larsson, T., Gustafsson, E., Karlsson K., Davidson, P. (2000).** Identification of protein vaccine candidates from Helicobacter pylori using a preparative two-dimensional electrophoretic procedure and mass spectrometry. *Anal. Chem* 72: 2148–2153.

93. **O'Farrell PH. (1975).** High resolution two-dimensional electrophoresis of proteins. *J Biol Chem* 250: 4007–4021.

94. **Özcengiz, E., Kilinç, K., Büyüktanir, Ö., Günalp, A. (2004).** Rapid purification of pertussis toxin (PT) and filamentous hemagglutinin (FHA) by cation-exchange chromatography. *Vaccine* 22: 1570–1575.

95. **Sebaihia, M., Preston, A., Murphy, L. D., Thomson, N., Harris, D. E., Holden, M. T., Churcher, C. M., Bentley, S. D., Mungall, K. L., Cerdeno-Tarraga, A. M., Temple, L., James, K., Harris, B., Quail, M. A., Achtman, M., Atkin, R., Baker, S., Basham, D., Bason, N., Cherevach, I., Chillingworth, T., Collins, M., Cronin, A., Davis, P, Doggett, J., Feltwell, T, Goble A, Hamlin N, Hauser H, Holroyd S, Jagels K, Leather S, Moule S,Norberczak H, O'Neil S, Ormond D,Price C, Rabbinowitsch, E., Rutter, S., Sanders, M., Saunders, D., Seeger, K., Sharp, S., Simmonds, M., Skelton, J., Squares, R., Squares, S., Stevens, K., Unwin, L., Whitehead, S., Barrell, B. G., Maskell, D. J.**

(2003). Comperative analysis of the genome sequences of *Bordetella pertussis, Bordetella parapertussis 2822* and *Bordetella bronchiseptica*, *Nat Genet*. 35: 32-40.

96. **Parton R. (1999).** Review of the biology of *Bordetella pertussis*. *Biologicals* 27: 71–76.

97. **Passerini de Rossi, B. N., Friedman, L. E., Gonzalez, F. L., Castello, P. R., Franco, M. A., Rossi J. P. (1994).** Characterization of the dermonecrotic toxin in members of the genus *Bordetella*. *Infect and Immun* 62: 3817-3828.

98. **Passerini de Rossi, B. N., Friedman, L. E., Gonzalez Flecha, F.L, Castello, P. R., Franco, M. A., Rossi, P. (1999).** Identification of *Bordetella pertussis* virulence-associated outer membrane proteins. *FEMS Microbiol Lett* 172: 9-13.

99. **Peng, X., Zijun, C., Bo, P., Sanying, W., (2004).** Rapid screening of highly efficient vaccine candidates by immunoproteomics. *Proteomics* 4: 3203–3213.

100. **Peng, X., Ye, X., Wang, S. (2004).** Identification of novel immunogenic proteins of *Shigella flexneri 2a* by proteomic methodologies. *Vaccine* 22: 2750-2756.

101. **Piatti, G. (1999).** Identification of immunodominant epitopes in the filamentous hemagglutinin of *Bordetella pertussis*. *FEMS Immunol Med Microbiol* 23: 235-241.

102. **Pinto, P., Chemale, G., Castro, L., Costa, A., Kich, J. D., Vainstein, M. H., Arnaldo, Z., Ferreira**, **H. B. (2007).** Proteomic survey

of the pathogenic *Mycoplasma hyopneumoniae* strain 7448 and identification of novel post-translationally modified and antigenic proteins. *Vet Microbiol* 121: 83–93.

103. **Preston, A., Parkhill, J., and J.Maskell D. (2004).** The *Bordatellae*: lessons from genomics. *Nature* 2: 379-390.

104. **Ramagli, L., S., Rodrigez, L., V., (1985).** Quantitation of microgram amounts of protein in two-dimensional polyacrylamide gel electrophoresis sample buffer. *Electrophoresis* 6: 559-563.

105. **Rane, J., Calvo, K. C. (1997).** Reversal of the nucleotide specificity of ketol acid reductoisomerase by site-directed mutagenesis identifies the NADPH binding site. *Arch Biochem Biophys* 338: 83-9.

106. **Rhomberg, T. A., Karlberg, O., Mini, T., Zimny-Arndt, U., Wickenberg, U., Rottgen, M., Jungblut, P. R., Jeno, P., Andersson, S. G., Dehio, C. (2004).** Proteomic analysis of the sarcosine-insoluble outer membrane fraction of the bacterial pathogen *Bartonella henselae. Proteomics* 4: 3021–3033.

107. **Ranford J. C., Henderson, B. (2002).** Chaperonins in disease: mechanisms, models, and treatments. *J Clin Pathol Mol Pathol* 55: 209–213.

108. **Rappuoli, R., Serruto, D. (2006).** Post-genomic vaccine development. *FEBS Lett* 580: 2985–2992.

109. **Rappuoli, R., Scarselli, M., Giuliani, M. M., Adu-Bobie, J., Pizza, M. (2005).** The impact of genomics on vaccine design. *Trends Biotechnol* 23: 84-91.

110. **Razin, E., Lee, Y. (2005).** Nonconventional involvement of LysRS in the molecular mechanism of USF2 transcriptional activity in FcεRI-activated mast cells. *Mol Cell Biol* 25:8904-12.

111. **Roe, F. H., Cho, E. J., Bae, J. B., Kang, J. G. (1996).** Molecular analysis of RNA polymerase alpha subunit gene from Streptomyces coelicolor A3. *Nucleic Acids Res* 24: 4565–457.

112. **Saha, S., and Raghava, G. P. S. (2006).** Prediction of Continuous B-cell Epitopes in an Antigen Using Recurrent Neural Network. *Proteins* 65: 40-48

113. **Santucci, A. Mini, R., Bernardini, G., Salzano, A. M., Renzone, G., Scaloni, A., Figura, N. (2006).** Comparative proteomics and immunoproteomics of *Helicobacter pylori* related to different gastric pathologies, *J Chromatogr B Analyt Technol Biomed Life Sci* 833: 63–79.

114. **Sato, Y., Arai, H. (1972).** Leucocytosis promoting factor of *Bordetella pertussis*. *Infect Immun 6*: 899–904.

115. **Sato, Y., Arai, H., Suzuki, K. (1974).** Leucocytosis promoting factor of *B. pertussis*. III. Its identity with protective antigens. *Infect Immun* 9: 801–810.

116. **Sellman, B. R., Howell, A. P., Kelly-Boyd C., Baker, S. M. (2005).** Identification of Immunogenic and Serum Binding Proteins of *Staphylococcus epidermidis*. *Infect and Immun* 73: 6591–6600.

117. **Sergueev K., Dabrazhynetskaya A., and Austin S. (2005).** Plasmid partition system of the P1*par* family from the pWR100 virulence plasmid of *Shigella flexneri*. *J Bacteriol* 187: 3369–3373.

118. **Severin, A., Nickbarg, E., Wooters J., Quazi, S. A., Matsuka, Y., Murphy, E., Ioannis M, Zagursky R J., and Olmsted S. B. (2007)** Proteomic analysis and identification of *Streptococcus pyogenes* surface-associated proteins. *J Bacteriol* 189: 1514-1522.

119. **Shafferman, A., Chitlaru, T., Gat, O., Grosfeld, H., Inbar, I., Gozlan, Y., (2007).** Identification of in-vivo Expressed Immunogenic Proteins by Serological Proteome Analysis of *Bacillus anthracis* Secretome. *Infect Immun* In press.

120. **Shin, G. W., Palaksha, K. J., Kim, Y. R., Nho, S. W., Kim, S., Heo, G. J., Park, S. C., Jung, T. S. (2007).** Application of immunoproteomics in developing a *Streptococcus iniae* vaccine for olive flounder (*Paralichthys olivaceus*). *J Chromatogr B.* In press.

121. **Shinnick, T. M. (1991).** Heat shock proteins as antigens of bacterial and parasitic pathogens. *Curr. Top. Microbiol. Immunol* 167:145–160.

122. **Sinha, R., Heath, P.T. (2005).** Pertussis. *Medicine* 33: 101-102.

123. **Spears, P. A. (2003).** Unexpected Similarities between *Bordetella avium* and Other Pathogenic Bordetellae. *Infect Immun* 71: 2591–2597.

124. **Stu, D., Fuchs, T. M., Schneider B., Bosserhoff, A. and Gross, R. (2005).** Identification and regulation of cold-inducible factors of *Bordetella bronchiseptica*. *Microbiology* 151: 1895–1909.

125. **Thodent, J. B and Frank, M. R. (1998).** Carbamoyl phosphate synthetase: a tunnel runs through it. *Curr Opin Struct Biol* 8: 679-685.

126. **Thoren, K., Gustafsson, E., Clevnert, A., Larsson, T., Bergstrom, J., Nilsson, C. L. (2002).** Proteomic study of non-typable Haemophilus influenzae. *J. Chromatogr. B Analyt. Technol. Biomed. Life Sci.* 782: 219–226.

127. **Tonon, S., Goriely, S., Aksoy, E., Pradier, O., Del Giudice, G., Trannoy, E., Willems, F., Goldman, M., De Wit, D. (2002).** *Bordetella pertussis* toxin induces the release of inflammatory cytokines and dendritic cell activation in whole blood: impaired responses in human. *Eur. J. Immunol.* 32: 3118–3125.

128. **Van Den Berg, B. M., Beekhuizen, H., Willems, R. J. L.″ Mooi, F. R., Van Furth, R. (1999)**. Role of *Bordetella pertussis* virulence factors in adherence to epithelial cell lines derived from the human respiratory tract, *Infect. Immun 67*: 1056–1062.

129. **Vatansever, U., Coplu, N., Oner, N., Sonmez, C., Karasalihoglu, S., Kurtoglu, D., Esen, B., Ekuklu, G. (2005).** Seroprevalance of *Bordetella pertussis* antibodies among healthy adolescent girls in Edirne. *Swiss Med Wkly* 135: 531–536.

130. **Vera, C. B., Cainelli Gebara, V. C., Risoleo, L., Lopes, A. P., Ferreira, V. R., Quintilio, W., Lepine, F., Silva, W. D., Raw, I. (2007).** Adjuvant and immunogenic activities of the 73 kDa N-terminal-domain of BrkA autotransporter and Cpn60/60 kDa chaperonin of *Bordetella pertussis*. *Vaccine* 25: 621-9.

131. **Vipond, C., Suker, J., Jones, C., Tang, C., Feavers, I. M. and Wheeler, J. X. (2006).** Proteomic analysis of a meningococcal outer membrane vesicle vaccine prepared fromthe group B strain NZ98/254. *Proteomics* 6: 3400–3413.

132. **Vytvytska, O., Nagy, E., Bluggel, M., Meyer, H. E., Kurzbauer, R., Huber, L. A., Klade, C. S. (2002).** Identification of vaccine candidate antigens of *Staphylococcus aureus* by serological proteome analysis. *Proteomics* 2: 580–590.

133. **Walker, M. J., Smith, A. M., Guzman, C. A. (2001).** The virulence factors of *Bordetella pertussis*: a matter of control. *FEMS Microbial Rev* 25: 309-333.

134. **Wasinger, V. C., Cordwell, S. J., Cerpa-Poljak, A., Yan, J. X., Gooley, A. A., Wilkins, M. R., Duncan, M. W., Harris, R., Williams, K.**

W., Humphrey-Smith, I. (1995). Progress with gene product mapping of the Mullicutes *Mycoplasma genitalium*. *Electrophoresis* 16: 1090–1094.

135. **Weber, S., Lottspeich, F., and Kohl, J. (1995).** An Epitope of Elongation Factor Tu Is Widely Distributed within the Bacterial and Archaeal Domains *J Bacteriol* 177. 1: 11–19.

136. **Westermeier, R. And Naven, T. (2002).** Proteomics in Practice, Wiley-VCH.

137. **Weiss, A. A. and Rambow A. A. (2004).** Temporal expression of pertussis toxin and Ptl secretion proteins by *Bordetella pertussis*, *J Bacteriol* 186: 43–50.

138. **Wickner, B., Eichler, J. (1998).** The SecA subunit of *Escherichia coli* preprotein translocase is exposed to the periplasm. *J Bacteriol* 180: 5776-9.

139. **Wirsing, K., Halperin, S., Riffelmann, M., and Guiso, N. (2002).** Pertussis of adults and infants. *Lancet Infect Dis* 2: 744–50.

140. **Wolfe, D. N., Kirimanjeswara, G. S., Harvill, E.T. (2005).** Clearance of *Bordetella parapertussis* from the lower respiratory tract requires humoral and cellular immunity. *Infect Immun* 73: 6508–6513.

141. **Wright, A. (2005).** Proteomic analysis of cell surface proteins from *Clostridium difficile. Proteomics* 5: 2443–2452.

142. **Yamamoto, T., Matui, H., Suzuki, M., Isshiki, Y., Kodama, C., (2003).** Oral Immunization with ATP-dependent protease-deficient mutants protects mice against subsequent oral challenge with virulent *Salmonella enterica* serovar typhimurium. *Infect Immun* 71: 30-39.

143. **Ying, T., Wang, J., Wang, H., Feng, E., Wei, K., Huang, L., Huang, P., Huang, C. (2005a).** Immunoproteomics of membrane proteins of *Shigella flexneri* 2a 2457T. *World J Gastroenterol* 11: 6880-6883.

144. **Ying, T., Wang, H., Li, M., Wang, J., Shi, Z., Erling, F., Xiankai, L., Su, G., Kaihua, W., Xuemin, Z., Peitang, H. and Liuyu, H. (2005b).** Immunoproteomics of outer membrane proteins and extracellular proteins of *Shigella flexneri* 2a 2457T. *Proteomics* 2005 5: 1-17

145. **Youngren, B., Radnedge, L., Hu, P., Garcia, E., Austin, S. (2000).** Plasmid Partition System of the P1-P7*par* Family from the pMT1 Virulence Plasmid of *Yersinia pestis*. *J Bacteriol* 182: 3924–3928.

APPENDIX A

COLLOIDAL COOMASSIE BLUE (CCB) STAINING
AND
STOCK SOLUTIONS

1-Coomassie Brilliant Blue (CBB) Stock

Coomassie Brilliant Blue G-250	5g
dH2O	100 mL

2-Fixation

40 % Ethanol	125 mL
10 % Acetic Acid	25 mL
50 % dH2O	100 mL

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

3-Washing

The gel is washed with dH2O 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

The gel is put in 200 mL CCB dye solution then 50 mL methanol is added and the gel is kept in this solution for 24 to 48 hours.

6-Washing

The gel is washed with dH2O until protein spots are clearly visible.

7- Acrylamide/Bis

Acrylamide	146 g
N.N'-Methylene-bis Acrylamide	4 g

Distilled water to 500 mL. Filtered and stored at 4 C. Protected form light.

8- Tris HCl (1.5 M)

Tris base	54.45 g
dH_2O	150 mL

pH is adjusted to 8.8 with HCl, distilled water to 300 mL and stored at 4° C.

9- Tris HCl (0.5 M)

Tris base	6 g
dH_2O	60 mL

pH is adjusted to 6.8 with HCl, distilled water to 100 mL and stored at 4° C.

10- Tris-EDTA Buffer (TE)

Tris	10 mM
EDTA	1 mM

pH is adjusted to 8.0 with HCl.

11- Running Buffer (5X)

Tris base	15 g
Glycine	72 g
SDS	5 g

Distilled water to 1 L. Stored at 4° C.

APPENDIX B

CHEMICALS AND THEIR SUPPLIERS

Chemicals	Chemical 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
CH_3CN	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	BIO-RAD
KH_2PO_4	Merck
Methanol	Merck
$MgSO_4$ 7H2O	Merck
Molecular Weight Standard (14,400-116,000)	Fermentas
NaOH	Merck
NH4Cl	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

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 sterilized at 121^{O} C for 45 min (Sato et al., 1972).

Modified Morse Bray Medium

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
$MgCl_2.6H_2O$	0.4 g/l
$CaCl_2$	0.01 g/l
Glutamic Acid	0.2 g/l

Dissolved in 600 ml of dH_2O. PH was adjusted to 7.4 and volume was completed to 975 ml with dH_2O and the mixture was autoclaved.

B

Component	Concentration
Glutation	100 mg/l
$FeSO_4.7H_2O$	10 mg/l
L-Cysteine	40 mg/l
Ascorbic Acid	20 mg/l
Nicotonic Acid	4 mg/l
L-Prolin	120 mg/l

Dissolved in 10 ml of dH_2O and volume was completed to 25 ml with dH_2O. The mixture was filtered through 0.22 µm membrane and added to A (Sato et al., 1974).