STUDIES ON NOVEL IMMUNOGENIC PROTEINS OF CLOSTRIDIUM CHAUVOEI

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STUDIES ON NOVEL IMMUNOGENIC PROTEINS OF
CLOSTRIDIUM CHAUVOEI

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CLOSTRIDIUM CHAUVOEI

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ABSTRACT

STUDIES ON NOVEL IMMUNOGENIC PROTEINS OF

CLOSTRIDIUM CHAUVOEI

Coral Didem

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Clostridium chauvoei is a gram-positive, spore-forming anaerobic bacterium. It is the pathogenic agent of blackleg, a disease causing serious toxemia and high mortality in cattle, sheep and many other domestic and wild animals. It is considered the most important *Clostridium* producing economic losses in livestock. Typically, animals infected with blackleg die rapidly without any signs of illness. Animals quickly die within 12 to 48 hours after contracting the disease. Therefore, the control of this disease is done by commercial vaccines consisting of whole formolized cultures. Immunity against *C. chauvoei* is associated with whole cell, including its somatic and flagellar antigens while in other clostridial diseases, protective immunity is obtained by the use of vaccines containing toxoids. Moreover, it is essential to obtain new information about the somatic antigens of *C. chauvoei*.

Proteomics is the study of the proteome, the protein complement of the genome. The proteome has been defined as the entire complement of proteins expressed by a cell, organism, or tissue type, and accordingly, proteomics is the study of this complement expressed at a given time or under certain environmental conditions. 2-DE with Immobilized pH Gradients (IPGs) combined with protein identification by Mass Spectrometry (MS) is currently the workhorse for proteomics. Much of information about immunogenic component can be derived from proteomics coupled to Western blotting, namely immunoproteomics.

Our study constitutes the first immunoproteomic analysis of *C. chauvoei* to identify candidate immunogenic antigens for development of new vaccines. Analyses were performed by Western blot and dot blot techniques against the whole cell extract proteins of *C. chauvoei* separated by 2-DE. Firstly, the growth conditions of two different strains, *C. chauvoei* ATCC 11957 and *C. chauvoei* 20 were optimized. After mice immunization studies with experimental vaccines prepared, sera were obtained for evaluation of the immunoglobulin G antibody level by ELISA. After high level of antibody response determination, 1-DE, 2-DE and immunoblot studies were performed for the characterization of immunogenic proteins.

In the study, a total of 460 protein spots could be detected on the 2-DE gels by the help of Delta2D image analysis software and 30 of them were reacted with polyclonal antibodies against inactivated whole cells of *C. chauvoei*. Among these 30 spots, and 8 of them could be characterized by MALDI-TOF MS analyses. Of these 8 spots revealed four different gene products (distinct ORFs). Ornithine decarboxylase, methionine adenosyltransferase, glucose-6-phosphate isomerase, and flagellin protein FliB (C) are the characterized proteins. Glucose-6-phosphate isomerase has been identified as an

immunogenic protein for a pathogenic microbe and in *C. chauvoei* for the first time. Methionine adenosyltransferase and ornithine decarboxylation were identified as immunogenic for *C. chauvoei* for the first time. The last defined protein is the flagellin protein FliB(C) which is known to be major immunogenic protein of *C. chauvoei*.

Key words: Protein, Proteome, *Clostridium chauvoei,* 2-DE, Immobilized pH Gradients, MALDI TOF MS, Immunoproteomics, Immunoblotting, Western Blot, Dot Blot, ELISA.

ÖZ

CLOSTRIDIUM CHAUVOEI'NİN YENİ İMMUNOJENİK PROTEİNLERİ ÜZERİNE ÇALIŞMALAR

Coral, Didem

Yüksek Lisans Biyoteknoloji Bölümü

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Clostridium chauvoei, gram pozitif, sporlu, anaerobik bir bakteri türüdür. Bu bakteri, ciddi toksemik etkisi olan ve büyük baş hayvan, koyun ve diğer evcil ve yabani hayvanlarda yüksek oranda ölüme neden olan yanıkara hastalığının etkeni olarak bilinmektedir. Çiftliklerde ekonomik olarak kayba yol açan en önemli *Clostridium* türünün *C. chauvoei* olduğu düşünülmektedir. Genel olarak, enfeksiyona yakalanan hayvanlar, hiçbir belirti göstermeden 12-48 saat içinde ani bir şekilde ölürler. Bu nedenle, hastalığın kontrolünde aşı tercih edilmektedir. Bu aşılar, toksoid içeren diğer *Clostridium* türleri için kullanılan aşılardan farklı olarak, formalin ile inaktive edilmiş, hücresel ve flajella antigenleri de içeren aşılardır. Bununla beraber, bu bakterinin hücresel antijenleri için yeni bilgilere ihtiyaç duyulmaktadır.

Proteom çalışması olan proteomik, genomun protein seviyesinde ifadesi için tamamlayıcısıdır. Proteom, hücre, doku yada organizma tarafından üretilen bütün proteinler olarak tanımlanmaktadır ve buna göre, proteomik belirli zaman ve belirli çevre koşullarında üretilen bütün proteinlere ait global bir çalışmadır. 2-DE ve IPG (Immobilized pH Gradient) lerin protein tanımlaması için MALDI TOF MS analizleri ile birleşimi proteomik'in en geleneksel araçlarıdır. Patojenlerin immunojenik yapılarıyla ilgili en kapsamlı bilgi, proteomik teknolojisinin, Western blot analiziyle birleşmesini içeren, immunoproteomik yöntemlerle elde edilebilir.

Bu çalışma, *C. chauvoei* için yeni aşıların geliştirilmesinde kullanılabilecek aşı adayı antijenlerin tanımlanması amacıyla yapılan ilk *C. chauvoei* immunoproteomik analizini içermektedir. *C. chauvoei'* nin tam hücre ekstratının 2-DE'de ayrılmış proteinlerine karşı Western blot ve dot blot teknikleri kullanılarak analizler gerçekleştirilmiştir. Bu amaçla, iki farklı suşun (*C. chauvoei* ATCC ve Pendik) üreme koşulları optimize edilmiş ve hazırlanan tam hücre deneysel aşılarla farelerde yapılan immunizasyon çalışmaları sonrasında ELISA yöntemiyle IgG antikor düzeyleri incelenmiştir. Yüksek oranda antikor yanıtı oluşumunun belirlenmesinden sonra immunogen proteinleri tanımlamak için 1-DE, 2-DE ve immunblot çalışmaları yapılmıştır.

Çalışmamızda 2-DE jellerinden Delta2D görüntü analiz yazılımı kullanılarak 460 protein spotu belirlenmiştir ve 30 spot *C. chauvoei'* nin inaktif tam hücre poliklonal antikorları ile immunreaksiyon vermiştir. Bu 30 spotun 8 tanesi MALDI-TOF MS ile analiz edilmiştir. Bu 8 spotun 4 farklı gen ürününü (farklı ORF'leri) temsil ettiği görülmüştür. Ornitin dekarboksilaz, metionin adenosiltransferaz, glukoz-6-fosfat isomeraz, ve flagellin protein FliB (C) proteinleri tanımlanan proteinlerdir. Bunlardan glukoz-6-fosfat isomeraz ilk

kez patojenik bir organizmada immunojenik protein olarak bulunmuştur. Diğer yandan, bu çalışmada tanımlanan immunojenik proteinlerinden methionin adenosiltransferaz ve ornitin dekarboksilaz'ın immunojenik olduğu *C. chauvoei* özelinde ilk kez bu çalışmada gösterilmiştir. Son olarak, *C. chauvoei'* nin en immunojenik proteini olarak bilinen flagellin protein FliB(C) tanımlanmıştır.

Anahtar kelimeler: Protein, Proteom, *Clostridium chauvoei,* 2-DE, IPG (Immobilized pH Gradient), MALDI TOF MS, Immunoproteomik, İmmunoblotting, Western blot, Dot blot, ELISA.

To My Grandmother

ACKNOWLEDGEMENTS

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CHAPTER 1

INTRODUCTION

1.1 Genus *Clostridium*

Clostridia are spore-forming, obligatory anaerobic bacteria in the Phylum *Firmicutes*. Most clostridia will not grow under aerobic conditions and vegetative cells are killed by exposure to O_2, but the endospores of many species are extremely sturdy and survive extended boiling in water and exposure to air. Spores germinate under conditions favorable for vegetative growth, such as anaerobiosis and presence of organic substrates. They usually stain gram positive especially in young cultures. Cells are rod shaped with rounded or pointed ends. For motility, peritrichous flagella usually present. They form oval or spherical endospores which distend the cell terminally or subterminally. (Cato *et al.*, 1986)
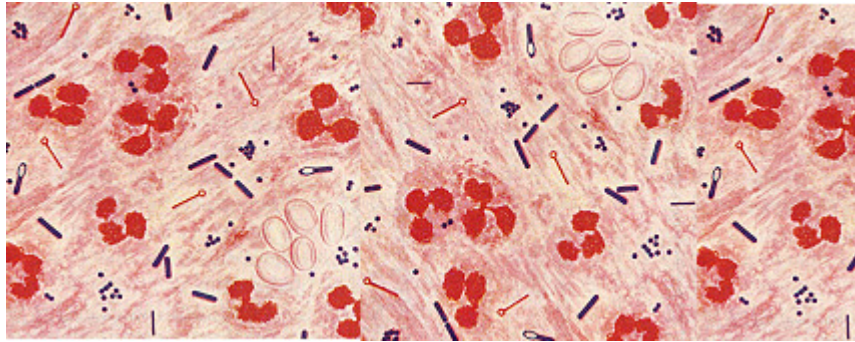
Figure 1: Stained pus from a mixed anaerobic infection. At least three different clostridia are apparent.

This genus comprises about 150 metabolically diverse species of anaerobes that are widespread in the environment. They are found in soil, sewage, marine sediments, and the intestinal tracts of humans and other animals and in decaying animal and plant products. Because of the ubiquity of these organisms, they find their way into wounds, foods, and feeds and some are pathogenic for animals, usually but not always mediated by their toxins.

From the evolutionary perspective, clostridia are considered to be the most ancient bacteria. It is believed that present day *Mollicutes* (*Eubacteria*) have evolved regressively (i.e., by genome reduction) from gram-positive clostridia-like ancestors with a low GC content in DNA. Several species of clostridia (e.g., *C. perfringens*, *C. botulinum*, *and C. tetani*) are known opportunistic toxin-producing pathogens in animals and humans. Some species are capable of producing organic solvents (acetone, ethanol, etc), molecular hydrogen and other useful compounds. There are also species that can fix molecular nitrogen and thus are important participants in biological turnaround of nitrogen compounds in nature. 20 of 83 *Clostridium* species listed in Bergey's Manual of Systemic Bacteriology are pathogenic or

else encountered clinical or environmental specimens relating to illness or infections in humans or other animals (Cato *et al.*, 1986).

1.2 Veterinary Clostridial Diseases

Veterinary Clostridial diseases are usually acute and are not contagious. Thus, common predisposing causes result in to outbreaks. The bacteria responsible are frequently found in soil with high organic content as well as intestinal tracts of healthy animals. They are very resistant to the many environmental conditions, and organisms are able to lie dormant for years. The source of the infection is almost always their presence in the environment, not another animal (Kayne *et al.*, 2004).

The important veterinary clostridial diseases are; Blackleg caused by *C. chauvoei*, Bacillary haemoglobinuria caused by *C. haemolyticum*, tetanus caused by *C. tetani*, malignant edema, braxy caused by *C. septicum*, black disease caused by *C. novyi* type B, pulpy kidney or overeating disease caused by *C. perfringens* type D, lamb dysentery caused by *C. perfringens* type B, big head and malignant edema caused by *C. novyi* type A, struck caused by *C. perfringens* type C, abomasitis caused by *C. sordellii*, and botulism caused by *C. botulinum*. In each case, one or more exotoxins or bacterial membrane proteins lead to the pathogenesis. These organisms are listed in Table 1, along with their major toxins and their activities and the diseases they cause.

Table 1. Pathogenic clostridial diseases summary

Clostridium Species	Major Toxins/ Antigenic Proteins	Protein Size (kDa)	Activity	Disease	Host
C. tetani	Neurotoxin	150	Tetanus	Tetanus	Horses, ruminants, humans and other animals
	Tetanolysin	48	Oxygen Labile hemolysin		
C. botulinum (types A-F)	Neurotoxin	150	Botulism	Botulism	Many animal species and man
	Component I	50	ADP-ribosylation Binding		
	Component II	105	Binding		
C. chauvoei	Flagella	47	Major virulance factor	Blackleg	Cattle, sheep, pigs
	Hemolysin	27	Oxygen-labile hemolysin		

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Table 1 Cont'd

Clostridium Species	Major Toxins/ Antigenic Proteins	Protein Size (kDa)	Activity	Disease	Host
C. septicum	Alpha	46	Lethal, necrosis, hemolytic	Malignant Edema, Braxy,	Cattle, Sheep, pigs Sheep
C. novyi					
Type A	Alpha	280	Lethal, edematizing, cytotoxic activity	Sheep	Big head of rams
	Beta	43	Phospholipase C, lecithinase activity	Cattle, sheep	Gas gangrene
	Gamma	30	Phospholipase C, hemolysis		
Type B	Alpha	280	Lethal, edematizing, cytotoxic activity	Sheep, cattle	Black Disease (Necrotic hepatitis)
	Beta	43	Phospholipase C, lecithinase activity		

Table 1 Cont'd

Clostridium Species	Major Toxins/ Antigenic Proteins	Protein Size (kDa)	Activity	Disease	Host
C. haemolyticum	Beta	45	Phosholipase C,hemolysin	Cattle, sheep	Bacillary Haemoglobuniria
C. sordelli	Alpha	43	Phospholipase C	Cattle, sheep, horses	Gas gangrene
	Beta HT (hemorogic toksin) LT (lethal toxin)	300 240	Lethal		
	Hemolysin	43	Oxygen-labile hemolysin		
C. perfringens					
Type B	Alpha	43	Alpha	Lambs (under 3 years old) Neonatal calves, foals	Lamb dysentery Enterotoxemia
	Beta	40	Beta		
	Beta-2	28	Beta-2		
	Epsilon	40	Lethal, permease/ enterotoxemia		

Table 1 Cont'd

Clostridium Species	Major Toxins/ Antigenic Proteins	Protein Size (kDa)	Activity	Disease	Host
Type C	Alpha	43	Phospholipase C/myonecrosis	Piglets, calves, foals, lambs	Haemorrhagic enterotoxemia
	Beta	40	Lethal, necrotic/ enterotoxemia		Struck
	Beta-2	28		Adult sheep	Necrotic entiritis
				Chickens	
Type D	Alpha	43	Phospholipase C/myonecrosis	Sheep	Pulpy kidney diseases
	Epsilon	40	Lethal, permease/ enterotoxemia	Goat, calves	
	Toxin B	360-470	Cytotoxin/AAPMC		

(Constructed from Hatheway, 1990; Quinn, 1994)

1.3 *C. chauvoei* and Blackleg

C. chauvoei is a gram positive, motile, obligately anaerobic rod that produces subterminal or subcentral spores (Hirsh D. *et al.,* 2004) *Clostridium chauvoei* requires strict anaerobic conditions and media rich in cysteine and water soluble vitamins (Dwight *et al.*, 2004)

Figure 2: *C. chauvoei* spores in a tissue smear. They are oval, central to subterminal and bulge the mother cell. The citron (lemon-shaped) forms are characterisitic (Gram stain x1000) (Quinn, 1994).

C. chauvoei and *C. septicum* are similar organisms and considered by some authors as the members of the same species (MacLennan, 1962; Willis, 1969). They are difficult to distinguish from each other on the basis of their physiological and toxigenic characteristics. The organisms are gram positive rods (often gram negative in older cultures). Both organisms ferment

glucose, fructose, lactose, maltose, and mannose. Both produce acetic and butyric acids as metabolic end products. It has been reported that long chains of bacilli or long filaments in the serous cavities and on the liver surface in infected animals are formed by *C. septicum,* but not by *C. chauvoei* (Heller, 1920; Willis, 1969).

The major distinction is the difference in the diseases they cause. These organisms were encountered early in the history of microbiology because of their involvement in diseases of cattle and sheep (Heller, 1920). In the middle of the 19th century, blackleg (Rauschbrand, Gerausch, charbon symptomatique) and malignant edema were confused with anthrax (Milzbrand, charbon). All three diseases under their various names were considered as variants of one and the same disease. Bollinger reported in 1875 that numerous short bacteria observed microscopically in tissues from cattle dying from Gerausch had no similarity what so ever with the filamentous anthrax bacillus. Arloing, Cornevin, and Thomas described the blackleg organism in some detail in 1880. The name *Clostridium chauvoei* was given in honor of the French veterinarian Auguste Chauveau (Willis, 1969).

C. chauvoei belongs to the histotoxic clostridia and it is the pathogenic agent of blackleg, is a disease characterized by emphysematous inflammation and sero-hemorrhagic content in large muscle masses, and it is classified as one of the gangrenes. It causes serious toxemia and high mortality in cattle, sheep and many other ruminants associated with spore contaminated soil. It is considered the most important clostridium producing economic losses in livestock (Smith and Williams, 1984; Corpus *et al.*, 2008). Animals other than ruminants are rarely infected. Young growing ruminants on pasture are especially sensitive to *C. chauvoei*. Survival of *C. chauvoei* in soil may be a

significant factor, since the disease occurs year-after-year on the same premises, usually in well-fed cattle less than 3 years old. These clostridia are normally present in the digestive tract of many animals, and enter the soil from the carcasses of animals that die of clostridial infection. The bacteria exist in high numbers in soil where cattle graze. This infection begins when the susceptible animal ingests the endospores. The endospores then cross over the gastrointestinal tract and enter the bloodstream. Endospores are deposited in tissue throughout the animal body. They lie dormant in the tissue until they become activated and trigger the disease. Under certain conditions such as bruising of muscle, these organisms begin to multiply, produce toxins and other antigenic components that may be highly fatal (Richey, 2004).

Conditions favoring spore germination, bacterial growth, and toxin production cause formation of local lesions marked by edema, hemorrhage and, myofibrillar necrosis. The centers of lesions become dry, dark emphysematous due to bacterial fermentation, while the periphery edematous and hemoragic. A rancid butter odor is typical (Hirsh D. *et al.*, 2004).

The mechanism of infection is not clearly understood, but ingestion is probably the most common route of exposure in cattle: various tissues, especially skeletal muscle, are seeded with spores from the intestine. The organism remains dormant until the muscle provides conditions that favor germination, multiplication, and toxin production (Floyd, 1994). Clinically, there is high fever, anorexia, and depression acute lameness and death. The onset is sudden. Animals may be found dead without premonitory signs.

1.4 Blackleg Vaccine

Since the disease is often rapidly fatal and usually affects cattle 6 months to 2 year of age, vaccination is accepted as cheap insurance. Commercial vaccines consisting of whole formalized cultures are used for controlling of the disease. These vaccines are generally presented as polyvalent formulations with other *clostridia* species (Crichton *et al.*, 1990). Intramuscular injection of clostridial vaccines causes significant damage to muscle so that blackleg vaccines are recommended to administer subcutaneously (under the skin) in the neck area. Injection in this area prevents injection site damage. Many clostridial vaccines require revaccination 4 to 6 week following the initial treatment (Compendium of Beef Products, 1993). These whole formalized culture vaccines are successful in protection of the animals.

Immunity of *C. chauvoei* is considered to be mainly somatic and flagellar antigens being the most widely studied for the vaccine development. Since blackleg is considered an enzootic disease, the incorporation of local strains with high immunoprotective capacity in vaccine formulations is encouraged (Chandler and Hamilton, 1975; Tamura *et al.*, 1984; Matter *et al.*, 2001).

The commercial vaccines must meet strict legal requirements stated by current pharmacopeia and manufacturing regulations. Current standards (British Pharmacopeia (Veterinary) 1985) for vaccines containing *C. chauvoei* require a potency test based on a challenge assay in guinea-pigs. Most veterinary clostridial vaccines are multi component, requiring assays in rabbits to test the potency of components other than *C. chauvoei*. Potency tests for vaccines against the exotoxin producing species of clostridia are based on measuring the antitoxic response induced by the vaccine in rabbits.

The individual antitoxins produced by the rabbits are assayed in mice by comparing the specific toxin neutralizing activity of the test sera with the neutralizing activity of reference sera of defined potency. As immunity to *C. chauvoei* is generally considered to be antibacterial rather than antitoxic, an assay of the type used to measure the response to an exotoxin is inappropriate. As a result, potency tests for vaccines containing *C. chauvoei* antigens are based on a challenge assay in guinea-pigs (Crichton *et al.*, 1990; Morris, 2005).

For the production of *C. chauvoei* vaccine, it is essential that a well characterized seed strain capable of yielding safe and immunogenic vaccine that meets the requirements as laid down in the British Pharmacopoeia (Veterinary), 1985, have been used. Organisms which grow during the course of infection in animals possess full antigenic components. This full antigenic pattern has been maintained as nearly as possible in any seed strain to be used for production of vaccine. Repeated passage of organisms in artificial medium results in loss of both virulence and antigenicity so keeping cultures within three passages in artificial media from the original seed obtained from infected cattle, sheep or guinea pigs is necessary. For this reason, a large stock of freeze-dried seed is prepared. Production of *C. chauvoei* vaccine is required to be based on seed-lot systems. It is crucial that the seed lots are subjected to a full range of control tests, i.e. identity, safety and potency, prior to use in vaccine production.

1.5 Virulence Factors of *C. chauvoei*

1.5.1 Flagella

Flagella are best known for their role in bacterial motility. In certain pathogenic bacteria, flagella also appear to play an important role in the interaction of the parasite with its animal host. Many studies on the flagella of *Vibrio cholerae* (Attride and Rowley, 1983), *Salmonella typhimurium* (Carsiotis *et al.*, 1984), *Pseudomonas aeruginosa* (Montie *et al.*, 1982) and *Campylobacter jejuni* (Morooka *et al.*, 1995) suggested that the flagella may be important as a virulence factor in the pathogenesis of infection caused by these organisms. Moreover, the studies of the flagella of *V. cholerae* (Yancey *et al.*, 1979) *and P. aeruginosa* (Montie *et al.*, 1987) suggest that flagella may elicit protective antibodies against the respective infections.

C. chauvoei is peritrichously flagellated. Moussa (1959) reported that *C. chauvoei* possessed a single O-antigen, and could be divided into two groups based on two H-antigens which were distinct from those of *Clostridium septicum*.

Over the past few years, it has been shown that the flagella of *C. chauvoei* are important as a protective antigen flagella and it has been considered as one of the protective antigens contained in the vaccine against blackleg (Tamura *et al.*, 1984).

1.5.2 Toxins

C. chauvoei produces at least five soluble antigens (toxins) that include an oxygen-stable hemolysin (alpha), a Dnase (beta), a hyaluronidase (gamma),

an oxygen labile hemolysin (delta) and a neuroaminidase (Cortinas *et al.*, 1999). These toxins and other factors have been associated with pathogenicity of *C. chauvoei*.

1.5.2.1 Hemolysin

Bacteria are known to produce hemolysin, which may lyse erythrocytes from various species of animals. Studies have shown that hemolysin-producing bacteria are more virulent to animals than hemolysin lacking bacteria (Cavalieri and Snyder, 1982; Smith and Linggood, 1971). It is well known that *C. chauvoei* produces hemolysin in the culture medium (Bruner and Gillespie, 1966). The hemolysin is suspected as one of the virulence factors of *C. chauvoei* infection in animals. Some of the biological characteristics were reported by Moussa (1958) and Kerrin (1934) that hemolysin of *C. chauvoei* was filterable, heat-labile, actively antigenic and can be precipitated by ammonium sulfate. Hang'ombe *et al.* (2006) reported the molecular mass of hemolysin, determined by SDS-PAGE, was found to be approximately 27 kDa. Hemolysis produced by the delta toxin occurs more rapidly than that produced by the alpha toxin (Hatheway, 1990).

1.5.2.2 Beta-toxin

The beta-toxin from this organism is a DNase. Princewill and Oakley (1976) found that the beta toxin is heat stable, activated or inhibited by metal ions, inhibited by chelating agents, and having precipitability with ammonium sulfate. The beta-toxin may be responsible for degenaration of muscle cell nuclei (Ramachandran, 1969).

1.5.2.3   Gamma-toxin

The gamma-toxin of *C. chauvoei* is a hyaluronidase, an enzyme that hydrolyzes glycosidic bonds between N-acetylglucosamine and glucuronic acid residues of hyaluronic acid which is the base substance in connective tissue. Bacterial hyaluronidases are produced by a number of pathogenic Gram-positive bacteria that initiate infections at the skin or mucosal surfaces. Gram-positive organisms capable of producing hyaluronidase include various species of *Streptococcus*, *Staphylococcus*, *Peptostreptococcus*, *Propionibacterium*, *Streptomyces* and *Clostridium*. Among the *staphylococci*, hyaluronidase production has been shown for pathogenic strains of *Staphylococcus aureus* and *Staphylococcus hyicus* subsp. *hyicus* (Skalka, 1985). Most, if not all, of these Gram-positive genera capable of elaborating hyaluronidase are able to cause infections initiated at a mucosal or skin surface of either humans or animals. Many pathogenic bacteria able to establish infections at the mucosal or skin surface produce the enzyme hyaluronidase. Since hyaluronate is a major constituent of the ground substance of most connective tissues, particularly the skin, hyaluronidase may be an essential component in enabling the spread of the pathogens from an initial site of infection (Hynes, and Walton, 1999) The role played by this toxin in the pathogenesis of blackleg is also undefined.

1.5.2.4   Neuraminidase

The neuraminidase of *C. chauvoei* removes sialic acid residues from glycoconjugates on cell walls of eucaryotic cells resulting in distruptions of the intercellular matrix. The molecular weight of C. *chauvoei* neuraminidase is 65 kDa and it is reported that native enzyme exist as a dimeric protein as 135 kDa protein (Useh *et al.*, 2006). It is reported that *C. chauvoei*

neuraminidase has the capacity to hydrolyse red blood cells and it could account for the hypovolemia observed in blackleg (Singh *et al.*, 1993; Useh *et al.*, 2006).

1.6 Proteomics

Proteomics is the study of the proteome, the protein complement of the genome. The terms "proteomics" and "proteome" were coined by Marc Wilkins and colleagues in 1995, mirror the terms "genomics" and "genome" and proteomics was defined as the large-scale characterization of the entire protein complement of a cell line, tissue, or organism.

The proteomics, now evokes not only all the proteins in any given cell, but also the set of all different proteins, the interactions between them, the structural description of proteins and their higher-order complexes (Tyers and Mann, 2003). Different proteins include not only the primary polypeptides created by the individual genes, but also all the co- and post-translational modifications of a protein. Different proteins can occur in different cell types and different subcellular compartments and there can be differences in protein composition and concentration for a distinct cell type at different life stages or during any functional process taking place in the cell. Therefore, it is clear that the proteome is far more complex than the genome (Gauss *et al.*, 1999). The terminus genome is "used to refer to all the genes carried by a single gamete" (King and Stansfield, 1990) and, it is static since 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 differs from one cell type to another qualitatively as well as quantitatively, and, moreover, depends on the course of time (Gauss *et al.*, 1999).

1.6.1 Why Proteomics?

Analyzing gene expression on the transcript level and extrapolating that data to the protein level is not sufficient. There are a number of reasons why gene sequence information does not provide a complete profile of a protein's abundance or its final structure and state of activity (Banks *et al.*, 2000; Celis *et al.*, 2000).

Verification of a gene product by proteomic methods is an important first step in "annotating the genome." Proteins are frequently the functional molecules and, therefore, they reflect differences in gene expression. Genes may be present, they may be mutated, but they are not necessarily transcribed. Some messages are transcribed but not translated, and the number of mRNA copies does not necessarily reflect the number of functional protein molecules (Anderson and Seilhamer, 1997). In addition, proteomics addresses problems that cannot be approached by DNA analysis, namely, relative abundance of the protein product, posttranslational modification, subcellular localization, turnover, interaction with other proteins as well as functional aspects (Celis *et al.*, 2000).

It is therefore not surprising that proteome analysis has become a key enabling technology in the emerging science of systems biology. Proteomics may be defined as the direct qualitative and quantitative analysis of the full complement of proteins present in an organism, tissue, or cell under a given set of physiological or environmental conditions. The advantage of proteomics lies in the ability to directly examine the biomolecules and assemblies of biomolecules that are most responsible for the function of biological systems.

1.6.2 Proteomic Tool

The proteomic technology is complex, and comprises a plethora of state of the art techniques to resolve (high resolution two-dimensional gels), quantitate (phosphorimager, special scanners), identify and characterize proteins (microsequencing, mass spectrometry), as well as to store (two-dimensional polyacrylamide gel electrophoresis (2D PAGE) databases; http://biobase/dk/cgi-bin/celis; http://expasy.hcuge.ch/ sprot/sprot-top.html) communicate and interlink protein and DNA sequence and mapping information (bioinformatics) (Celis *et al.*, 1998; Dunn, 2000; Humphery and Smith, 1997; Huber, 1997).

For many years, high resolution 2D PAGE has been the technique of choice for analyzing the protein composition of cells, tissues and fluids, as well as for studying changes in global patterns of gene expression elicited by a wide array of effectors (Dunn, 2000; Celis, 1984). The technique, which was originally described by O'Farrell (O'Farrell, 1975) and Klose (Klose, 1975), separates proteins both in terms of their isoelectric point (pI) and molecular weight and provides high resolution for protein analysis.

1.6.3 Steps in Proteome Work

1.6.3.1   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). It makes use of the property of proteins that their net charges are determined by the pH of their local environments. Proteins carry 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. Proteins show considerable variation in isoelectric points, but pI values usually fall in the range of pH 3-12. 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, proteins move toward the electrode with the opposite charge and the protein will either pick up or lose protons. As it does, its net charge and mobility will decrease and the protein slow down. Migration stops when protein will arrive at the point in the pH gradient equaling its pI. 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.

For many years the 2D PAGE technology relied on the use of carrier ampholytes (amphoteric compounds) to establish the pH gradient. Carrier ampholytes are small, soluble amphoteric molecules with a high buffering capacity near their pI. Carrier ampholytes are composed of hundreds of individual polymeric molecules with their pIs spanning a specific pH range. However, this technique has proven to be difficult because of the lack of reproducibility created by uncontrollable variations in the batches of ampholytes used to generate the pH gradients. Lately, with the introduction of immobilized pH gradients (IPGs) (Bjellqvist *et al.*, 1982; Görg *et al.*, 2000), which are integral part of the polyacrylamide matrix, it has been possible to

obtain focusing patterns that can be easily reproduced. IPGs avoid some of the problems associated with carrier ampholytes such as cathodic drift and endosmosis, allow a higher loading capacity for micropreparative runs, and provide increased charge resolution when narrow pH gradients (0.03 pH unit/cm) are used (Görg *et al.*, 2000, Corthals, 2000).

After the first dimension is completed, the second dimension is achieved on the base of protein size using sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE).

1.6.3.2   Protein Detection and Image Analysis

Protein detection and visualization following 2 Dimensional Electrophoresis (2-DE) are carried out using nonfluorescent stains such as Coomassie Brilliant Blue (CBB); although this method is quick and easy and also compatible with MS analysis, it has limited sensitivity (200-500 ng of protein per spot). Alternatively, silver staining is 100 times more sensitive than CBB and can detect up to 0.1 ng of protein per spot (Rabilloud, 1999). Fluorescent stains based on use of fluorescent compound are known to have much larger linear dynamic range and are, therefore, more sensitive than nonfluorescent detection methods.

1.6.3.3   Mass Spectrometry Analysis

Methods of protein identification have included immunoblotting, Edman peptide sequencing, amino acid composition and more recently the use of mass spectrometry. The latter technique, which relies on the comparison of peptide mass fingerprints, is fast and requires only picomol amounts of proteins (Celis *et al.*, 2000).

The basic principle of mass spectrometry (MS) is to generate ions from sample of a compound, to separate these ions by their mass-to-charge ratio (m/z) and to detect them qualitatively and quantitatively by their respective m/z and abundance. Mass spectrometers can be divided into three fundamental parts, namely the ionization source, the analyzer, and the detector. The sample has to be introduced into the ionization source of the instrument. Once inside the ionization source, the sample molecules are ionized, because ions are easier to manipulate than neutral molecules. These ions are extracted into the analyzer region of the mass spectrometer where they are separated according to their mass (m) -to-charge (z) ratios (m/z). Although there are few available techniques, a time-of-flight (TOF) is one of the simplest mass analyzer. It measures the m/z ratio of an ion by determining the time required for it to transverse the length of a flight tube. From the MS/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   Immunoproteomics of Pathogenic Bacteria

Immunoproteomics could be defined as the combination of any proteomic technology with an immunological data presentation. Its development is vital in an age where it is increasingly becoming urgent to identify disease biomarkers and pathogenic target antigens for the development of new drugs and vaccines (Steel *et al.*, 2005). Among the current proteomic techniques available, 2-DE has often been chosen as the working tool in immunoproteomic applications in combination with Western blot (Klade, 2002).

Immunoproteomics takes advantage of the fact that higher organisms contain a highly sophisticated immune system that has the ability to distinguish 'normal' (self) from 'abnormal' (nonself) proteins. Proteins that are regarded as nonself by the immune system are called antigens (antibody generators) and can be defined as proteins that bind to immune receptors and elicit an immune response (Alberts *et al.*, 2002). For diagnostic purposes, it is important that circulating antibodies reflect a molecular imprint of those antigens. The key issue in immunoproteomics is to visualize what is seen by the immune system during the diseases (Tjalsma *et al.*, 2007).

Vaccination of individuals has been practiced for many years and has been one of the most effective methods of controlling infectious diseases. Louis Pasteur, who developed the first vaccine against rabies, established in 1881 the basic paradigm for vaccine development, which included the isolation, inactivation and injection of the causative microorganism. Unfortunately, even the success of the vaccine production methods and studies, society continues to suffer multi-billion dollar economic losses annually due to infectious diseases. One of the reasons for that development of infectious diseases is that the antibiotic resistancy of the bacteria that were previously susceptible to the large armory of antibiotics (Hiramatsu *et al.*, 2001; WHO, 2001; Appelbaum, 2002; Walsh, 2003). The other reason is progressive manipulations and aggressive treatments which cause immunological defense suppressions and it makes the infectious more serious and more difficult to treat. The losses occur in all animal species as well as in humans. According to a report by the WHO, infections by microorganisms are globally responsible more than 15 million deaths each year. In order to further reduce these losses, the multidisciplinary approaches to develop better and safer vaccines are employed. The disciplines include on advances in

molecular biology, chemistry, pharmacy, immunology, genomics, proteomics, and fermentation techniques. In the last decade, a revolution in vaccine design has emerged from the use of post-genomic technologies (Serruto and Rappuoli, 2006; Khan *et al.*, 2006).

All existing vaccines are based on killed or live-attenuated microorganisms or subunit vaccines. The former two classes are based on the whole cells of the pathogen, while subunit vaccines based on selected antigens and their respective genes are recognized as the safest type, although their efficacy is lower than those comprised of whole attenuated cells. The important point in the development of effective subunit vaccines is the choice of the antigens. For more than 100 years, diverse strategies have been applied to select candidates for subunit vaccine construction. In the beginning of the 20th century, bacterial virulence factors were identified, mainly by biochemical approaches. In the second half of the 20th century, progress in the field of vaccinology was possible owing to application of new recombinant DNA technologies. Another step forward in microbiology and medicine took place at the end of 20th century in 1995, when the first bacterial genome (*Haemophilus influenza*) was sequenced. Presently, the complete genetic information of approximately 1000 bacterial strains is available according to Genomes Online Database (2009). Development of rapid sequencing methods combined with novel bioinformatics has revolutionized the study of bacterial pathogenesis and facilitated the development of a new strategy for identification of novel antigens, termed "reverse vaccinology". Proteomics, large scale analysis of cellular proteins, is a powerful tool for studying protein identification, localization, modifications, function and possible interactions or complexes they can form. By this time, proteome databases of diverse pathogenic microorganisms including *Mycobacterium tuberculosis*, *Helicobacter pylori*, *Salmonella enterica*, *Bacillus anthracis* and many others

were established. Much of information about immunogenic components can be derived from immunoproteomics which has been successfully applied for the discovery of antigens from various bacterial pathogens. Using proteomics and immunoproteomics as a supporting approach ensures the reduction of the number of vaccine candidates, indicating the most abundant and highly seropositive proteins for the future studies (Katarzyna *et al.*, 2009).

1.7.1 Immunoproteomic approaches to pathogenic Clostridia Species

Proteome maps of sequenced organism are important research tools for the authentication of hypothetical proteins, the identification of components of different cellular proteome fractions and for yielding information concerning the occurrence and abundance of proteins. Such proteome maps in the public domain have been generated for many pathogens and are of great value in identifying new virulence factors and the antigens of potential diagnostic and curative value against infections with pathogens. Despite a sudden spurt of activity towards proteomic characterization of bacterial pathogens, for unknown reasons, clostridia have largely been ignored. *C. difficile* and *C. perfringens* are the only clostridial species for which analysis of proteome and immunoproteome has been carried out to some extent so (Calabi *et al.*, 2002; Drudy *et al.*, 2004; Pecine *et al.*, 2005).

C. difficile, an intestinal pathogen, is frequently recovered from the hospital environment. Patients and hospital staff have been implicated as a major source of nosocomial acquisition (McFarland *et al.*, 1989). There are known virulence factors and main ones are the toxins A (TcdA) and B (TcdB), which are expressed by the bacterium in the gastrointestinal tract. These toxins mediate destruction of the integrity of the epithelial cell barrier and induce a

variety of physiological effects on intestinal cells (Just & Gerhard, 2004; Poxton *et al.*, 2001; Voth & Ballard, 2005). The other potential candidate proteins for these activities include the surface-layer proteins (SLPs) (Calabi *et al.*, 2001), flagella (Tasteyre *et al.*, 2000), the chaperone GroEL (Hennequin *et al.*, 2001), the fibronectin- binding proteins (Hennequin et al., 2003) and the adhesion protein Cwp66 (Waligora *et al.*, 2001). Recently, Wright *et al.*, (2008) focused on immunoproteomic study of *C. difficile* to identify such proteins that are immunoreactive in humans. Cell wall proteins were separated by 2-D PAGE, and immunoreactive proteins were revealed by reaction with patient sera. The identity of immunoreactive proteins was established by MS. Forty-two different proteins were identified in total. All patient sera reacted with at least one component of the surface-layer protein (SLP), four reacted with both components (high- and low-molecular-mass SLPs), and five reacted with one other cell wall protein, suggesting that these are immunodominant antigens (Wright *et al.*, 2008). In further studies, the role of these proteins as potential vaccine candidates and their roles in pathogenesis should be investigated.

C. perfringens is a medically important clostridial pathogen and an etiological agent, causing several diseases in humans and animals; the former includes gas gangrene, food poisoning, necrotizing enterocolitis of infants and enteritis necroticans (MacLennan, 1943; Titball and Rood, 2001). A total of 22 surface-localized proteins and 10 cell envelope proteins were identified by proteomic analysis of *C. perfringens* ATCC 13124. In the study, two media, Cook Meat Media (CMM) and tryptose-yeast extract-glucose medium (TYGM), were compared and growth on CMM resulted in over expression of several proteins of which 11 most prominent ones have been identified. Choloylglycine hydrolase family protein (SP2), glutamate synthase (SP3), sucrose-6-phosphate dehydrogenase (SP4), and ornithine

carbamoyltransferase (SP15) were found to be immunogenic cell surface proteins as revealed by the Western blot analysis of 2-DE separated surface proteins. Ornithine carbamoyltransferase identified in this study can be putative vaccine candidates as it was over-expressed in CMM grown cells. The latter is immunogenic, and their homologs in other pathogenic bacteria have been shown to be immunogenic/virulence factor. In addition phosphoglycerate kinase, N-acetylmuramoyl-L-alanine amidase, and translation elongation factor Tu and EF-G could also be putative vaccine candidates as they were abundant on the cell surface fraction and their homologs in other Gram positive pathogenic bacteria have been shown to be immunogenic/virulence determinants (Alam *et al.*, 2009).

1.8 Aim and Scope of the present study

The aim of the present study was to characterize immunogenic proteins of *C. chauvoei* and investigate novel immunogenic proteins by using 1-DE and 2-DE followed by immunoblot techniques and MALDI-TOF MS analysis. In order to characterize these proteins, the optimum growth medium composition and corresponding growth curve in this medium was determined. After immunization of the mice with the inactivated whole cell culture in optimized growth media, sera were obtained for the evaluation of antibody response. The first immunoproteome map of *C. chauvoei* was constructed with the aim of providing further insight into the immunogenicity of this pathogen and improvement of new vaccines.

CHAPTER 2

MATERIALS AND METHODS

2.1 Bacterial Strains and Maintenance

The strain of *C. chauvoei* ATCC 11957 which was isolated from blackleg sheep and *C. chauvoei* 20 which was obtained from Pendik Veterinary Institute were used in this study. The strains were kindly provided for this study by Dr. Erkan Özcengiz (VBR Vaccine Res. Co., Ankara). For long term maintenance, the bacteria grown in Modified Clostridia Medium, M1-2-V, in an anearobic jar (appendix A) for 24 h were preserved in 20% glycerol, at -80°C.

2.2 Culture Media

The composition and the preparation of the culture media are given in Appendix A.

2.3 Buffers and Solutions

Buffers, solutions, their composition and preparation are listed in Appendix B.

2.4 Chemicals and Enzymes

The chemicals and their suppliers are listed in Appendix C.

2.5 Culture Conditions

C. chauvoei strains were cultured in our improved Clostridial medium, M1-2-V. The organism was incubated as pre-culture and grown overnight at 37°C in an anaerobic jar. Then, it was inoculated to sub-culture for 16 h under the same conditions. The day after, the organism was inoculated freshly to a 2-L fermentor (Sartorius BBI Systems, Melshungen, Germany) with 1.5 L of culture medium which was autoclaved at 121°C for 30 min. The B solution of the medium and vitamin solution were passed through filters (0.2) µm for sterilization and added shortly before the inoculation. The percentage of the inoculum was 1% (v/v). Culture was incubated at 37°C with free pH evolution after adjusted to pH 7.5 by using 2.5 M NaOH. pH was observed from the display screen of the fermentor. The anaerobic conditions required for growth of *C. chauvoei* was met by nitrogen tubes. Culture growth was monitored by optical density determinations at 630 nm (OD_{630}) with spectrophotometer (Hitachi, Japan).

2.6 Hemolytic Assay

C. chauvoei strains were cultured in the clostridia medium, M1-2-V in the fermentor. Starting from the growth, necessary amount of culture sample was taken for both hemolytic assay and optical density observation at every two hour till the end of the second day. After each sampling, the culture was centrifuged at 9000 rpm at 4°C for 20 minutes and the supernatant was

stored at 4°C. At the end of culturing in the fermentor, all samples were used in hemolysin test.

A mouse was bled for the test. The blood was taken to 0.1 ml %3.8 citrate solution to prevent coagulation of the blood. The blood sample was washed 2 times with phosphate-buffered saline (PBS; 10 mM NaHPO4 [pH 7.2], 5 mM KCl, 145 mM NaCl, 0.1% glucose) by centrifugation at 3000 rpm for 5 minutes at room temperature. Then, 0.1 ml of blood pellet was taken and diluted in 10 ml PBS, which became 1 % erythrocyte solution.

Hemolytic assay test was performed as described by Ballard *et al.,* 1991 and Hang'ombe *et al.*, 2006 with slight modifications. The sample was diluted with PBS into a final volume of 200 µl in the first tube, and to the remaining tubes, 100 µl of PBS was added. Serial 1:2 dilutions were performed by removing 100 µl of the first dilution, adding it to 100 µl of PBS in a subsequent tube, and mixing thoroughly. This was repeated until 7 to 8 dilutions had been made. To each tube, 100 µl of an erythrocyte solution was added, and the tubes were incubated at 37°C for 2 h and then centrifuged at 1000 rpm for 5 min at room temperature. The absorbance of the supernatant was measured at 595 nm. PBS and 1% Triton X-100 solution were used as control. In PBS, there have to be no hemolytic activity, so supernatant of this tube was used as blank. On the other hand, 1 % Triton X-100 has 100 % hemolytic activities in mouse erythrocytes. Results were evaluated as positive and negative according to blank and the highest dilution that gave positive value accepted as hemolytic unit (HU) of the sample.

2.7 Preparations of Protein Samples for 1-DE, 2-DE and ELISA Studies

2.7.1 Whole Culture Lysate

C. chauvoei strains were cultured in M1-2-V medium. Cultures were diluted 1:1 in rehydration buffer. Samples were vortexed 5 min and they were put into ice. These were repeated for 3 times. The protein concentration was measured and protein samples were stored at -20 °C. These protein samples were used in 1-DE SDS-PAGE studies.

2.7.2 Sonication

Sonication was performed as described by Mattar *et al.*, 2001 with slight modifications. *C. chauvoei* was cultured in M1-2-V medium. Cultures were centrifuged at 9000 rpm at 4°C for 20 min and washed twice with phosphate saline buffer (PBS). Then the cells were disrupted using a Vibra Cell sonicator (Cole-Parmer Ultrasonic Proccessor,USA), for 4 min at 20 kHz in a discontinuous way (50% duty cycles) while being cooled in an ice-water bath. Unbroken cells were removed by centrifugation at 13000 rpm for 20 min. The protein concentration was measured and protein samples were stored at -20°C. These protein samples were used in ELISA studies.

2.7.3 Whole Cell Extraction

Protein extraction was performed as described by Altındiş *et al.*, 2008 with slight modifications. For the preparation of whole cell lysates, the cells were collected and suspended in cold TE buffer and spinned at 9000 rpm for 20 min at 4°C in a centrifuge. The pellet was resuspended in 0.5 ml 0.04 M

lysosyme in TE buffer and incubated at 37°C at water bath for 35 min, followed by a centrifugation at 13000 rpm for 20 min at 4°C. The pellet was solubilized in 8M urea, vortexed at 4°C for 30 min. Unbroken cells were removed by centrifugation at 23000 rpm for 1 h at 4°C. The protein concentration was measured by using modified Bradford method. These protein samples were used in 2-DE SDS-PAGE studies.

2.8 Protein Estimation

To determine total protein concentrations, the Bradford assay described by Ramagli and Rodrigez (1985) was used. The Bradford assay, a colorimetric protein assay, is based on an absorbance shift in the dye Coomassie when the previously red form coomassie reagent changes and stabilizes into coomassie blue by the binding of protein. 1 mg/ ml Bovine Serum Albumin (BSA) protein is used to prepare calibration curves. Bradford stock reagent was diluted 1:5 volume and filtered by using Whatman No.1 filter paper. Standards and samples were prepared as shown in Table 2 in duplicate. Blank was prepared by using buffer that protein solubilizes in. Spectroscopic readings were done at 595 nm and calibration curve of absorbance versus protein concentration in microgram was drawn.

Table 2. Preparation of Standards for Bradford Assay

BSA	dH$_2$O	Bradford 1X
2µl	98µl	900µl
4µl	96µl	900µl
6µl	94µl	900µl
8µl	92µl	900µl
10µl	90µl	900µl
Sample	**dH$_2$O**	**Bradford**
2µl	98µl	900µl
Blank	**dH$_2$O**	**Bradford**
2µl	98µl	900µl

For 2-DE studies, Bradford assay was slightly different. While extracted proteins were solubilized in 8 M urea, calibration curve was prepared by using a mixture of BSA and 8 M urea in 1:1 ratio and the 8 M urea was used as blank.

2.9 1-D gel Electrophoresis and Western Blotting

A vertical mini electrophoresis apparatus was used for SDS-PAGE. SDS Polyacrylamide gel was prepared as shown in Table 3. Proteins to be loaded were diluted 3:1 ratio in a 4X Laemmli sample buffer and put into heat block at 90°C for 1 min. Samples were loaded 20 µg/well to the gel and run in running buffer at 16 mA until loading dye reaches end of the gel. Pre-staining protein marker (Fermentas) was loaded 3 µl to a well.

Table 3. SDS Polyacrylamide Gel Preparation

	Stacking Gel 0.125 M Tris, pH 6.8	**Separating Gel** 0.375 M Tris, pH 8.8
Monomer concentration	4.5%	12%
Acrylamide/bis	1.3 ml	4ml
dH$_2$O	6.1 ml	3.35 ml
1.5 M Tris-HCl, pH 8.8	-	2.5ml
0.5 M Tris-HCl, pH 6.8	2.5 ml	-
10% (w/v) SDS	100 µl	100 µl
10% Ammoniumpersulphate (fresh) TEMED	50 µl 10 µl	50 µl 5 µl
TOTAL MONOMER	10 ml	10 ml

SDS PAGE Gels were stained either by Coomassie Blue G-250 (Neuhoff *et al.*, 1988) or Silver Staining method (Switzer *et al.*, 1979). For coomassie staining, the gel was removed from the cassette and placed into a tray containing fixing solution. The gel was soaked in this solution at least 1 hour. Then, the gel was soaked in appropriate volume of freshly prepared Coomassie Blue G-250 stain after electrophoresis. The gel was put in 200 ml CCB dye solution then 50 ml methanol was added and the gel was kept in this solution for 24 to 48 h. The gel was washed with dH_2O until protein spots are clearly visible.

For silver staining, the gel was removed from the cassette and placed into a tray containing appropriate volume of fixing solution. The gel was soaked in this solution at least 1 h. Then, the fixative solution was discarded and the gel was washed in 50% ethanol for 20 min. This step was repeated at 3 times. After washing step, enough volume of the pretreatment solution was added and gently rotated for 1 min. The gel was washed twice, 20 s each time in distilled water. Then, impregnate solution was added and gel was shaked for 20 min. The gel was washed twice, 20 s each time in distilled water. The gel was rinsed shortly with the developing solution. The reaction was terminated with stop solution as soon as the desired intensity of the bands was reached. The solutions are listed in the Appendix B.

Western blotting was performed as described by Towbin *et al.*, 1979 with slight modifications. Whatman (3D) papers and nitrocellulose sheet (Biorad) cut to the size of SDS-PAGE gel were prewet in transfer buffer and placed onto semidry gel electrophoresis apparatus (Cleaver Scientific, England). 6 whatman papers were put onto bottom. Then, nitrocellulose membrane sheet was put and non-stained gel was placed onto it. Finally, 6 whatman papers were the upper side. Proteins were transferred onto nitrocellulose

membrane sheet at 1.5 µA / cm^2 for 1.5 h. The nitrocellulose sheet was incubated 2 h at 37 C° in 10% skim milk for blocking purposes to prevent non-specific binding of serum. After blocking, nitrocellulose sheet was washed 3 times in TBS with 0.5% Tween-20 (TTBS). Membrane was then incubated for 1 h at room temperature in Mouse anti-*C. chauvoei* serum diluted (1:200) in 5% skim milk with constant shaking. After this incubation, membrane was washed 3 times (5 min) in TTBS and incubated 1 h at room temperature with Alkaline Phosphadase conjugated anti-mouse IgG (Sigma St. Louis, MO, USA) which is diluted (1:15000) in 5% skim milk. Then, membrane was washed 3 times (5 min) in TBS solution. Biorad AP Substrate kit was used for detection of proteins colorimetrically. Membrane was incubated in substrate solution prepared according to the manufacturer's instructions 10-40 min until protein bands are visible. Color development was stopped with dH$_2$O.

2.10 Preparation of *C. chauvoei* Vaccines

A monovalent vaccine contained the formalin treated whole culture of both *C. chauvoei* strains were prepared. *C. chauvoei* strains were cultured in 2-L fermentor as mentioned before and 400 ml culture samples were taken inactivated by adding formaldehyde in a percentage of % 0.4 and incubated at 37°C for a week. Inactivity and sterility tests were applied by inoculation of inactive cultures to the Thioglycolate medium (BD), Nutrient Broth medium (NB) and Nutrient Agar medium (NA) for 3 days. No growth meant that the cultures were sterile and inactivated. Then, formalin inactivated whole cultures were concentrated about 20 times by using 10 kDa cassette in cross flow apparatus (Sartorious, Germany). These concentrated cultures were adsorbed aluminium hydroxide gel (alhydrogel) as adjuvant in a certain ratios. Vaccines were stored at 4°C.

2.11 Immunization for ELISA and Immunoblot Analysis

Five, 18-20g BALB/c mice were immunized twice subcutaneously with 0.5 ml of prepared vaccines at 2-week intervals. Two weeks after the final immunization, all mice were bled and blood samples were collected. These blood samples were centrifuged at 4000 rpm for 10 min and sera were collected, pooled and stored at -20°C. These sera were used in ELISA and immunoblot analysis.

2.12 ELISA

ELISAs were performed in 96-well micro-ELISA plates (Costar, Cambridge, MA, USA). Wells of ELISA plates were coated overnight at 4°C with 200 μl per well of sonicated proteins at a concentration of 20 μg ml^{-1} in bicarbonate buffer pH 9.6. The wells were washed 3 times with saline containing 0.05 % Tween 20, and then 100 μl of serum was serially diluted with PBS containing 2% fetal calf serum and 0.05% Tween 20, starting from 1:50. The sera were allowed to incubate for 2 h at room temperature. After being washed, the wells were incubated with 100 μl Alkaline Phosphatase conjugated anti-mouse IGg (1:1000 dilution in same diluent as mouse sera) (Sigma St. Louis, MO, USA) for 2 h at room temperature. The wells were washed and incubated with 100 μl of substrate solution kit (Biorad) for 30 min at room temperature in the dark and color development was terminated with 50 μl 1M NaOH solution. The optical density (OD) of each well was observed with micro plate reader (Rayto, Germany) at 490 nm. Negative reference serum of mouse was used as a reactivity control.

2.13 2D-PAGE and 2-DE Western Blotting

IPG strips were passively rehydrated by applying 380 µl of rehydration buffer (8M urea, 2M thiourea, 1% w/v CHAPS, 20 mm DTT and 0.5% v/v ampholyte 3-10) containing 400 µg protein sample for 15 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. Strips were covered with glycerol and wet filter papers were put into the IEF chamber and these papers were changed at least 2 times to prevent the drying of the gel. The following voltage profile was used for IEF: 5 h 50 V; 5 h 70 V; 1 h 100 V; 1h 300 V; 1h 600 V; 2 h 1000 V; 2 h 3000 V; 2 h 5000 V followed by a linear increase to 8000 V. The final phase of 8000 V was terminated after 50 000 Vh. The IPG strips were equilibrated for 30 min each in 5mL of solution 1 (6M urea, 50mM Tris-HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 50mg DTT) and then in 5mL of solution 2 (6M urea, 50mM Tris-HCl (pH 8.8), 30% v/v glycerin, 2% w/v SDS, 225mg iodacetamide. In the first equilibration step, DTT (1%) is added to the equilibration buffer for proper unfolding of proteins, and iodoacetamide (4%) during the second step to remove excess DTT held responsible for "point streaking" during silver staining (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 25 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.

2.14 Dot blot

The immunoreactivity of each spot that gave positive signal in Western blot analysis was verified through their excision from 2-DE gels followed by dot blotting. Each spot was cut by the help of a tip of micropipette and incubated overnight with 200 µl destaining solution in the V-bottomed micro titer plates with constant shaking. Spots were tested dublicately. After removing destaining solution, the spots were washed with distilled water for 10 min. 200 µl 10 % SDS was adding to each well after removing water and plate was shaked for 10 min. Then, spots were put into the nitrocellulose membrane for transfer. The procedure used for dot blotting after transfer was the same as that used for Western blotting.

2.15 Evaluation of 2-DE Data

Coomassie stained gels were digitized by using a scanner (HP Scanjet 4070 Photosmart scanner, USA). Spot pattern analyses were accomplished by using the 2-DE image analysis software Delta2D version 3.4 (Decodon, Germany).

2.16 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.

2.17 Database Searches

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. The spots were searched in NCBI database with the help of MASCOT software.

CHAPTER 3

RESULTS AND DISCUSSION

3.1 Cultural Growth

The cultural growth optimization was made for the *C. chauvoei* in a fermentor system. Growth of *C. chauvoei* was monitored during a time course of 48 h. Fermentation was at 37°C with an agitation rate of 250 rpm. Cultures were incubated without pH control after the medium pH was adjusted to pH 7.5 before the inoculation since the growth was much poorer when the pH was controlled. While *C. chauvoei* produces acetic and butyric acids as metabolic end products during growth, the pH of the culture dropped drastically during log growth time until stationary phase and then remained constant with a final pH 4.8 and 5.0. Decrease in pH is a display of growth due to acidic byproducts released by *C. chauvoei*. The pH of the medium which is left uncontrolled during fermentor studies was used to follow up bacterial growth in another measure. The relationship between bacterial growth and decrease in pH is shown in Figure 3.

The growth was exponential for about 5-8 h but its start point was dependent on the lag phase which could last for 12 hours. It was important that *C. chauvoei* cells from a logarithmic-phase culture were used for inoculation and it should not be exposed to oxygen before inoculation to

fermentor. Maximum growth was attained in late logarithmic phase. The culture was then lysed, followed by a long stationary phase.
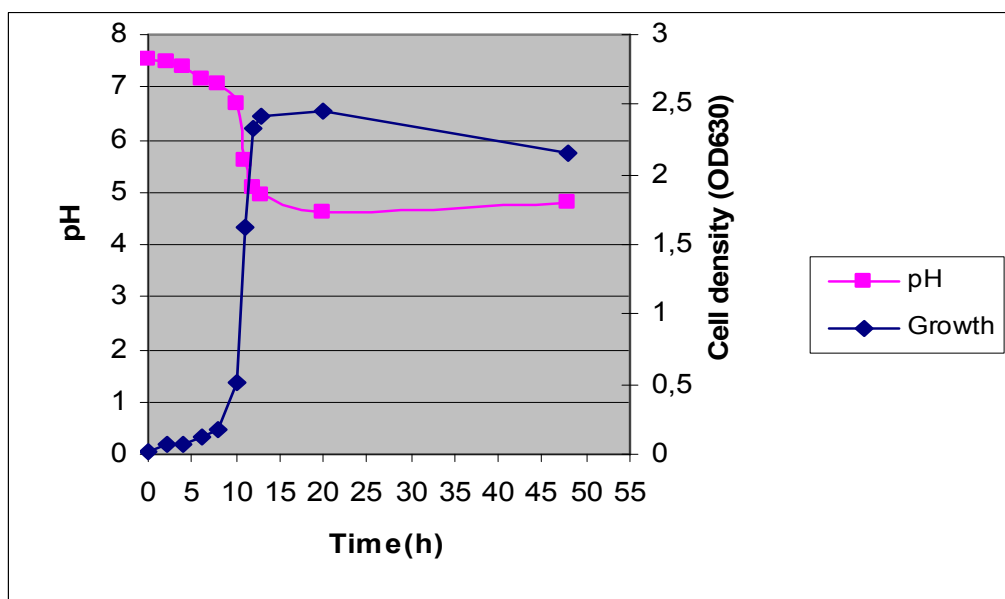
Figure 3: The course of growth (◊) of *C. chauvoei* ATCC 11957 and pH change (□).

3.2 Hemolysin Activity

Hemolysin activity was measured from cell-free supernatants at intervals between 2 and 72 hours. Highest titres were observed at late logarithmic phase corresponding to 10 to 12.5 h of incubation (Figure 4). The activity sharply declined and no hemolytic activity was observed after 48 h which was the late stationary phase. However, as based on the 1-DE Western

blotting data in Figure 4, we observed hemolysin (27 kDa protein) in all 3 days in whole culture lysate of *C. chauvoei*. Therefore, we concluded that the production of hemolysin activity in the *C. chauvoei* culture mainly occurred during the log phase growth and terminated at the transition from log to stationary growth phases, however the protein is not degraded, but it only looses its activity during the late stationary phase.
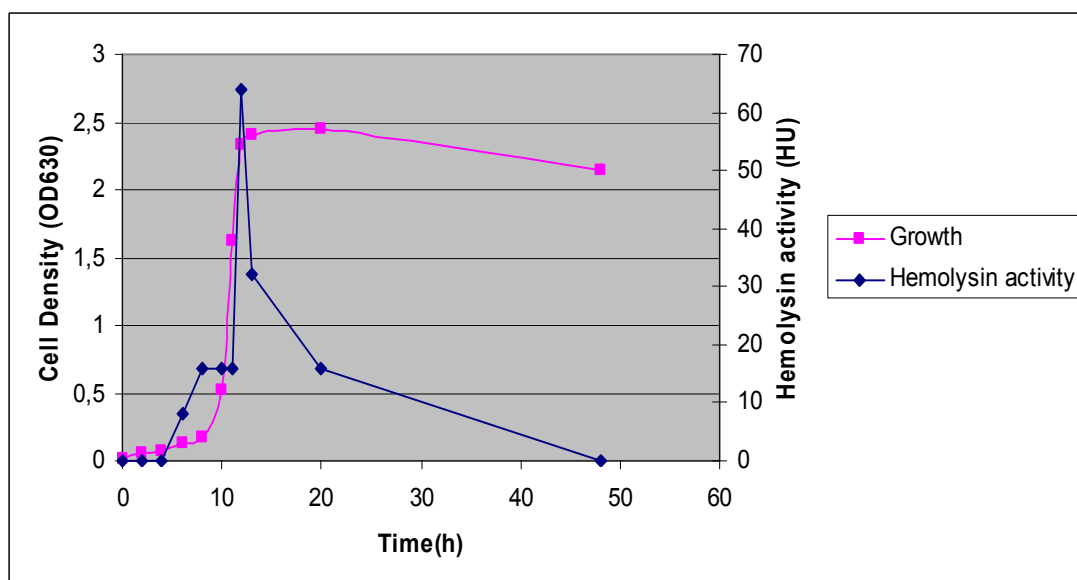
Figure 4: Growth of *C. chauvoei* and expression of hemolysin activity as functional of time.

3.3 Antibody Response

To evaluate the IgG levels of the sera of mice immunized with *C. chauvoei* vaccines, ELISA tests were performed as mention in Section 2.12. Sonicated cells of both *C. chauvoei* ATCC 11957 and 20 strains were used as coating antigens and sera collected from animals immunized with experimental vaccines of ATCC 11957 and 20. Each serum was reacted with its own antigen and cross reactivity was also determined. According to the results that are shown in Figure 5, immunized sera had a high titer of IgG. Therefore, it was demonstrated that these sera were appropriate for 1-DE and 2-DE immunoblotting analyses of this study.
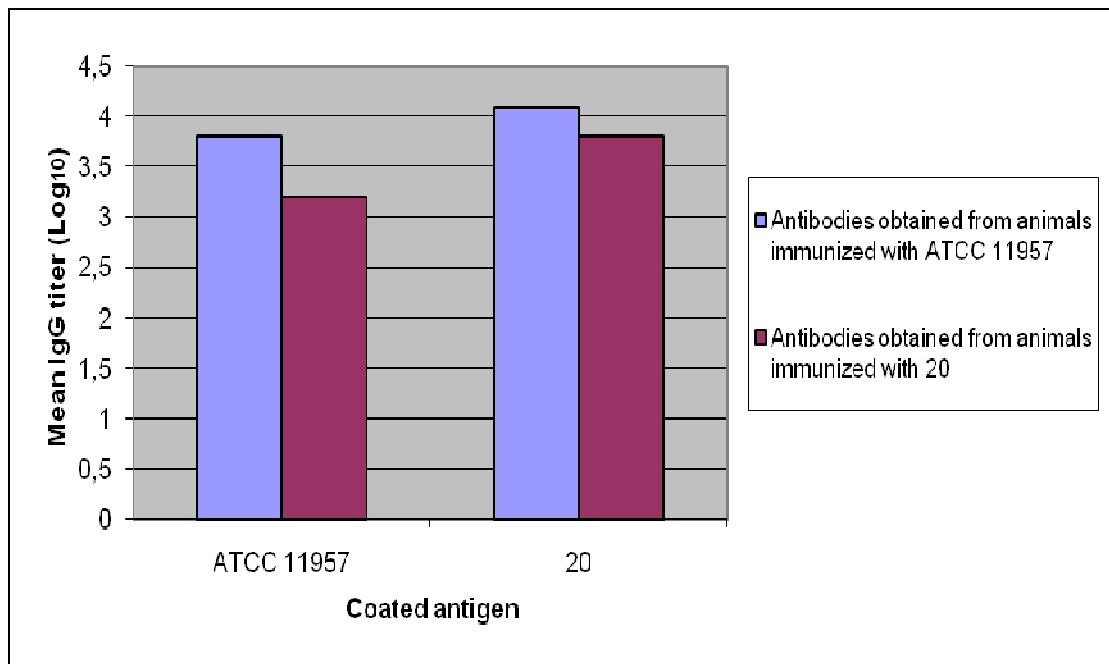
Figure 5: IgG levels induced by *C. chauvoei* strains as determined by ELISA tests when mice were immunized with experimental vaccines of strains ATCC 11957 and 20.

3.4 Protein Profiles in 1-DE and Immunoblots

Before 2-DE analyses, the 1, 2, and 3 days whole culture lysate samples of two strains subjected to SDS-PAGE and immunoreactivity in Western blotting. The protein profiles, analyzed by one dimensional, Silver stained SDS-PAGE gels (Figure 6-A and 7-A) showed high number of protein bands (20-25) that ranged from 150 to 15 kDa in size. The protein profiles of the two *C. chauvoei* strains were similar. Also protein profiles of the cultures of different age were almost the same. In addition, Figure 9 shows protein pattern of whole culture lysates of *C. chauvoei* ATCC 11957 strain at different

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times during culture. Samples were loaded in equal volumes (20 µg) to each well, but the protein amounts were not calibrated. Therefore, the evaluation of this data was only qualitative. There were not exactly reflecting any significant differences in protein profile at different hours of the culture.

Determination of immunoreactivity of proteins, Western blotting was performed for both strain extracts. Serum that obtained from immunized mice was used. In addition to homolog reactivity, cross reactions between two strains were observed. Figure 6 and 7 show the results of Western blotting by using *C. chauvoei* ATCC 11957 immunization serum. Figure 8 shows the Western blot analysis result of two strains extracts by using *C. chauvoei* 20 immunization serum.

The 46 kDa flagella protein, known to be immunogenic (Tamura et al., 1984; Tamura and Tanaka, 1984), verified in our 1-DE Western blot analyses for both strains. Besides that, 27 kDa hemolysin protein, suspected to be a major virulence factor (Hang'ombe *et al.*, 2006), also appeared 1-DE Western blot analyses for both strains (Figure 6-B, 7-B and 8). Moreover, six bands of relative molecular masses of 135, 130, 105, 91, 70 and 60 kDa were recognized by sera. In previous studies, Matter *et al.*, (2001) showed that 130, 91 and 72 kDa proteins of *C. chauvoei* 10092, 8, 5078 and 17 strains were immunogenic. We have also found immonogenic proteins with similar sizes in *C. chauvoei* ATCC 11957 and 20 strains in our Western blot analyses. These proteins should further be identified to clarify their functions in pathogenicity of *Clostridium chauvoei* strains.
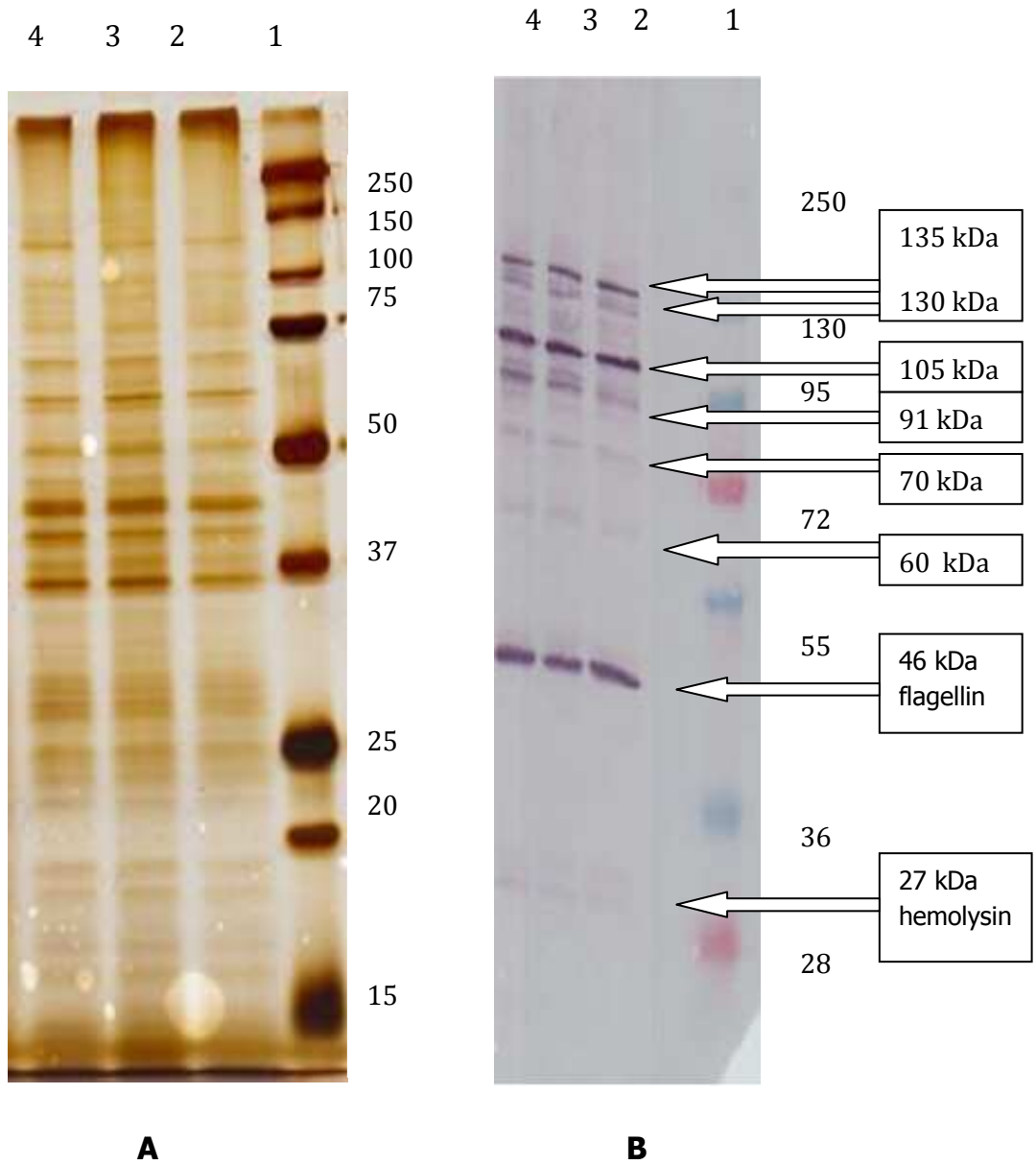
Figure 6: Silver-stained 1-DE SDS-PAGE gel of whole culture lysate of *C. chauvoei* ATCC 11957 (A) and Western blot analysis of whole culture lysates of *C. chauvoei* ATCC 11957 by using serum obtained from mice immunized with *C. chauvoei* ATCC 11957-1 day grown formalin inactivated whole cell vaccine (B). For A and B; the lane 1 represented the prestained marker and the lanes 2 to 4 represented 1, 2, and 3 days cultures of *C. chauvoei* ATCC 11957, respectively.
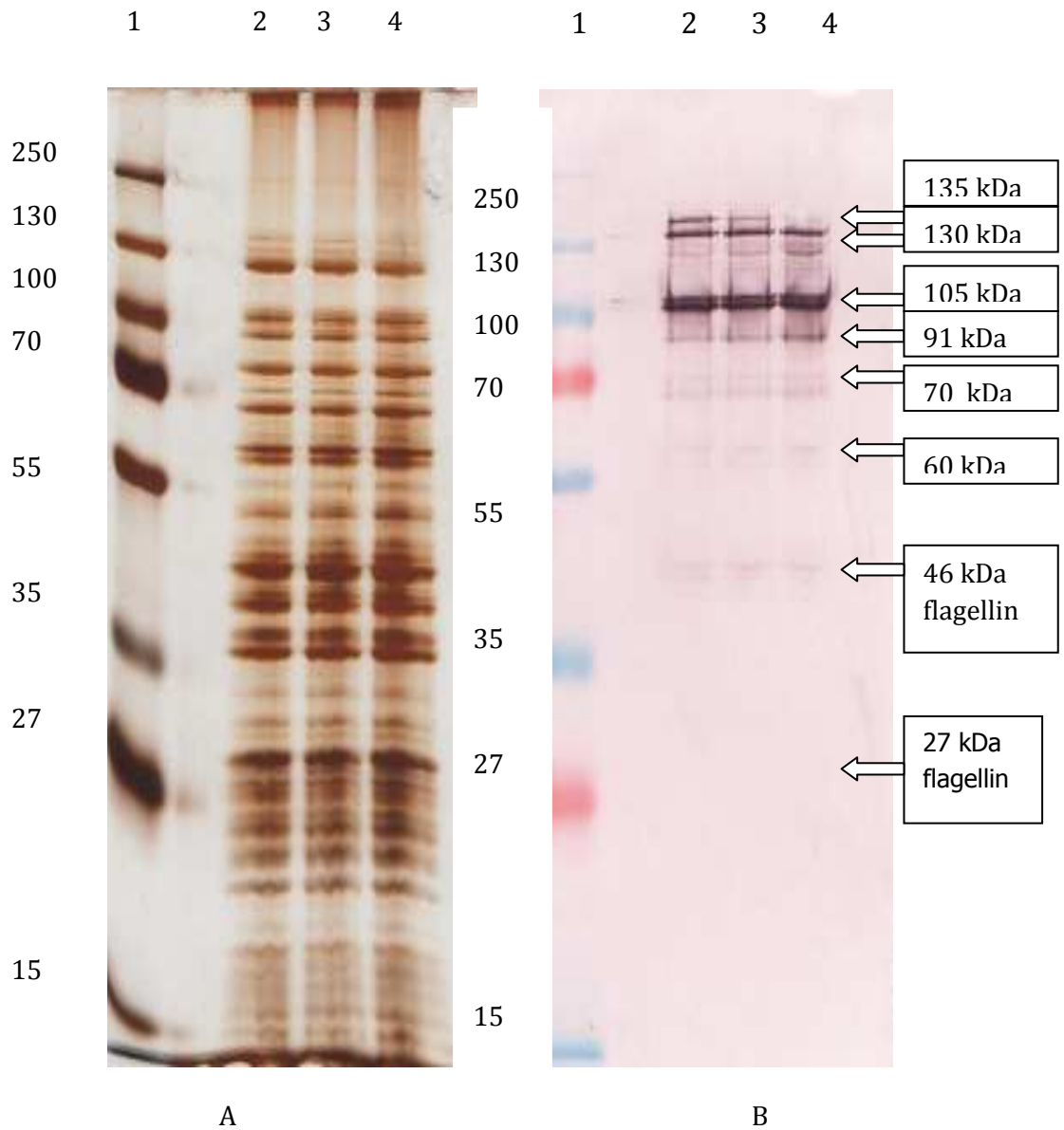
Figure 7: Silver-stained 1-DE SDS-PAGE gel of whole culture lysate of *C. chauvoei* 20 (A) and Western blot analysis of whole culture lysate of *C. chauvoei* 20 by using serum obtained from mice immunized with *C. chauvoei* ATCC 11957-1 day grown formalin inactivated culture vaccine (B). For A and B; the lane 1 represented the prestained marker and the lanes 2 to 4 represented 1, 2, and 3 days cultures of C. chauvoei 20, respectively.
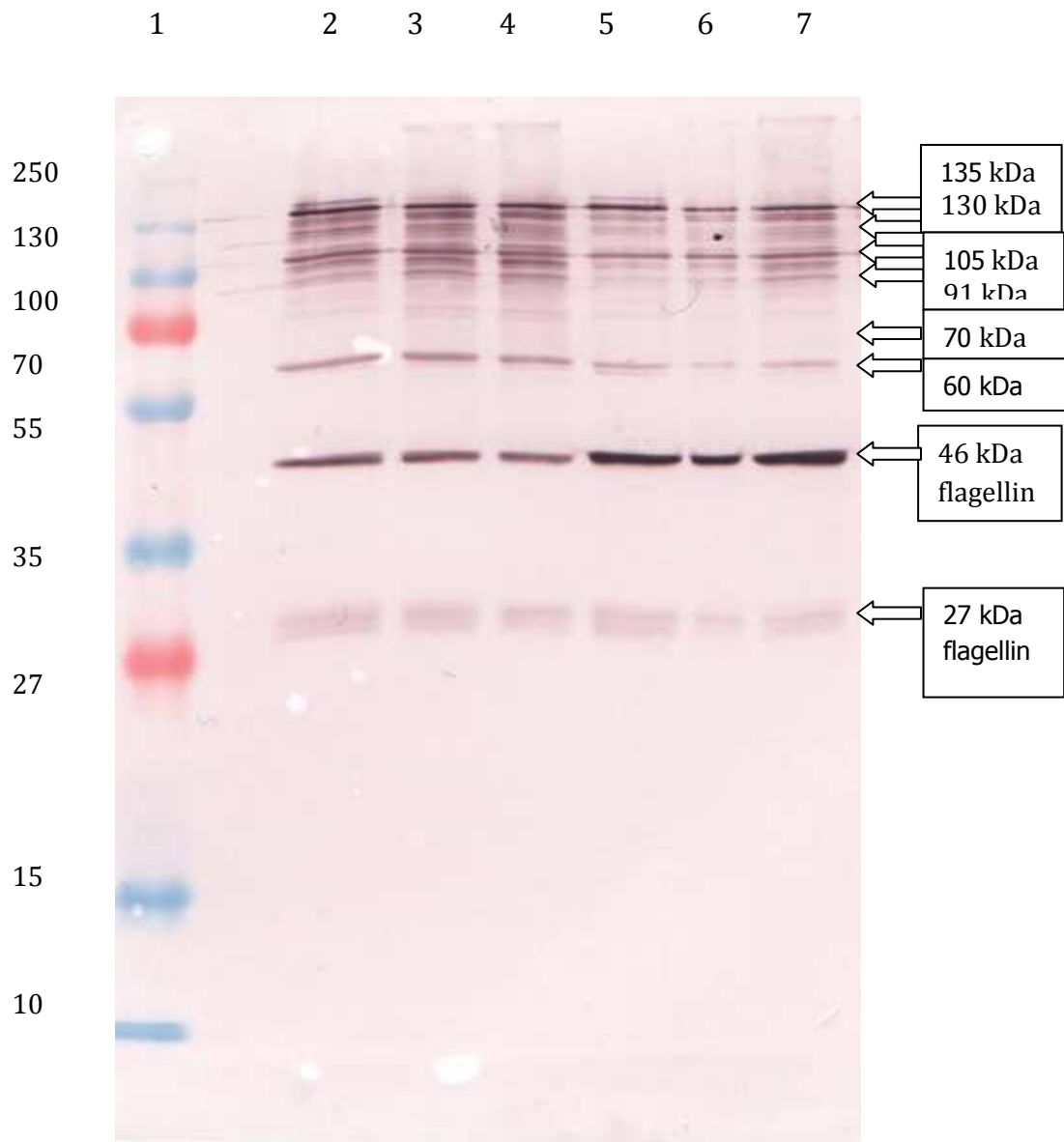
Figure 8: Western blot analysis of whole culture lysates of both *C. chauvoei* ATCC 11957 and *C. chauvoei* 20 by using serum obtained from mice immunized with *C. chauvoei* 20-1 day grown formalin inactivated whole cell vaccine. The lane 1 represented the prestained marker. The lanes 2 to 4 represented 1, 2, and 3 day cultures of *C. chauvoei* ATCC 11957, respectively. The lanes 5 to 7 represented 1, 2, and 3 day cultures of *C. chauvoei* 20, respectively.
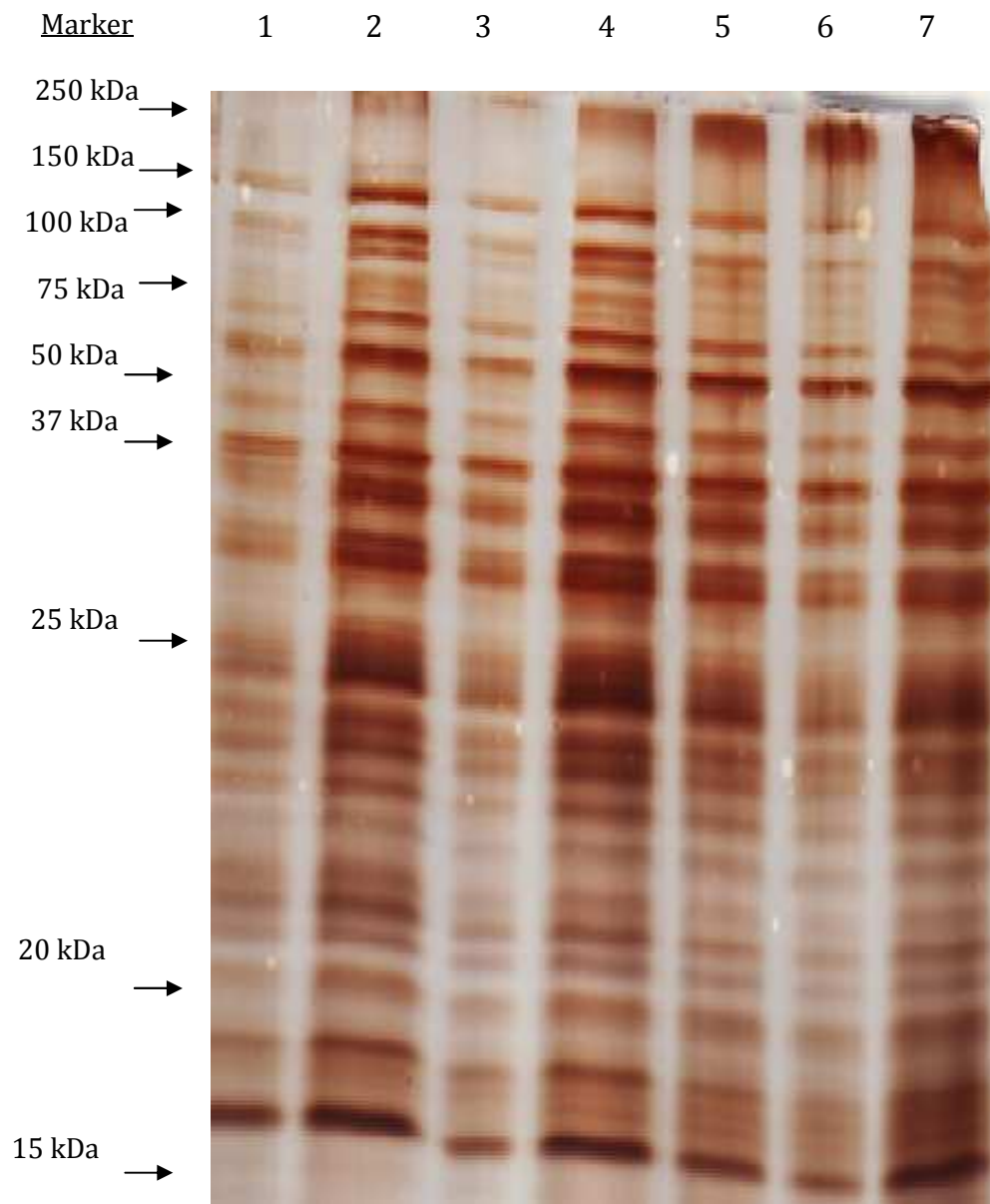
Figure 9: Silver-stained SDS-PAGE gel of whole culture lysates of *C. chauvoei* ATCC 11957 at different times during cultivation. The lanes 1-7 represented 8, 10, 12, 14, 20, 24, 48 and 72 h whole culture lysates, respectively.

3.5 Preparation of Proteome Samples for 2DE

One of the most important steps in the 2-DE PAGE technology is preparation of proteome samples. Due to the diversity of proteome samples, the best combination of steps and reagents must be determined empirically for each sample type. Whole cell extracts of *C. chauvoei* was obtained by both sonication method detailed in section 2.7.2 and whole cell extraction method of Altındiş *et al* (2008) as detailed in part 2.7.3. It has been demonstrated, in previous studies, which the protein profiles from sonicated cells showed less bands in 1-DE SDS-PAGE gels than whole cell (Chandler and Gulasekharam, 1974). Similarly, better pattern and better focusing of proteins were achieved by whole cell extraction method of Altındiş *et al.*, 2008 than sonication method in our study. In this method, the protein samples must be freshly prepared and should be no warmer than $30^{\circ}C$ to avoid carbomylation reaction which causes incorrect results in MALDI-TOF analysis due to urea (Westermeier *et al.*, 2002). Even by using this method, there were some focusing problems which appeared as horizontal streaks in 2DE gels. Commonly, 2-DE experiments begin with extraction into a solution containing at least 7 M urea. Solubilization of bacterial surface (cell wall and membrane-associated proteins) for 2-DE is challenging, particularly in the case of Gram-positive bacteria. This is primarily due to strong protein association with the cell wall peptidoglycan and protein hydrophobicity. We solubilized whole cell proteins for 2-DE from the Gram-positive pathogen *C. chauvoei* using 8M urea instead of 7M that is used for gram-negative bacteria. Moreover, centrifugation in high speed (23000 rpm) for 1 h was added to the protocol to remove insoluble material and also remove possible contamination of DNase, RNase, salt and lipid by the help of urea in the buffer. These contaminations and insoluble material could be the reason of streaks in the

gels. Therefore, after these optimizations, the protein separation on 2-DE gels was highly good.

3.6   Proteome and Immunoproteome of *C. chauvoei*

The profile of whole cell proteins of *C. chauvoei ATCC 11957* separated by 2-DE gel by using IPG strips in a pH range from 3-10 for the first dimension and stained by Comassie G-250 is shown in Figure 9. A total of 460 protein spots could be detected on the 2-DE gels by the help of Delta2D (Decodon, Germany) image analysing software. 30 of them reacted with polyclonal antibodies against inactivated whole cells of *C. chauvoei ATCC 11957* (Figure 11). Among these 30 proteins, 17 (34 spots as a total from the master gel duplicates) could be cut and analyzed by MALDI-TOF MS and 8 of them could be characterized. Of these 8 spots, 4 corresponded to *C. chauvoei* flagellin protein FliB(C) which is known to be major immunogenic protein. The reason for multiple spots for this protein is likely post translational modification events such as enzymatic cleavage or chemical modifications.

It has been reported in the literature that the protective antigens of *C. chauvoei* are placed on flagella and cell walls of the bacterium (Chandler and Hamilton, 1975). Then, it was reported that the flagella of *C. chauvoei* are important for protective immunity in mice in that purified flagella elicit a protective effect, and non-flagellated mutants give 100-fold less protective immunity than the flagellated parental strain (Tamura *et al.*, 1984; Tamura and Tanaka, 1984). On the other hand, Tamura and Tanaka (1984) have demonstrated that anti-flagellar serum prevents bacterial growth in the liver, but not in infected muscle against intramuscular challenge with calcium chloride activated spores in normal mice. They suggested that the anti-flagellar serum exerts its effect in an opsonic function and that opsonised *C.*

chauvoei is eliminated mainly by polymorphonuclear leukocytes. Passive immunization studies were performed to see whether antigens may be useful as protective immunogens (Tamura and Tanaka, 1984; Chandler, 1975; Menzies and Kernodle, 1996). Micallizzi and Guzman (1997) showed that passive immunization of mice provided by anti-flagellar sera elicits 75% protection. In addition, Tanaka *et al*. (1997) reported that vaccines with poor flagella, which were prepared by dilution or heat treatment of vaccines showed lower protective effects in the mouse protection test. In conclusion, flagella of *C. chauvoei* have shown a good performance in terms of protection, so it is a highly immunogenic protein by itself. As verified by our results, flagella can be a good vaccine candidate for *C. chauvoei*.

On the other hand, 2 protein entries, methionine adenosyltransferase and glucose-6-phosphate isomerase, matched well with those of other Clostridium species which are *Clostridium botulinum* Bf and *Clostridium* sp. 7_2_43FAA, respectively. 2 proteins which existed in multiple spots corresponded to another bacterial protein, ornithine decarboxylase and this protein matched with *Pectobacterium carotovorum* subsp. *Carotovorum* WPP14. This protein was detected in two spots on the gel, indicating post-translational modification. Therefore, these 3 proteins constituted novel immunogenic proteins for *C. chauvoei*. The characterized proteins were listed in Table 5 and shown in Figure 12.

Well-separated master gel presented in Figure 10 constituted first experimental reference proteome map for *C. chauvoei* from which we obtained first immunoproteomic study for *C. chauvoei*. In the study, 4 immunogenic proteins were identified in 8 spots of which 3 immunogenic proteins are novel for *C. chauvoei*. Besides, the identification of every other immunogenic protein spots has not yet been accomplished. Genome project

of *C. chauvoei* has been recently completed by the group of Hattori in Tokyo University, however it is still in draft form and not published yet according to Genomes Online Database (GOLD) (2009). Until now, the proteome studies of *C. chauvoei* have been carried out on NCBI's general protein databases. The studies are under way to define the whole proteome of the organism, which will form a basis for physiological proteomic studies of the pathogen which is planned to be the next step of the current research. For the future studies, we are planning to compare the theoretical proteome map, which is derived from the genome sequence of the organism, with the experimental results. We will integrate the genetic information with data obtained from protein studies of *C. chauvoei* in order to confirm the immunogenic proteins by epitope prediction. Therefore, availability of complete genome of C. chauvoei will provide us more accurate, more reliable, high percentile and fast protein identification in the future.
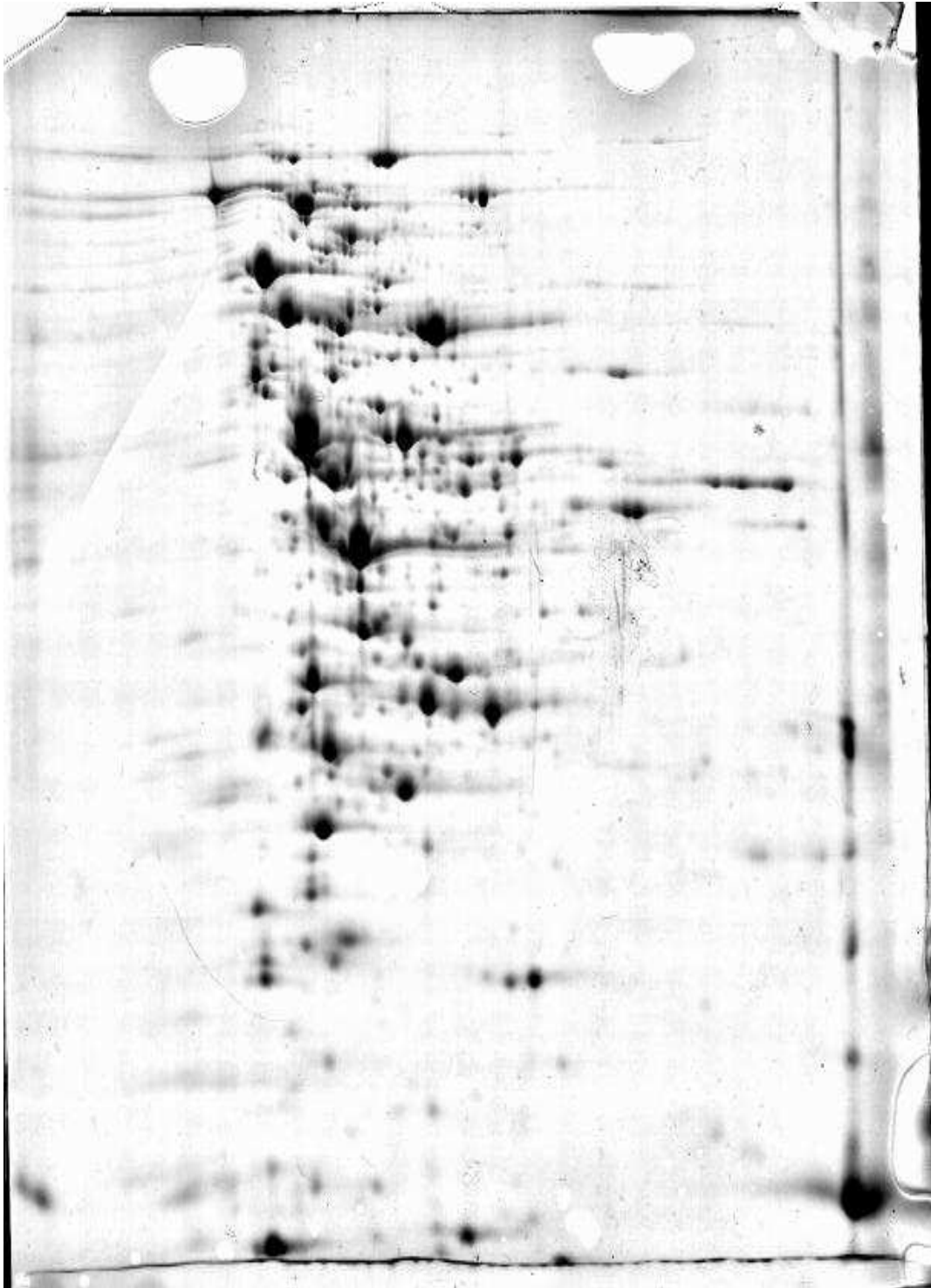
Figure 10: A reference 2-DE map (master gel) of total proteome of *C.chauvoei* ATCC 11957 (pI 3-10).
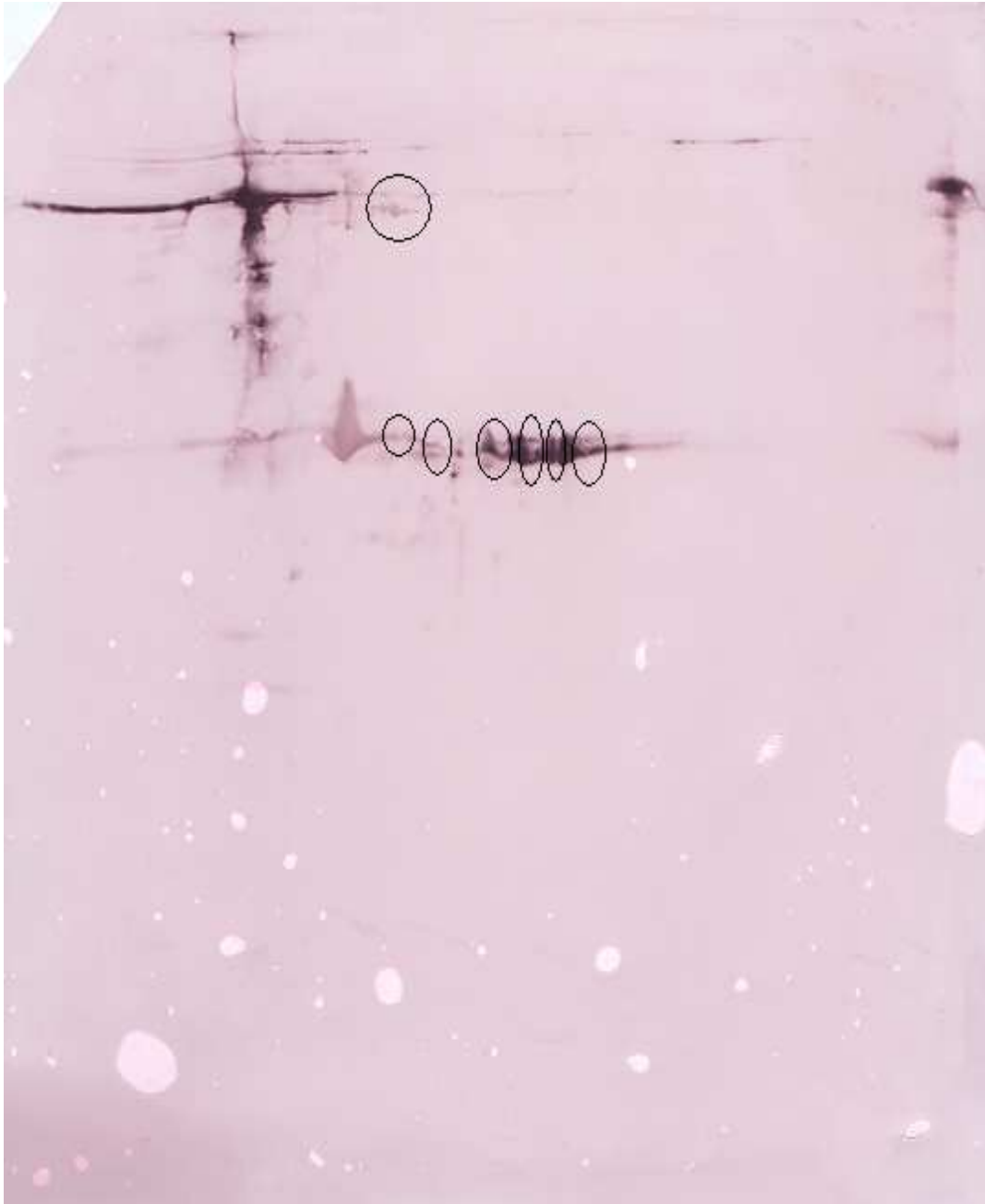
Figure 11: Western blot analysis of the whole cell proteome of *C. chauvoei ATCC 11957.* The immunogenic proteins are identified in circle. The serum was obtained from mice immunized with inactivated whole cells of *C. chauvoei* ATCC 11957.
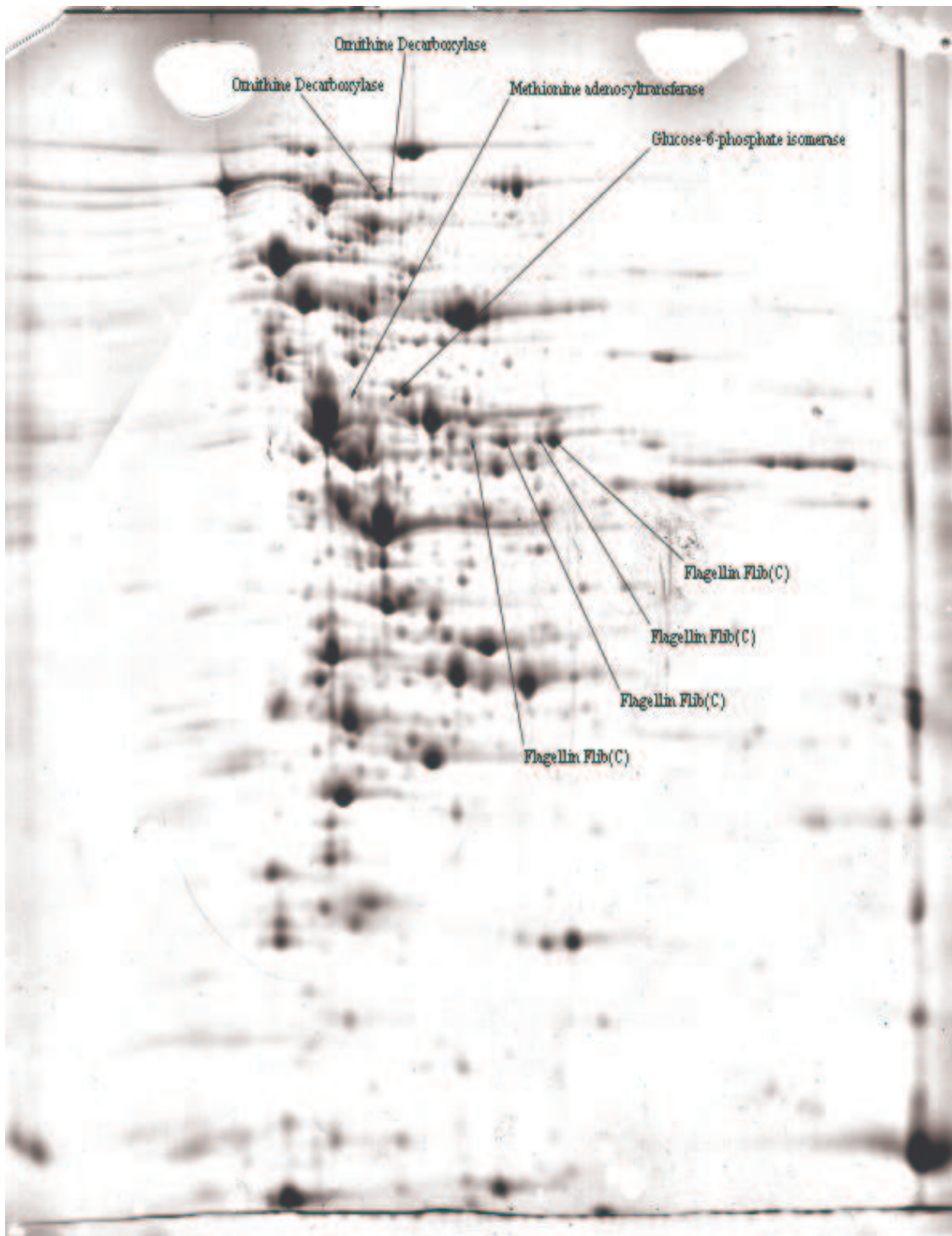
Figure 12: Representation of identified immunogenic proteins of *C. chauvoei* ATCC 11957 2-DE gel.

Table 4. List of identified immunogenic whole cell proteins of *C.chauvoei ATCC 11957*

Protein Name	Species	Accession Number	Protein MW	Protein pI	Peptide Count	Protein Score	Multiple spots
Ornithine decarboxylase	*Pectobacterium carotovorum* subsp. carotovorum WPP14	gi|227327684	80202,5781	5,58	6	129	2
Methionine adenosyltransferase	*Clostridium botulinum* Bf	gi|168184770	41927,7109	5,8	9	169	1
Glucose-6-phosphate isomerase	*Clostridium* sp. 7_2_43FAA	gi|254517666	49930,2383	5,22	12	272	1
Flagellin protein FliB(C)	*Clostridium chauvoei*	gi|19910959	43518,1289	6,17	22	459	4

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

3.7 Evaluation of Immunogenic Proteins

3.7.1 Methionine Adenosyltransferase

Methionine adenosyltransferases (MATs), also called S-adenosylmethionine synthetase, are the family of enzymes that synthesize the main biological methyl donor, S-adenosylmethionine (AdoMet) from L-methionine and ATP (Catoni, 1953). Methionine adenosyltransferase (MAT) catalyzes the synthesis of s-adenosylmethionine (AdoMet), a metabolite that plays a major role in the metabolism of cells in all living organisms (Tabor and Tabor, 1984) and it is a key compound in the trans-methylation reactions (Lu, 2000). It is an intermediary in the trans-sulfuration pathway to cysteine, which is one of the three amino acids involved in glutathione and trypanothione synthesis (Fairlamb and Cerami 1992; Mato *et al.*, 2002; Tabor and Tabor, 1985). In addition, AI-2 protein which is an autoinducer protein in quorum sensing activity is produced from s-adenosylmethionine. Quorum sensing, or the control of gene expression in response to cell density and it has been shown that many pathogens use quorum sensing to regulate virulence. Quorum sensing involves the production and detection of extracellular signalling molecules called autoinducers. It is used by both Gram-negative and Gram-positive bacteria to regulate a variety of physiological functions. In general, Gram-negative bacteria use acylated homoserine lactones as autoinducers while Gram-positive bacteria use processed oligo-peptides to communicate. On the other hand, highly conserved *luxS* homologues have now been identified in both Gram-negative

and Gram-positive bacteria species including *Escherichia coli*, *Salmonella typhimurium*, *Salmonella typhi*, *Salmonella paratyphi*, *Haemophilus influenzae*, *Helicobacter pylori*, *Bacillus subtilis*, *Borrelia burgdorferi*, *Neisseria meningitidis*, *Neisseria gonorrhoeae*, *Yersinia pestis*, *Campylobacter jejuni*, *Vibrio cholerae*, *Deinococcus radiodurans*, *Mycobacterium tuberculosis*, *Enterococcus faecalis*, *Streptococcus pneumoniae*, *Streptococcus pyogenes*, *Streptococcus mutans*, *Staphylococcus aureus*, *Clostridium perfringens*, *Clostridium difficile*, *Shewanella putrefaciens*, *Klebsiella pneumoniae,* and *Pasteurella multocida* (Surette *et al.*, 1999) Most of the species of bacteria possessing a *luxS* gene have been shown to produce AI-2 activity (Bonnie and Bassler, 1999). This result suggests that communication via an AI-2 signal response system could be a common mechanism (Bassler, 1999; Surette *et al.*, 1999; Bassler et al., 1997). AI-2 has been shown to control a variety of cellular processes, such as production of pathogenicity factors in *Streptococcus pyogenes* (Lyon *et al.*, 2001), toxin production in *Clostridium perfringens* (Ohtani *et al.*, 2002), and formation of mixed biofilm between the two oral bacteria *Streptococcus gordonii* and *Porphyromonas gingivalis* (Mcnab *et al.*, 2003).

The immunogenicity of *S*- adenosylmethionine synthatase was previously shown in a *H. pylori* immunoproteomics study (Lin *et al.*, 2006) and also Altındiş *et al.* (2009) showed that this enzyme is an immunogenic protein of *Bordetella pertussis* in immunoproteomics studies. Herein, we demonstrated that it is an immunogenic protein of *C. chauvoei*.

3.7.2 Glucose-6-phosphate Isomerase

Glucose-6-phosphate isomerase (GPI) or phosphoglucose isomerase (PGI) is universally distributed among eukaryotes, bacteria and some archaea. It

catalyzes the reversible isomerization of glucose-6-phosphate and fructose-6-phosphate, which is an essential reaction involved in both catabolic glycolysis and anabolic gluconeogenesis. Many GPIs have been reported from plants to animals and GPI has been one of the evidences for evolution theory (Grauvogel *et al.*, 2007; Katz, 1996).

It has been reported that GPIs are involved in many metabolic pathways, for example, they may be one of the messengers to response to the stimulation of environment factors such as nitrate in plants (Munjral *et al.*, 2007; Wang *et al.*, 2003), and GPIs also act as an autocrine motility factor (AMF) that can promote tumor cells metastasis and invasion by binding to their receptors (AMFR) in animals (Chiu *et al.*, 2008; Funasaka, 2007). A study by Pazour *et al.* (2005) showed that the flagellar proteome of *Chlamydomonas reinhardtii* (*C. reinhardtii*) contained most of enzymes of the glycolytic pathway, suggesting GPIs, a rate-limiting enzyme in glycolytic pathway, may be related to the flagella of *C. reinhardtii* (Pazour *et al.*, 2005). While *Chlamydomonas reinhardtii* is an eukaryotic organism, a homology may be considered between two GPI proteins of *C. reinhardtii and C. chauvoei*. For that reason, flagella protein of *C. chauvoei* can also be related to glucose-6-phosphate isomerase. Besides that, motif searches can lead to important functions of proteins. Motif found in GPI enzyme of *Vibrio vulnificus*, a pathogenic bacteria, includes flagellar basal body rod proteins signature and this supports our theory (Richard, 2004).

Our study was the first implication of the existence of this protein in the immunoproteome of a bacterial pathogen.

3.7.3 Ornithine Decarboxylase

Polyamines constitute a class of positively charged molecules found in all organisms. They are required for cell growth and reported to have various functions in stabilizing DNA or membrane structure, and in resistance to oxidative stress. Ornithine decarboxylase (ODC) catalyzes the decarboxylation of L-ornithine to form diamine putrescine, a rate limiting step in the polyamine synthesis pathway (Williams *et al.*, 1992). The expression of ODC is activated in macrophages by infection with *S. typhimurium* or treatment with *S. typhimurium*-derived LPS (Prosser *et al.*, 1984). Moreover, *Helicobacter pylori* infection causes chronic inflammation of the gastric mucosa that result from an ineffective immune response. It has been demonstrated that one underlying mechanism is induction of macrophage apoptosis mediated by polyamines. The transcription factor c-Myc has been linked to induction of ornithine decarboxylase (ODC), the rate-limiting enzyme in polyamine synthesis (Cheng *et al.*, 2005)

Bailey *et al.* (2000) studied on a knockout strain of *Stagonospora* (*Septoria*) *nodorum* lacking the single ornithine decarboxylase (ODC) allele and pathogenicity studies on these mutants showed that they are greatly reduced in virulence compared with non-disrupted transformants.

The present article constitutes the first report on the immunogenicity of ornithine decarboxylase for *C. chauvoei*.

3.7.4 Flagellin

The flagellum is the appendage responsible for motility in the majority of bacterial species. The structure of a bacterial flagellum can be divided into

three parts: the basal body, the hook and the flagellar protein. Flagellin is the structural protein that forms the major portion of flagellar filaments. The flagellar filament is composed of approximately 20 000 subunits of a unique protein, known as flagellin (Ramos *et al.*, 2004). Although most of the secreted flagellin is usually assembled in the flagellum, flagellin can also accumulate in the bacterial environment as a result of leaks and spillover during the construction of flagellae (Komoriya *et al.*, 1999). Flagellar motility allows bacteria to respond to favourable or unfavourable stimuli in their environment, and is also strongly related to the infectivity of some pathogenic bacteria (Finlay *et al.*, 1997; Kersten *et al.*, 2001).

Flagella have been implicated as virulence determinants in several enteric pathogens. The flagella appear to be necessary for *Vibrio cholerae* to attach itself to the intestinal mucosa to deliver its toxin efficiently (Attridge and Rowley, 1983; Guentzel, and Berry, 1975). Immunization with crude flagellum vaccines decreases the number of radiolabeled vibrios attached to the mucosae of challenged rabbits (Yancey *et al.*, 1979). Flagella have been reported to enhance pathogenicity in *Salmonella typhimurium* (Carsiotis *et al.,* 1984; Weinstein *et al.,* 1984), probably by increasing survival time within macrophages. Moreover, the groups of mice immunized with *Pseudomonas aeruginosa* flagellar antigen preparations showed higher survival rates when they were challenged locally in the burned area, and the protection appears to be due to immobilization of the microorganisms in the burned skin tissue (Holder *et al.*, 1982). Motility functions of *Helicobacter pylori*, *Pseudomonas aeruginosa* and *Vibrio cholerae* are crucial for infection of the stomach, lungs and intestinal mucosa, respectively (Drake and Montie, 1988; Ottemann and Lowenthal, 2002; Krukonis and DiRita, 2003). Colonization of rabbit appendix by *Salmonella enterica* serovar Typhimurium also depends on accessibility and motility (Marchetti, *et al.*, 2004). Because motility increases the

occurrence of host–pathogen interactions, this feature contributes to the main role of the flagellum in pathogenesis. Crude flagella from the opportunistic pathogen *Clostridium difficile* bind to cecal mucus of germ-free mice (Tasteyre *et al.*, 2001). In addition, non-flagellated *C. difficile* associate with the cecal tissue at a rate that is 10-fold lower than that of a flagellated strain, highlighting the in vivo role of flagella in adherence to mucus.

In this study, the flagellin fliB(C) protein of *C. chauvoei* ATCC 11957 strain was identified as 43.5 kDa protein. It is smaller than that obtained with different strains used in other previous studies. Genero *et al.* (1999) determined the *C. chauvoei* strain 5078 flagella protein as a 47 kDa one. While *C. chauvoei* Okinawa strain flagellar protein was defined as 56 kDa by Tamura *et al.* (1995), it was found as 46 kDa by Kojima *et al.* (1999). Besides that, it was reported that DNA sequence analysis of *C. chauvoei* Okinawa strain fliC gene encoding the flagellin protein revealed an open reading frame of 413 amino acid residues with a calculated molecular mass of 43,8 kDa (Kojima *et al.*, 2000). There might be some strain based differences. Moreover, our study is the first proteomic study of *C. chauvoei* and first MS analysis of its immunoreactive proteins. Therefore, any discrepancies between experimental and theoretical masses might have been caused by post-translational proteolytic processing and modifications. Strain based variations that have been frequently encountered must also be taken into account.

CHAPTER 4

CONCLUSION

- Hemolysin protein has been suspected to be a major virulence factor of *C. chauvoei*. In the study, hemolysin activity of the strains *C. chauvoei ATCC 11957* and *20* were observed highest at the late logaritmic and early stationary phases. There was almost no hemolytic activity to be remained during the late stationary phase though the protein still existed in cultures. Therefore, experimental vaccines were prepared by using one day grown inactivated whole cells which were expected to contain hemolysin.

- Antibody response evaluation by ELISA indicates that sera obtained from mice immunized with experimental vaccines had a high titer of IgG. As expected, these sera were effectively used in immunoblotting analysis.

- 1-DE SDS-PAGE and Western blot analysis for protein profiles of *C. chauvoei* ATCC 11957 and 20 strains showed that there is no significant difference between these strains with respect to the protein profile. Also, the age of the respective cultures did not influence the protein profiles.

- In the present, study, first successful 2-DE gel representation of *C. chauvoei* was constructed and a total of 460 protein spots could be detected.

- The present study constituted the first immunoproteomics analysis of *C. chauvoei*. A total of 4 immunoreactive proteins of *C. chauvoei* (ornithine decarboxylase, methionine adenosyltransferase, glucose-6-phosphate isomerase, and flagellin FliB (C)) protein were identified. Glucose-6-phosphate isomerase was identified as immunogenic as the first time in a pathogenic microbe and methionine adenosyltransferase and ornithine decarboxylation were identified as immunogenic for *C. chauvoei* for the first time. Other identified protein flagellin FliB (C) is known to be major protective antigen and virulence factor of *C. chauvoei*. This protein appeared in 4 parallel spots, strongly suggesting posttranslational charge modification of this protein. 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.

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APPENDIX A

Composition and Preparation of Culture Media

M1-2-V MEDIUM

A SOLUTION (Autoclave separately)

Tryptone	10 g/l
Yeast Extract	5 g/l
NaCl	5 g/l
Soluble Starch	1 g/l
Peptone	4 g/l
Casamino Acids	6 g/l

pH: 7,6

B SOLUTION (Filter sterilization)

L-Cysteine HCl	6	g/l
Dextrose	0.6	g/l

VITAMIN SOLUTION (Filter sterilization) (4, 4 ml to 1 L medium)

Calcium pantothenate 4 mg

Thiamin-HCL   1 mg

Pyridoxine-HCl   1 mg

Riboflavin   1 mg

Biotin solution   90 µl

Ethanol   4.6 ml

H_2O 13.1 ml

Biotin Solution: 2, 5 mg/ml Biotin

Nutrient Broth Medium (NB)

Beef Extract   3 g/l

Peptone   5g/l

pH: 6,8±0,2

Nutrient Agar Medium (NA)

Beef Extract   3 g/l

Peptone   5g/l

Agar   20g/l

pH: 6,8±0,2

APPENDIX B

Solutions and Buffers

Coomassie Brilliant Blue (CBB) Staining Solutions

Coomassie Brilliant Blue (CBB) Stock Solution

Coomassie Brilliant Blue G-250	5g
dH_2O	100 ml

Fixation Solution

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

CCB Dye Solution

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

CCB Staining Solution

CCB dye solution	200 ml
Methanol	50 ml

Silver Staining Solutions

Fixation Solution

50 % Methanol	100 ml
12% Acetic Acid	24 ml
0.05% Formaldehyde	100 µl

Pretreatment Solution

0.02% Sodium Thiosulfate

Impregnate Solution

0.2% $AgNO_3$

Development Solution

2.25% Sodium Thiosulfate

0.05% Formaldehyde

2% Pretreatment solution

Stop Solution

%50 Methanol

%12 Acetic Acid

SDS-PAGE SOLUTIONS

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.

Tris HCl (1.5 M)

Tris base	54.45 g
dH$_2$O	150 ml

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

Tris HCl (0.5 M)

Tris base	6 g
dH$_2$O	60 ml

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

Tris-EDTA Buffer (TE)

Tris	10 mM
EDTA	1 mM

pH is adjusted to 8.0 with HCl.

Running Buffer (5X)

Tris base	15 g
Glycine	72 g
SDS	5g

Distilled water to 1 L Stored at 4°C.

Sample Buffer

dH$_2$O	3 ml
Tris HCl (0.5 M)	1 ml
Glycerol	1.6 ml
SDS (10%)	0.4 ml
β- mercaptoethanol	0.4 ml
Bromophenol blue	(0.5%, w/v) (in water) 0.4 mL

WESTERN and DOT BLOT SOLUTIONS

Transfer Buffer

Methanol	200 ml
Tris-Base	3,63 g
Glycine	14,4 g
SDS	0,37 g
dH$_2$O	up to 1 Liter

TBS

Tris-Base	2,42 g
NaCl	29,24 g

Destaining Solution

Methanol	100 ml
Glacial Acetic acid	100 ml
dH_2O	800 ml

Bradford Reagent 5X

%85 H_3PO_4	100 ml
Methanol	50 ml
Comassie blue G	100 mg
dH_2O	up to 200 ml

Rehaydration Buffer

Urea	8 M
Thiourea	2 M
Ampholite	0,2 M
DTT	28 mM
CHAPS	2% m/v
dH_2O	up to1 0 ml

APPENDIX C

Chemicals and Their Suppliers

Chemicals	Chemical Supplier
Acetic acid	Merck
Acetone	Merck
Acrylamide	Sigma
Ammonium sulphate	Merck
Ampholanes pH (3-10)	Fluka
Beef Extract	BD
Bis-acryamide	Sigma
Biotin	Sigma
Bovine Serum Albumin (BSA)	Sigma
Calcium pantothenate	Sigma
Casamino Acids	BD
CH_3CN	Applichem
CHAPS	Merck
Comassie Brillant Blue G 250	Sigma
DTT	Fluka
Dextrose	BD
Ethanol	Merck
Glycerol	Merck
Glycine	Merck

HCl	Merck
IPG strips	BIORAD
KH_2PO_4	Merck
Methanol	Merck
$MgSO_4\ 7H_2O$	Merck
Molecular Weight Standard	Fermentas
NaOH	Merck
NH4Cl	Merck
Peptone	BD
Pyridoxine HCl	Fluka
Riboflavin	Sigma
SDS	Sigma
Soluble Starch	BD
TEMED	Sigma
TFA	Applichem
Thiamine HCL	Fluka
Thiourea	Fluka
Trichloroacetic acid (TCA)	Merck
Tris- HCl	Sigma
Tryptone	BD
Urea	Fluka
Yeast Extract	BD