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CHARACTERIZATION OF *CLOSTRIDIUM PERFRINGENS* BETA2 TOXIN

A Thesis in

Pathobiology

by

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ABSTRACT

Clostridium perfringens, a gram positive, sporulating anaerobic bacterium is responsible for a wide spectrum of diseases in animals and humans. *Clostridium perfringens*-mediated hemorrhagic enteritis and enterotoxemias in livestock, domestic animals, aquatic species and birds have imposed significant economic impact on animal husbandry. The anaerobic, nutrient-enriched environment of the mammalian gastrointestinal tract serves as an ideal habitat for the *C. perfringens* to proliferate and attain a disease inducing potential. The pathogenesis of the *C. perfringens* mediated enteric diseases can be attributed to the well characterized, lethal membrane active toxins such as alpha, beta, epsilon, iota, enterotoxin and perfringolysin O.

In recent years, a variant of the *C. perfringens* beta toxin known as beta2 has been widely reported as one of the causative agents in *C. perfringens* related enteric diseases. The clinical symptoms of beta2 toxin induced enterotoxemias are characterized by watery sporadic diarrhea, edema of affected tissues and loss of body weight. The role of beta2 toxin in the causation of enterotoxemias and or haemorrhagic/necrotic enteritis in humans, domestic animals, birds and aquatic species has generated considerable interest in the field of human and veterinary medicine.

Clostridium perfringens beta2 has been reported as an accessory toxin and may act in synergy with other major toxins of the *C. perfringens* in the production of necrotic and hemorrhagic enteritis. The beta2 toxin may play a role in hindering the process of nutrient absorption in the intestine and thereby debilitating affected animals.

It is strongly speculated that a sudden change in the gut Microbiolflora or the intestinal physiological equilibrium may lead to clostridial disease outbreak. Studies that report on *C.*

perfringens beta2 producing isolates from healthy animals suggest that beta2 toxin as a potential health hazard.

Very few studies have reported the biophysical and molecular biological properties of the beta2 toxin in detail. It has been hypothesized that the mechanism of the beta2 toxin induced enteritis could include direct effects on transport of ions in the intestinal epithelial cells through membrane binding or pore formation. It is felt that experiments that focus on studying the cytotoxicity of beta2 toxin could lead to better understanding of beta2 toxin in context of *C. perfringens* related enterotoxemias. Based on these reported findings, elucidation of the molecular and biological properties of beta2 toxin would be of significant value to researchers towards developing a targeted epitope vaccine.

Clostridium perfringens are toxinotyped into types A-E by conventional bacteriology and PCR based confirmation techniques. Owing to the widespread distribution of *C. perfringens* toxinotypes and its epidemiological significance a highly sensitive diagnostic tool that replaces the conventional PCR is required. In food animal practice, active immunoprophylaxis against *C. perfringens* is achieved by use of commercial toxoid vaccines against types B, C and D. There are no published reports on vaccines conferring immunity against beta2 toxin.

The primary objectives of this dissertation are **(1) develop a molecular based toxin typing technique for *C. perfringens* which has practical application in a diagnostic laboratory and aid in conducting molecular epidemiological studies, (2) Elucidate the structure and function of beta2 toxin, 3) Evaluate in vitro the cytotoxic properties of beta2 toxin, and 4) Evaluate the role of beta2 synthetic lipopeptide vaccine in a challenge study in the mouse model.**

The results of our study revealed a high prevalence and distribution of *C. perfringens* beta2 toxin encoding strains in feces of lactating cattle. Among the five toxinotypes, toxinotype A was widely distributed among seven dairy herds screened by multiplex Real time PCR assay. We successfully used a GST fusion system for expression of recombinant beta2 toxin. The purified recombinant toxin was shown to be cytotoxic to *CaCo2* cell line at concentration of more than 10 µg/ml. the toxin treated cells exhibited cytopathic effects such as cell rounding, membrane blebbing and leakage of cytoplasm. These results could suggest that the *C. perfringens* beta2 toxin could act as one of the pore forming toxins. A monoclonal antibody raised against beta2 toxin completely neutralized the cytopathic effects of the toxin on *CaCo2* cells.

To study the physical structure of the beta2 toxin, recombinant beta2 toxin was crystallized in buffer comprising of 32-36% PEG in one week. Preliminary crystallographic analysis of the recombinant beta2 toxin suggested the crystals of the toxin belonged to space group R3. The triangular prism shaped crystals diffracted up to 2.9Å resolution and measured up to 200 Microbiolns in size. A novel approach of developing and evaluating a synthetic lipopeptide vaccine against *C. perfringens* beta2 toxin was demonstrated as part of this study. The lipopeptide conjugate chemically linked to Pam2Cys, successfully elicited a strong protective response in subcutaneously immunized mice. The immune response was characterized as mixed Th1 and Th2 response to the synthetic lipopeptide vaccine.

It is anticipated that the results of our study will provide animal agriculture with an alternate approach to protect food producing animals against *C. perfringens* infections. The study will lead to the development and the standardization of a real-time PCR based technique to identify and quantify the various toxigenic strains of *C. perfringens* directly from clinical

samples. The structure and function studies demonstrated in our study will provide a better understanding of the molecular pathogenesis of beta2 toxin and the development of a safe and effective vaccine against beta2 toxin.

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CHAPTER 1

Statement of the Problem

The emergence of *Clostridium perfringens* beta2 toxin over the last decade has presented a new paradigm on *C. perfringens* related enteric diseases. Reports of beta2 toxin as a causative agent of clostridial enteric diseases in various animal and aquatic species have generated considerable awareness in the field of veterinary and human medicine. Vital scientific data on (i) the molecular epidemiology of beta2 toxin, (ii) its effect and changes (cytotoxicity) on host cells and (iii) its ability to elicit an immune response, is essential for understanding the significance of beta2 toxin on *C. perfringens* toxin mediated enteric diseases in animals and humans.

CHAPTER 2

REVIEW OF LITERATURE

2.1 Clostridia

The genus *Clostridium* was first described by Prazmowski in 1880. The genus consists of Gram positive, anaerobic, heat resistant endospores forming phylogenetically indifferent organisms. The members of genus *Clostridium* are considered descendants of a common ancestor that emerged early in the evolution of gram positive bacteria (Stackebrandt and Rainey, 1997). Nearly two-thirds of the clostridial species are harmless saprophytes residing in the environment, animals and putrefying vegetation. The genus is comprised of 120 different species, 35 of which are considered pathogenic in humans and animals (Stackebrandt and Rainey, 1997). The importance of the genus clostridia in humans and veterinary medicine is attributed to the variety of proteinacious toxins produced by the pathogenic clostridial species (Shimizu et al., 2002).

Homology studies using 23S rRNA molecules have divided 56 clostridial species into four major groups (Johnson and Francis, 1975). Groups I and II consist of well defined species with a low G+C content (24-32%), while group III consists of low G+C content species that did not fit into another group. Group IV consists of organisms with a high G+C content (41-42%) (Lyras and Rood, 2006).

Based on the 16S rDNA sequencing, the genus *Clostridium* is comprised of 120 accurately described species (<http://www.bacterio.cict.fr/c/Clostridium.html>). The phylogenetic classification of genus *Clostridium* based on 16S rDNA has been widely reported by several groups (Collins et al., 1994; Stackebrandt et al., 1999; Stackebrandt & Rainey, 1997). Stackebrandt et al. (1999) described 19 different clusters for the genus *Clostridium* based on the 16S rDNA analysis. The phylogenetic analysis demonstrated that the genus consisted of a

heterogenous group of organisms (Figure 2.1). *Clostridium botulinum* and *C. perfringens* are foodborne clostridial pathogens belonging to the same cluster (cluster 1, Figure 2.1).

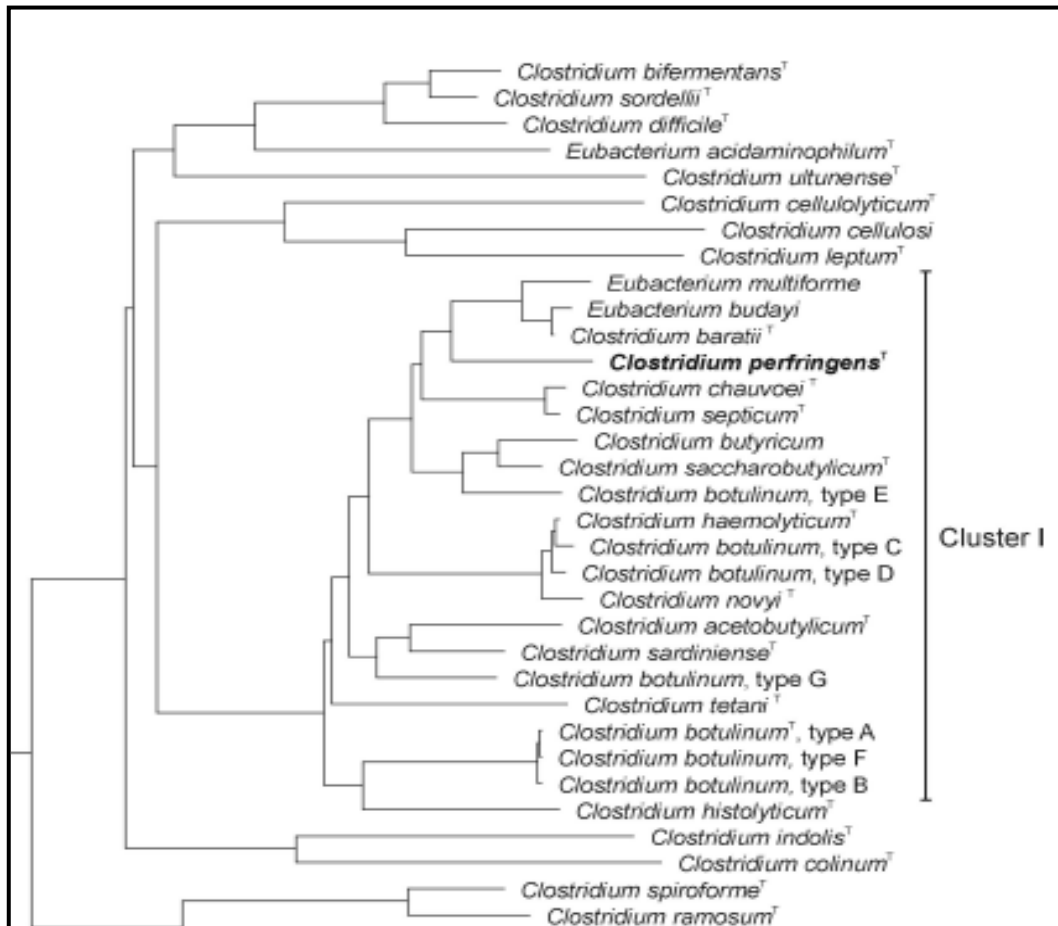


Figure 2.1. Phylogenetic relationship of Clostridia. Taken from Johanson et al., (2006).

The genus clostridia produce more toxins than any other bacterial genus (Fonstein and Haselkorn, 1995). Extracellular enzymes and more than 20 toxins have contributed to the virulence associated with *Clostridium* species. The genes that encode for several clostridial toxins are located on nonintegrative lysogenic bacteriophage or on plasmids (Johnston and Barry, 1997). The presence of virulence genes on extrachromosomal elements results in

phenotypic properties such as genetic instability and horizontal gene transfer to nontoxigenic organisms leading to dispersion of the toxigenicity. The potent extracellular toxins produced by the pathogenic clostridia cause cell and tissue damage, which is a common characteristic of pathogenic clostridial species.

Pathogenic clostridia can be classified broadly into three groups based on their host tissue affinity. The first group consists of neurotoxic clostridia that produce toxins affecting the nervous system. The second group is made up of histotoxic clostridia, characterized by the production of toxins that affect the structural and functional integrity of the host cells located at or near the site of infection. The third group consists of enterotoxigenic clostridia which produce toxins that affect the gastrointestinal tract (Lyras and Rood, 2006).

In summary of the above literature, the Clostridial genus includes important pathogenic species like *C. botulinum*, *C. perfringens*, *C. difficile*, and *C. tetani*. The pathogenic species within the genus clostridia are known to produce potent toxins having lethal effects on the host tissue. The genes encoding for these virulence factors are located on plasmids and acquired through horizontal gene transfer. These zoonotic organisms are primarily soilborne and under opportunistic conditions can result in infection/toxicosis in a wide variety of mammalian and avian species.

2.2 *Clostridium perfringens*

2.2. 1. General characteristics

In 1892 American Microbiologists, Welch and Nuttall, isolated a Gram positive, anaerobic organism from gangrenous wounds. The organism was later identified as *Clostridium perfringens* (Fisher et al., 2006). The organism has been described by several names such as

Bacillus aerogenus capsulatus, *Bacillus perfringens*, *Bacillus welchii*, and *Clostridium welchii*.

The organism is now known as *Clostridium perfringens*, a causative agent of clostridial myonecrosis (death of muscle tissue). Based on its high growth rate, oxygen tolerance and manipulation of its genetic machinery, *C. perfringens* has been described as a prototype species within the toxigenic clostridia (Rood, 1998).

Clostridium perfringens belong to the class clostridia within the phylum Firmicutes (gram positive cell wall bacteria). The other classes of bacteria within the phylum that cluster with clostridia are Bacillales, Lactobacillales, and Mollicutes (Fisher et al., 2006). *C. perfringens* is located in the order Clostridiales, family Clostridiaceae along with other important bacterial pathogens such as *C. difficile*, *C. septicum*, *C. botulinum*, and *C. tetani*. The bacterium is widely spread in the environment throughout soil and water and is commonly found in the intestines of animals. *Clostridium perfringens* does not exhibit adherence and invasive properties towards healthy intestinal mucosa. An alteration in the physiological equilibrium of the intestine and resident Microbiota due to antibiotic therapy, management related stress, the ingestion of soil and feces contaminated feed, or improperly fermented silage or haylage allows colonization by toxigenic clostridia leading to enterotoxemia and hemorrhagic enteritis.

Soil, sewage and digestive tracts of humans and animals are some of the ecological niches of *C. perfringens*. *Clostridium perfringens* is classified as an anaerobe, occasionally oxygen tolerant, gram positive, rod shaped bacteria that is capable of forming endospores (Quinn et al., 1994). They have large rods (0.6-2.4 x 1.3-9.0 µm), are encapsulated and non-motile (Cato, George and Fingergold, 1984). Most *C. perfringens* isolates are catalase negative, oxidase negative and grow optimally around 42-50°C (Adams & Moss, 1995). The growth is characterized by a high amount of gas (H₂) production (Bryant & Stevens, 1997).

Clostridium perfringens, as an anaerobic organism, is devoid of the citric acid cycle and does not possess genes required for ATP generation through aerobic respiration (Shimizu et al., 2002). *Clostridium perfringens* derives its energy through anaerobic fermentation resulting in acetic, butyric, and lactic acid as fermentation end products (Fisher et al., 2006).

The growth of *C. perfringens* on egg yolk agar media plates is characterized by lecithinase activity, while the growth on Shahidi Ferguson Perfringens (SFP) agar is characterized by sulphite reduction producing black colonies along with the lecithinase reaction (Allen et al., 2003, Cato et al., 1984) (Figure 2.2). Cato et al., (1984) demonstrated that *C. perfringens* colonies produce double zones of hemolysis on blood agar plates; this reaction is used for presumptive identification of *C. perfringens* (Figure 2.3).



Figure 2.2 *Clostridium perfringens* growth on Shahidi Ferguson perfringens agar characterized by sulphite reduction producing black colonies and lecithinase activity producing an opaque halo.

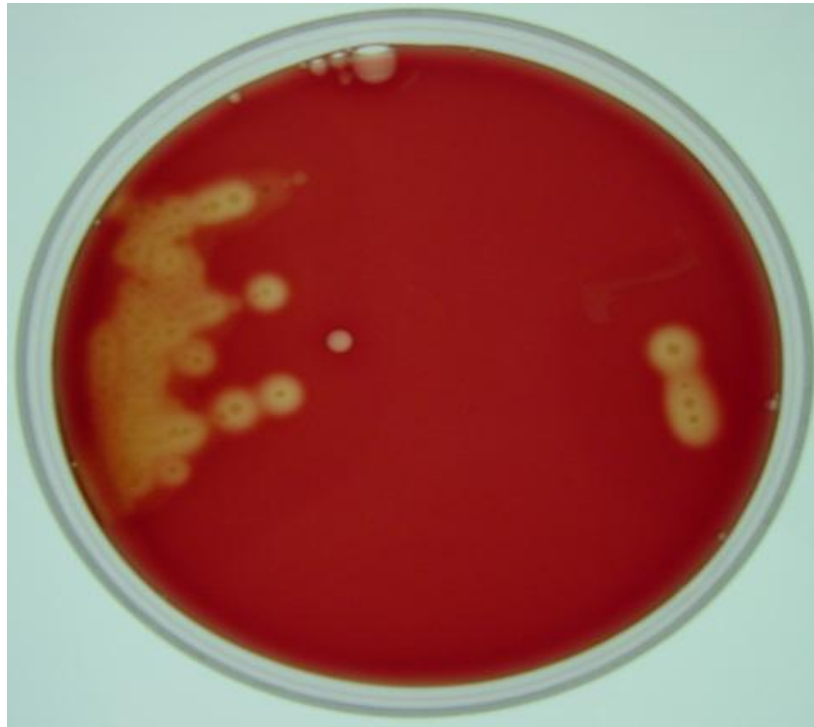


Figure 2.3 *Clostridium perfringens* growth on Blood agar characterized by double zones of hemolysis.

Clostridium perfringens derives its energy from the putrefaction of available nutritional resources and the formation of acidic byproducts. This anaerobic lifecycle of the bacterium has led to development of various *C. perfringens* related pathologies. The mammalian gastrointestinal tract offers a good carbon source, high temperature, and other nutritional factors that are required for optimum growth and proliferation of the organism. Collectively, the alteration in the gut physiological equilibrium enables the *C. perfringens* to multiply and produce an array of pathogenic toxins.

2.2.2 Genetics of *Clostridium perfringens*

The genome size of clostridial species ranges from 2 to 6.5 Mbp (Cato et al., 1984). Myers et al., (2006) compared the genome sequence of three *C. perfringens* strains to those of other pathogenic clostridia and showed that the average GC content for the three strains of *C. perfringens* strains was about 28%. The genome size of *C. perfringens* can range from 3-4 Mbp. The variation in the genome size could be due to mobile genetic elements such as phage like sequence inserts in the genome (Myers et al., 2006).

Shimizu et al., (2002) showed that the virulence related genes of *C. perfringens* were associated with colonization factors. Five putative hemolysin genes that resembled hemolysins described in other bacteria were detected. They also found similarities in the fibronectin binding protein genes of *C. perfringens* to those of *Listeria monocytogenes* and *Bacillus subtilis*. The genome sequence analysis of *C. perfringens* (Myers et al., 2006) revealed that *C. perfringens* lacked pathogenicity islands. It was hypothesized that different toxintypes of *C. perfringens* may have evolved through the acquisition of extrachromosomal elements such as plasmids and transposons (Johansson et al., 2006).

Clostridium perfringens has the ability to produce many extracellular toxins and enzymes including alpha toxin, beta toxin, theta toxin, iota toxin, epsilon toxin, kappa toxin and as well as sialidase (Rood, 1998). In addition to the above mentioned toxin production, some strains of *C. perfringens* also can produce enterotoxin and a novel beta2 toxin (Gurjar et al., 2007).

The alpha and theta toxin encoding genes are located on the chromosome, while the other toxin-encoding genes are present on large plasmids (Table 2.1). The location of enterotoxin gene (*cpe*) can be either chromosomal or plasmid. The location of *cpe* gene in food poisoning isolates is chromosomal (Brynestad & Granum, 1999), while non-food-poisoning human gastrointestinal

disease isolates and veterinary isolates carry the *cpe* gene on large plasmids (Cornillot et al., 1995).

Table 2.1 Toxins of *Clostridium perfringens* (Modified from Petit, Gibert & Popoff, 1999, ; Johansson, 2006).

Toxin	Gene Location	Biological activity
Alpha	Chromosome	Cytolytic, hemolytic, dermonecrotic, lethal
Theta	Chromosome	Hemolytic, modulation of host inflammatory response
Enterotoxin	Chromosome/ Plasmid	Cytolytic, lethal, leakage of water and ions
Beta	Plasmid	Cytolytic, dermonecrotic, lethal
Epsilon	Plasmid	Edematous in liver, kidney, nervous system
Iota (Ia & Ib)	Plasmid	Disruption of actin cytoskeleton and affects cell membrane integrity
Beta2	Plasmid	Cytotoxic, lethal

To date, the two component system (VirR/VirS) is known to regulate the transcription of many toxin genes (alpha, theta, beta2) and thus contribute to the pathogenicity of *C. perfringens*. The two components comprising the system are a sensor histidine kinase (VirS), and a response element (VirR) (Ba-Thein et al., 1996). The beta2 toxin gene is transcribed during vegetative growth, especially during the exponential growth phase (Ohtani et al., 2003).

In summary, horizontal gene transfer of extrachromosomal elements such as insertion sequences and plasmids could be attributed to variation in the genome size of *C. perfringens*. The extrachromosomal elements confer fitness and pathogenic potential for growth and survival of the organism. The regulation of the extracellular toxin production in *C. perfringens* is globally controlled by the VirR/VirS system. Presence of the various virulence (toxin) genes on the chromosome and the extrachromosomal elements has resulted in the classification of the organism into different toxintypes. The ability to affect various organ systems through its pathogenic toxins has made *C. perfringens* one of the most pathogenic species within the genera Clostridia.

2.2.3 *Clostridium perfringens* virulence factors

Clostridium perfringens has been shown to produce an array of different toxins. Five of the toxins (α -, β -, ϵ -, i -, and enterotoxin) are responsible for tissue lesions and host death (Daube, 1992; Songer, 1996; Petit et al., 1999). Four of the above toxins: iota (i), alpha (α), beta (β), and epsilon (ϵ) have been used for classification of *C. perfringens* into five isotypes (A, B, C, D, and E) with each type carrying a different combination of the toxin genes (Al-Khalidi et al., 2004) (Table 2.2). The presence of alpha toxin gene (*cpa*) is common to all five *C. perfringens* types (A, B, C, D, and E), while the beta toxin (*cpb*) is associated with types B and C. The epsilon toxin (*etxD*) also can be found in types B and D. The iota toxin (*iA*) has been shown to be present in type E only.

Table 2.2 *Clostridium perfringens* toxintypes and disease manifestation in different hosts.

<i>C. perfringens</i> Toxintype	Hosts	Disease Manifestation
A	Humans, Dogs, Pigs, Fowl, Lambs	Gangrene, Food poisoning, Diarrhea, Necrotic Enteritis, Enterotoxaemic Jaundice
B	Lambs, Sheep, Neonatal Calves, Foals	Lamb Dysentery, Enterotoxaemia
C	Piglets, Calves, Lambs, Foals, Adult Sheep, Chickens	Haemorrhagic Enterotoxaemia, Struck, Necrotic Enteritis
D	Sheep, Goat, Calves	Pulpy Kidney disease
E	Calves, Rabbits, Lambs	Enterotoxaemia

The tissue pathology associated with alpha toxin is due to the enzyme phospholipase C. The alpha toxin has lethal, necrotizing, hemolytic, and cytolytic activities. The beta toxin is produced by type B and C strains of *C. perfringens* and causes necrotic enteritis characterized by hemorrhagic mucosal ulceration or superficial mucosal necrosis of the small intestine (Niilo, 1986; Sakurai and Fujii., 1987).

The presence of trypsin or protease inhibitors in the gut has been shown as the most important cofactor in beta toxin induced necrotic enteritis. Current literature strongly suggests that beta-toxin is a pore forming toxin which oligomerises to form channels in susceptible

membranes, resulting in the increase in membrane permeability and cell death. It is hypothesized that once absorbed from the intestines into the circulation beta-toxin might exert neurological effects on the host by affecting the distribution of cations across the membranes of susceptible nervous systems cells (Smedley et al., 2004).

Epsilon toxin is the third most lethal toxin of all clostridial toxins, behind only tetanus and botulinum neurotoxin. The epsilon-toxin causes fatal enterotoxemias mainly in goats and lambs and also is considered to be a potential bioterrorism agent. The toxin is absorbed across the mucosal barrier and enters the blood stream, either through direct damage of the gut wall or through a change in the intestinal permeability. Goats and lambs fed on high fiber diets are at a higher risk of Epsilon toxin induced enterotoxemia. Once the toxin reaches the target organs (brain, kidneys and lungs), it interacts with the appropriate receptors on vascular endothelial cells. It forms a nonselective pore leading to the absorption of large amounts of toxin into the systemic circulation. This results in a cascade of molecular events that begins with loss of transepithelial resistance and terminates with edema of the intoxicated tissues (Smedley et al., 2004).

Clostridium perfringens type A strain also produces an enterotoxin (*cpe*) that is responsible for gastrointestinal symptoms. Type A strains containing the *cpe* gene have frequently been isolated from food poisoning outbreaks and from livestock such as foals, and fattening pigs with diarrhea (Smedley et al., 2004).

Clostridium perfringens type E produces iota-toxin. This is a binary toxin that consists of two non-linked proteins implicated in sporadic outbreaks of diarrhea in animals. The binding component (Ib) is required for cells to internalize an enzymatic component (Ia) that ADP-

ribosylates G-actin. This toxin has been shown to cause enterotoxaemia in calves and lambs (Hatheway, 1990).

In addition to the above mentioned major toxins, there are other toxins which play an important role in many human and animal diseases. Gibert et al. (1997) identified the *cpb2* gene, located on a large plasmid which encoded a necrotizing toxin named beta2 (β_2). Several studies have indicated the association of beta2 positive *C. perfringens* isolates with enteritis in piglets, typhocolitis in horses, diarrhea in dogs, ulcerative enteritis in an african elephant, and enterotoxaemia in horses (Waters et al., 2005). Since its recognition as a separate toxin producing strain, *C. perfringens* that encodes for beta2 gene has been also isolated from various avian and aquatic species (Schotte et al., 2004). Fisher et al. (2005) suggested the association of beta2 toxin as an accessory toxin in *C. perfringens* enterotoxin linked with antibiotic associated diarrhea (AAD) and sporadic diarrhea (SD). The authors demonstrated that more than 75% of the AAD isolates carried the beta2 toxin gene and >97% of these isolates produced beta2 toxin.

Theta toxin, also called perfringolysin O, is another important extracellular protein involved in pore formation and lysis of red blood cells (Tweten, 1997). Awad et al. (2001) demonstrated the ability of theta toxin in conjunction with the alpha toxin to modulate the host inflammatory responses, thereby causing leukocyte accumulation within blood vessels and preventing the influx of phagocytic cells into infected host tissue.

Conventional bacteriology and PCR based confirmation techniques were employed to identify the five toxinotypes of *C. perfringens* (Augustynowicz et al., 2000). Real-Time PCR technology allowed for the simultaneous detection and amplification of specific gene targets, thereby providing immediate identification of pathogenic bacteria. In addition, real-time PCR provided higher sensitivity over conventional PCR and was less time consuming as post-PCR

processing was avoided. Kalender et al. (2005) have reported typing of isolates of *C. perfringens* from healthy and diseased sheep by conventional multiplex PCR. Wise et al. (2005) performed quantitative detection of *C. perfringens* in the broiler fowl gastrointestinal tract by real-time PCR. The assay could detect approximately 50 fg of *C. perfringens* genomic DNA. The assay could detect up to 10^2 CFU/g of *C. perfringens* from ileal content and 10^4 CFU/g from cecal samples.

In summary, the pathogenicity of *C. perfringens* can be attributed to the toxins and enterotoxin it is able to produce when provided with suitable conditions for sporulation in the intestine of the host. *Clostridium perfringens* elicits a host response through the toxins it releases while sporulating. Based on these observations it can be said that *C. perfringens* has a less complex mode of action on the host tissue as compared to other foodborne pathogens such as *Salmonella* spp., *Listeria monocytogenes* and enteropathogenic *Escherichia coli*.

2.2.4 *Clostridium perfringens* diseases in animals

The pathogenicity of *C. perfringens* is associated with the production of proteinacious toxins. Most of the *C. perfringens* toxins (α -, β -, ϵ -, and enterotoxin) interact with the cell membrane with the exception of the iota toxin which acts intracellularly (Songer, 1996). The enteric diseases caused by *C. perfringens* are characterized by necrosis and intense inflammation of the intestinal mucosa with bleeding into the lumen (McClane, 2001).

Clostridium perfringens colonizes the intestinal mucosa, multiplies and causes damage to the target cells (Manteca et al., 2001). When bacterial toxins come in contact with host cells, they either inhibit protein synthesis or disrupt cellular membranes by forming membrane pores for delivery of toxic components to specific cellular sites (Tilley and Saibil, 2006).

Clostridium perfringens type A causes lamb enterotoxemia in suckling lambs (McGowan, Moulton & Rood, 1958). Researchers have reported *C. perfringens* type A diseases characterized by profuse watery diarrhea, high mortality in many domestic species of animals especially horses (Wierup, 1977; Wierup & DiPietro, 1981). Mild necrotic enterocolitis was observed in suckling and fattening pigs infected with *C. perfringens* type A isolates (Collins et al., 1989; Waters et al., 2003). In the environment, *C. perfringens* type A isolates are the predominant isotypes (>95%) as compared to type B-E (Bueschel et al., 2003). Type A *C. perfringens* contaminated feed and the environment are the major sources of necrotic enteritis in poultry (Craven et al., 2001). In humans, *C. perfringens* Type A isolates are important etiological agents of myonecrosis (Rood, 1998). The enzyme phospholipases C associated with alpha toxin are responsible for tissue damage. The alpha toxin has lethal, necrotizing, hemolytic, and cytolytic activities.

Songer et al. (1996) reported *C. perfringens* type B as a primary causative agent of dysentery in newborn lambs and chronic abdominal pain without diarrhea in older lambs. Affected animals showed signs of systemic toxicity caused by beta and epsilon toxins. These toxins were hypothesized to alter the intestinal membrane barrier and produce toxicity (Fisher et al., 2006). The beta toxin is produced by type B and C strains of *C. perfringens*.

Trypsin inhibitors present in the diet acts as a cofactor in beta toxin induced necrotic enteritis (Hunter et al., 1993). Current literature strongly suggests that β -toxin is a pore forming toxin which oligomerises endothelial cells to form channels in susceptible membranes, resulting in the loss of membrane permeability and cell death (Smedley et al., 2004).

Clostridium perfringens type C affects a wide range of host species including cattle, sheep, pigs and dogs (Songer et al., 1996). A lack of competitive gut flora makes neonates or

young ones prone to infection (Johansson et al., 2006). The infection is characterized by hemorrhagic or necrotic enterotoxemia accompanied by blood and necrotic fragments of the intestinal tissue in the feces (Songer et al., 1996). Lawrence (1997) reported that type C isolates were the only non-type A isolates to cause enterotoxemia in animals and humans. In adult sheep, type C isolates caused rapid, fatal enterotoxaemia referred to as Struck.

Niilo (1986) reported that young pigs with type C enterotoxemia resulted in 50 and 100% mortality and morbidity, respectively. Reports of fatal enterotoxemia were reported by other researchers in neonatal calves and foals (Griner & Bracken, 1953; Griner & Johnson, 1954).

In humans, Type C clostridium isolates cause enteritis necroticans (EN). The disease has been reported in countries including Germany (Lawrence, 1997), Papua New Guinea (Lawrence et al., 1979) and Cambodia (Johnson et al., 1987). Researchers have reported cases of EN in the US in patients with pancreatic diseases (Severin et al., 1984; Petrillo et al., 2000; Tonnellier et al., 2001; Gui et al., 2002; Li et al., 2004). Devitt and Stamp (1983) reported that cases of type C EN in humans were more prevalent in developing countries and rare in developed countries. Isolates of Type D clostridium cause enterotoxaemia in sheep referred to as Pulpy Kidney disease. It affects lambs up to one year of age due to overeating (Popoff, 1984).

Clostridium perfringens Type D isolates produce predominantly epsilon toxin. The epsilon-toxin causes fatal enterotoxemias mainly in goats and lambs and is considered to be a potential bioterrorism agent.

Clostridium perfringens type E produces iota-toxin, a binary toxin that consists of two non-linked proteins implicated in sporadic diarrheic outbreaks among animals. This toxin was implicated in calf and lamb enterotoxemia (Hatheway, 1990).

Based on the reported findings, toxins produced by *C. perfringens* can cause enteric diseases in wide range of animal species. The enteric diseases are characterized by watery sporadic diarrhea, edema of affected tissues and loss of body weight. The mechanism of the toxin to induce enteritis includes direct effects on the transport of ions in the intestinal epithelial cells through membrane binding or pore formation. *Clostridium perfringens* and its repertoire of toxin induced pathologies in a wide range of species are a major area of concern for veterinarians, producers and researchers.

2.3 *Clostridium perfringens* beta2 toxin

Over the past decade a variant of the beta toxin known as beta2 (*cpb2*) has been associated with enteric diseases in a wide range of animals including swine, cattle, poultry, sheep, horses, dogs, avian and aquatic species (Boujon et al., 2005; Klaasen et al., 1999; Garmory et al., 2000; Waters et al., 2003). Beta2 toxin has been implicated as an accessory toxin in *C. perfringens* mediated antibiotic associated diarrhea and sporadic diarrhea (Fisher et al., 2005).

Summary of epidemiological studies linking *C. perfringens* beta2 toxin to human and animal gastrointestinal diseases are listed below in Table 2.3.

Table 2.3. Epidemiological Studies linking beta2 toxin and diseases in different species

Toxinotype	Species	Pathology	Reference
A	Swine	Necrotic enteritis	Gibert et al. (1997)
A	Swine	Diarrhea	Klaasen et al. (1999)
A	Swine	Diarrhea, gastroenteritis	Waters et al. (2003)
A	Swine	Diarrhea	Bueschel et al. (2003)
A	Swine	Enteritis	Jost et al. (2005)
A	Swine	Enteritis	Jost et al. (2006)
A	Bovine	Enterotoxaemia	Manteca et al. (2002)
A	Bovine	Hemorrhagic bowel syndrome	Dennison et al. (2005)
A	Bovine	Enterotoxaemia	Lebrun et al. (2006)
A	Bovine	Hemorrhagic bowel syndrome	Ceci et al. (2006)
A	Bovine	Hemorrhagic enteritis	Songer & Miskimins (2005)
A	Equine	Fatal and Non fatal Typhlocolitis	Herholz et al. (1999)
A	Equine	Typhlocolitis	Bacciarini et al. (2003)
A	Equine	Type A myonecrosis	Choi et al. (2003)
A	Equine	Gastrointestinal diseases	Waters et al. (2005)
A	Equine	Typhlocolitis	Vilei et al. (2005)
A	Equine	Enterocolitis	Timoney et al. (2005)
A	Caprine	Enterotoxaemia	Dray et al. (2004)
A	Cervidae	Clostridial enterotoxaemia	Embury-Hyatt et al. (2005)
A	Humans	Antibiotic associated diarrhea	Harrison et al. (2005)
A	Humans	Food poisoning	Fisher et al. (2005)
?	Ursidae/Bear	Necrotic and Hemorrhagic enteritis	Greco et al. (2005)
A	Poultry	Necrotic enteritis	Gholamiandekhordi et al. (2006)
?	Ciconiidae/White Stork	Enterotoxaemia	Boujon et al. (2005)
A	Pisces/Atlantic cod	?	Ashfalk & Muller (2002)

Based on these studies, *C. perfringens* beta2 toxin affects a variety of animal species and birds. In humans the toxin has been associated with enterotoxin in antibiotic associated diarrhea. The clinical signs observed in *C. perfringens* beta2 toxin mediated enteric diseases ranged from pasty to watery diarrhea with blood in feces, abdominal pain, and loss of body condition. It is hypothesized that beta2 toxin may act in synergy with other major toxins of *C. perfringens* in the production of necrotic and hemorrhagic enteritis. The isolation of beta2 toxigenic *C. perfringens* from healthy animals is considered a risk and an indicator of a potential health hazard. Circumstances provoking enteric dybiosis, and other predisposing factors could lead to clostridial disease outbreak.

2.3.1 Pathology of beta2 *Clostridium perfringens* enteric diseases

Klaasen et al. (1999) reported the pathology of beta2 toxin producing *C. perfringens* strain infection in piglets. The postmortem changes were predominantly in the small intestine, which ranged from pasty or watery contents to hemorrhages. The piglets had signs of necrotic enteritis and other pathological changes mostly in the small intestine (mild fibrinous enteritis).

Manteca et al. (2001) conducted necropsies of 78 cattle that died either suddenly or very rapidly due to beta2 toxin producing *C. perfringens*. Hemorrhagic enteritis, mainly of the small intestine, was observed. Microbiologically, the intestinal lesions included necrosis of the top of the intestinal villi, intestinal epithelial cells and/or necrosis of the intestinal villus vascular axis.

Gross pathological lesions in the gastrointestinal tract of horses suspected to be infected with *C. perfringens* showed massive congestion, edema, and hemorrhage of various parts of the intestine. Histological changes included extensive necrosis to autolysis of the intestinal tissue (Bacciarini et al., 2003). Choi et al. (2003) described a case of Clostridial myonecrosis in a horse

in Korea. Macroscopic lesions included acute hemorrhagic myonecrosis accompanied by severe interfascicular and intrafascicular edema and hemorrhage of the right gluteal area. Microbiologically, the lung showed severe hemorrhage with mild neutrophilic infiltration.

Enterotoxaemia in a 5-week old goat associated with *C. perfringens* type A and beta2 toxin revealed serosanguinous fluid in the peritoneal cavity on postmortem examination. The small and large intestines showed signs of diffused congestion, edema and contained a large amount of hemorrhagic fluid. There was also enlargement and congestion of the mesenteric lymph nodes (Dray, 2004).

Embury-Hyatt et al. (2005) reported a *Clostridium perfringens* type A associated sudden death syndrome in farmed deer. Necropsy of the affected animals revealed splenomegaly, petechial intestinal hemorrhages and mucosal necrosis. Other changes like abomasal hemorrhage, random hepatic necrosis, and multifocal hepatic congestion also were observed.

Songer and Miskimins (2005) described a case of *Clostridial* abomasitis in a cloned gaur calf. At necropsy, the abomasum was distended and contained clotted milk and bloody fluid. The abomasal and the omasal walls were thickened and hemorrhagic. The duodenum showed hemorrhagic and emphysematous lesions at the proximal end. Histological lesions included gram positive rods in association with acute, hemorrhagic mucosal inflammation.

Greco et al. (2005) reported enterotoxemia associated with the presence of *C. perfringens* producing beta2 toxin in the tissues and intestinal contents of Asiatic black bears. Macroscopic changes associated with the disease consisted of necrotic and hemorrhagic enterocolitis. The intestinal mucosa was dark and filled with watery, blood-stained fluid. The myocardium showed petechial and ecchymotic hemorrhages, lungs were congested and edematous, and the liver was pale and friable.

Songer and Uzal (2005) reviewed Clostridial enteric infections in pigs. At necropsy, prominent lesion in animals affected with clostridial enteric diseases were observed in the small intestine, especially jejunum, and occasionally in the large intestine. Other lesions observed were intestinal and mesenteric hyperemia, and diffuse or segmental fibrinonecrotic enteritis with emphysema and bloody gut contents. Lymph nodes were swollen and hemorrhagic and the presence of hemorrhagic peritoneal and pleural fluid also was observed. Microbiologically, hemorrhagic necrosis of the intestinal wall, affecting all the layers starting from mucosa was observed. The luminal surface was covered by a pseudomembranous layer composed of degenerated and necrotic desquamated epithelial cells, cell debris, and inflammatory cells including neutrophils, lymphocytes, plasma cells, macrophages, fibrin, various large, thick bacteria and a few subterminal spores.

Clostridium perfringens was the most commonly identified Microbiolorganism in neonatal diseases in lambs and kids (Greco et al., 2005). Necropsy of the affected animals revealed the presence of peritoneal and pericardial bloody effusions, congested edematous lungs, and a pale friable liver. There was prominent catarrhal and hemorrhagic enterocolitis. Other postmortem lesions included ecchymotic hemorrhages on the myocardium and kidney cortices, and necrotic enteritis. Blood clots in both of the ureters also were observed (Greco et al., 2005).

Ceci et al. (2006) demonstrated the association of *C. perfringens* type A in hemorrhagic bowel syndrome in dairy cattle. Hemorrhagic enterocolitis in the small and large intestine was the most prominent macroscopic lesions observed. Large blood clots that had adhered to the mucosa also were observed in the jejunum. Few animals showed the presence of hematomas into the mucosa and sub-mucosa of the jejunum. Histological examination revealed moderate to severe necrotic hemorrhagic enteritis especially in the jejunum. Proliferation of lymphocytes,

plasma cells, eosinophils and neutrophils into the lamina propria was observed. Signs of focal necrosis of the mucosa and desquamation of the epithelium were evident. Other signs included complete destruction of the mucosa and severe hemorrhages and hematomas in the submucosa, muscular tunica, and serosa.

Based on the reported findings related to the gross pathologies of *Clostridium perfringens* beta2 toxin induced enteritis seen in food producing and wild animals, the gross pathology is characterized by hemorrhage and necrosis of the small and large intestine. Other prominent gross pathologic lesions include swollen, hemorrhagic mesenteric lymph nodes, and thickened and congested abomasal walls. The characteristic histological lesions include degenerated and necrotic desquamated epithelial cells, cell debris, inflammatory cells, fibrin and variable number bacteria and spores. The pathologic lesions observed suggest *C. perfringens* beta2 toxin is a primary enteric toxin affecting the nutrient absorptive capacity of the intestine, thereby debilitating the affected animal.

2.3.2 Vaccination control of *Clostridium perfringens* enterotoxemias

Enteric diseases of livestock and poultry remain economically important causes of production loss. A survey conducted by the National Animal Health Monitoring System in 2002 (<http://www.aphis.usda.gov/vs/ceah/ncahs/nahms/dairy/#dairy2002>) indicated the continuing importance of enteric diseases caused by *E. coli*, *Salmonella*, *Clostridium perfringens* and *Mycobacterium paratuberculosis*, as major sources of morbidity, mortality, and associated economic costs in animal agriculture. With the continued concern over the use of antibiotics in animal agriculture, there is an urgent need to find safe and practical alternatives to prevent and control enteric diseases (Gurjar et al., 2007). Sound biosecurity measures play an important role

in preventing many enteric diseases, but a lacunae exists in our understanding of enteric disease complexes and the ecological and host interactions that lead to disease and production losses.

Clostridium perfringens type B, C, and D are the most commonly encountered toxinotypes in veterinary medicine (Ebert et al., 1999). The major lethal toxins produced by these types are beta and epsilon. Necrotic enteritis in pigs, lambs and calves, caused by type C beta -toxin, and enterotoxaemia (pulpy kidney disease, lamb dysentery) caused by type D epsilon-toxin has been found worldwide. Prevention of these diseases through active immunization using toxoids has been successful to some extent.

Vaccination against clostridial enterotoxemias was initiated in the early 1970's. Feedlot lambs were immunized against clostridial enterotoxemia using enterotoxemia toxoid and bacterin (Pierson, 1968). The objective of the trial was to analyze differences in the immune response elicited by the two. Based on the results of the trial, the researchers determined that there was no significant difference between a toxoid and bacterin vaccine. Both had the same level of efficacy in protecting lambs against enterotoxemia.

Oxer et al. (1971) conducted vaccination trials of commercially available *C. welchii* type D vaccines alone or in combination with other clostridial vaccines in sheep and lambs. They observed a passive transfer of antibodies from the vaccinated dams to the new born lambs. The antibodies conferred protection up to 8 weeks of age in lambs against pulpy kidney disease, and in a few cases, the protection extended to 16 weeks of age.

Cameron (1980) described an effective immunization strategy in lambs against enterotoxemia. The administration of 2 injections of oil adjuvant vaccine or one injection of oil adjuvant followed 4 weeks later by an injection of alum-precipitated toxoid elicited protective

antitoxin levels that persisted for 8 months. A booster injection of alum-precipitated toxoid given at this stage resulted in an immunity that lasted for at least 1 year.

Blackwell et al. (1983) compared two commercial enterotoxemia vaccines in goats. Regardless of the vaccine used or dosage administered local tissue reactions at injection sites persisted for six months in 53% of the goats. Fleenor and Scott (1983) analyzed isotypic antibody response against *C. perfringens* beta toxin in dams. The results of the study demonstrated that pathogen-specific IgM response was predominant in dams' sera, while pathogen-specific IgA response was predominant in colostrum and neonatal sera. A similar observation was made by Clarkson et al. (1985) who showed that antibodies in colostrum provided passive immunity to the lambs against clostridial antigens.

Odendaal et al. (1988) studied the protective effect of semi-purified hyperimmune serum against *C. perfringens* type D in weaned lambs. Their study revealed that hyperimmune serum provided passive protection in vaccinated lambs. In another study, they showed that immunization increased the epsilon antitoxin titer in immunized lambs and had no negative effects on the development of an initial active immunity (Odendaal et al. 1989). Vaccination of ewes before lambing imparted passive protection in lambs up to 12 weeks of age while vaccination of young lambs did not provide any supplementary protection. (de la Rosa et al., 1997).

Uzal and Kelly (1998) evaluated the protective efficacy of *C. perfringens* type D epsilon toxoid in goats. They did not observe any clinical or postmortem lesions in vaccinated animals after challenge. Moderate to high serum levels of anti-epsilon antibody were observed in the vaccinated animals. These antibodies appeared to protect the goats against both the systemic and the intestinal effects of *C. perfringens* type D toxins. In another study, Uzal et al. (1998)

examined serum antibody titers in goat kids to commercial *C. perfringens* epsilon toxoid vaccine. The study demonstrated that the antibody levels were below the protective level (0.25 IU/ml) 98 days after vaccination.

Troxel et al. (1997) conducted vaccination trials to determine the efficacy of the multicomponent clostridial vaccine in beef cows and calves. The results of the study showed that the titers for clostridial diseases in 50 to 53 day old calves could be enhanced if dams were vaccinated approximately 4 months before calving. They also demonstrated that an interval of 120 days between vaccinations was too long to confer adequate protection.

Djurickovic et al. (1975) studied the levels of antitoxin titers in milk and colostrum of sows vaccinated with a toxoid of *C. perfringens* type C strain. Vaccination of sows with a primary dose five weeks prior to farrowing, followed by a booster dose two weeks later led to optimal antibody titers in the milk and colostrum of sows. The serum of the piglets had antitoxic activity that could have occurred due to passive transfer of antibodies through milk and colostrum.

Mietzner et al. (1992) immunized mice using a synthetic peptide homolog of *C. perfringens* type A enterotoxin (CPE) conjugated to a thyroglobulin carrier. The immunized mice produced antibodies that neutralized native enterotoxin cytotoxicity. Small fragments of the *C. perfringens* alpha toxin representing the N-terminal (1-249) and C-terminal (247-370) domains of the toxin were evaluated for their immunoprotective efficacy against experimental gas gangrene. Antibodies against C-terminal fragment could neutralize both the phospholipase C and hemolytic activities of the alpha toxin. It also provided protection in a mouse model against at least 10 LD₁₀₀ doses of *C. perfringens* type A challenge (Williamson and Titball, 1993).

Recombinant vaccinia virus expressing the nontoxic C-domain of *C. perfringens* alpha-toxin was constructed and used as a vaccine against *C. perfringens* alpha toxin. The recombinant vaccinia virus elicited an antibody mediated response against a lethal challenge with alpha-toxin (Bennett et al., 1999).

Schoepe et al. (2001) examined the ability of a naturally occurring *Clostridium perfringens* mutant strain 121A/91 as a vaccine candidate against alpha toxin associated diseases. This strain lacks the phospholipase C and hemolytic activity exhibited by wild type strains. The alpha toxin mutant strain successfully induced antibodies that significantly reduced the activity of wild-type toxin. The same investigators in another study demonstrated the reversion of an arginine residue at position 212 to histidine in the alpha toxin variant strain enhanced protective antibody production against wild-type alpha toxin of *C. perfringens* (Schoepe et al., 2006).

The protection against *C. perfringens* mediated enteritis and enterotoxemias conventionally has been achieved by using antitoxin toxoids and bacterins. The toxoids have been successful in transferring passive immunity to newborns through colostrum and sera of vaccinated dams. The antitoxin antibodies offered protection against clostridial enteric diseases in the first few weeks in the newborns. Traditionally, multicomponent clostridial vaccines that confer protection against *C. perfringens*, *C. septicum*, *C. novyi*, and *C. chauvoei* have been used successfully to protect animals against clostridial infections. The adjuvants incorporated in the vaccines elicit strong inflammatory reactions. These side effects have led to development of new technologies for the development of vaccines against *C. perfringens* mediated enteritis. To overcome the side effects of toxoid based vaccine, researchers have examined recombinant virus, mutant strains, and synthetic peptide technologies to develop efficient vaccines against *C. perfringens* toxins. The new methodologies have been successful in eliciting a protective

immune response in animal models. Although the commercially available vaccines confer protection against the well-defined major *C. perfringens* toxins, evidence of these vaccines providing protection against novel beta2 toxin has not been reported. With the reports of *C. perfringens* beta2 toxin as a potential accessory toxin in *C. perfringens* induced enterotoxemias, efforts should be directed in developing vaccines conferring protection against beta2 toxin.

2.4 New methodologies in vaccine design

Historically, vaccination has been considered a public health tool aimed at populations rather than individuals (Lombard et al., 2007). The benefits of veterinary vaccines rise above the limits of one species as many of them provide protection to humans from anthroozoonoses. The focus in veterinary vaccinology has changed from animal diseases to animal infections (Pastoret et al., 2007). Currently available vaccines are based on killed or live-attenuated Microbiolorganisms, on toxins detoxified by chemical treatment, on purified antigens, and on polysaccharide conjugated to proteins (Scarselli et al., 2005).

Based on the knowledge of pathogenesis of the Microbiolorganisms and their primary virulence factors, second generations of vaccines are being developed. The model approach in vaccine design primarily has been based on various molecular biology techniques. The techniques such as DNA Microbiolarray, two dimensional gel electrophoresis and mass spectrometry have enabled the manipulation of the genome of pathogenic Microbiolorganisms (Scarselli et al., 2005).

Completed genome sequences for bacterial pathogens of food animals and of public health importance are being analyzed to identify genes associated with virulence and their products that could elicit a host immune response (Gay et al., 2007). Cattle pathogens such as

Bacillus anthracis, *Mycobacterium avium* subspecies *paratuberculosis*, *Brucella abortus*, and *Leptospira*, as well as swine and poultry pathogens including *Pasteurella multocida* and *Bordetella avium* are among the veterinary pathogens with published genome sequences (Gay et al., 2007).

The most striking feature of the whole genome analysis of pathogenic organisms is targeted selection of protective antigenic determinants. These genomic technologies have allowed the researchers to move away from empirical approaches in vaccine development towards a more focused, logical development and discovery of protective DNA segments (Gay et al., 2007).

A pragmatic change in vaccine discovery has been the advent of bioinformatics. Application of bioinformatic algorithms to analyze gene products has provided a more powerful approach in vaccine design and discovery (Rappuoli, 2000). The use of *in silico* analysis of the genetic information instead of the pathogenic organism itself has proved very promising. Prediction of proteins (secreted or surface) through bioinformatics tools used to screen whole genomes of pathogens could be considered as vaccine candidates (Reid et al., 2001). The application of a pan-genome approach permits identification of candidate vaccines by comparative genomics (Gay et al., 2007).

Application of bioinformatics to predict immunodominant epitopes for the design of epitope vaccines has modernized vaccine design (Korber et al., 2006). ‘Immunoinformatics’, a science of epitope prediction and vaccine design, has been successfully adapted in the new era of vaccinology. The prediction of T-cell epitopes is based on anchor motifs in the binding groove of major histocompatibility complex molecule (Nielsen et al., 2004), while the prediction of

discontinuous B-cell epitopes is primarily based on the 3D structure of the target protein (Haste et al., 2006).

Multiepitope synthetic lipopeptide vaccine against foot and mouth disease virus (FMDV) was successfully designed. The vaccine contains seven T-cell epitopes, an immunostimulating principle of bacterial lipoproteins and linear B-cell epitopes of FMDV (Hohlich et al., 2003). Vaccinated cattle demonstrated protection against viral challenge and a strong T-cell response against at least one of the peptides used for immunization. The results successfully demonstrated the ability of synthetic lipopeptides to generate an antigen-specific response and protection.

Production of antigens and antibodies in plants has been under development for past few years (Floss et al., 2007). Ma et al. (2005) summarized the benefits of using plant system including product safety, production scale and economy, and storage distribution and safety. Curtiss and Cardineau (1990) described the expression of *Streptococcus mutans* surface protein antigen A (SpaA) in transgenic tobacco plants. Veterinary candidate vaccines have been produced using transgenic plants and engineered plant virus. Usha et al., (1993) successfully expressed a peptide representing an epitope of the VP1 envelope protein of the Foot-and-Mouth-Disease Virus (FMDV) on the surface of a plant virus particle.

Plants like *Arabidopsis thaliana* and tobacco are considered suitable candidates for generating transgenic plants expressing proteins of interest (Floss et al., 2007). The advantages of using transgenic plants as vehicle of antigen delivery are: (i) ease of oral delivery, (ii) induction of efficient mucosal immune response, and (iii) protection against pathogens interacting with host mucosal surfaces (Streatfield, 2005). Lamphear et al., (2004) developed a corn based delivery system for animal vaccines. Transgenic corn expressing Porcine Transmissible Gastroenteritis Virus (PTGV) was used to vaccinate gilts. The vaccine elicited

antibody levels in serum, colostrum and milk of immunized animals. Passive transfer of immunity was also demonstrated using suckling piglets.

Adjuvants have played a very important role in the efficacy of vaccines. Several criteria govern the choice of a safe and efficient adjuvant including target species, type of antigens, duration of immunity, type of immune response, and route of inoculation (Aucouturier et al., 2001). Emulsions historically have been used as common adjuvants but with strong local reactions.

Audibert and Lise (1993) classified adjuvants based on their source. Vegetal adjuvants included saponine and glucan extract, bacterial such as choleric toxin, lipopolysaccharides, chemicals such as aluminium hydroxide, and surfactants, cytokines such as IFN- γ or GM-CSF and hormones including dihydroxyepiandrosterone (DHEA).

The design of recombinant immunogens as future vaccines requires an adjuvant to improve their immunogenicity. In the era of Immunoinformatics germane to the issues of immunodominance and epitope identification is the identification of a potent and safe adjuvant. Bacterial lipoproteins and their synthetic analogues are considered strong immune modulators of early host responses (Sette and Fikes, 2003). The use of synthetic lipopeptides with emphasis on mucosal delivery was studied by BenMohamed et al. (2002). Lipopeptide based transgenic vaccine against melanoma was successfully used in mice (Sette and Fikes, 2003). The vaccine induced a strong cytotoxic T-lymphocyte response against the target antigen.

Several immunogenic vaccination strategies have effectively controlled several infectious agents. The traditional immunization arsenal includes the use of live attenuated vaccines, inactivated organisms, and conventional whole proteins. The widespread use of these conventional vaccines may be precluded from a safety and practicality standpoint. Recombinant

DNA vaccines, lipopeptide vaccines and transgenic plant based vaccines offer several potential advantages over conventional vaccines. The use of strong immunoadjuvants along with these immune determinants is of conventional necessity. Based on recent studies, the greatest advantage of lipopeptides is that they obviate the requirement of an adjuvant. Recent advances in our understanding of Microbiolbial genomics and development in peptide based vaccines indicates that reverse vaccinology is a promising approach.

2.5 References

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CHAPTER 3

Real-Time Multiplex PCR assay for Rapid Detection and Toxinotyping of *Clostridium perfringens* Toxin Producing Strains in Feces of Dairy Cattle.

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3.1 Abstract

Clostridium perfringens is an anaerobic, gram positive, spore-forming bacterium associated with a wide variety of diseases in domestic animals and humans. We have developed dual-labeled fluorescence hybridization probe (TaqMan®) based real time multiplex PCR assay for detection of toxin genes alpha (*cpa*), beta (*cpb*), iota (*ia*), epsilon (*etx*), beta2 (*cpb2*) and enterotoxin (*cpe*) of *C. perfringens* directly from cattle feces. The assay was standardized using ATCC reference strains of *C. perfringens* producing alpha, beta, iota, epsilon and enterotoxin respectively. The assay for detection of beta2 toxin gene was standardized using a field strain of *C. perfringens* producing beta2 toxin. Each real time assay was able to detect as low as 10 pg of DNA for all the six toxin producing strains of *C. perfringens*. A total of 307 fecal samples collected from 7 dairy herds in Pennsylvania were analyzed using the multiplex assay. The real time PCR assay revealed that *cpa*, *cpb*, *ia*, *etx*, *cpb2* and *cpe* were detected in 68 (28.2%), 6 (2.5%), 6 (2.5%), 4 (1.6%), 164 (68%) and 11 (4.5%) of 241 PCR positive samples, respectively. The findings of the study revealed that *C. perfringens* beta2 toxin producing strains were widely prevalent in lactating cows in Pennsylvania and they may play an important role in *C. perfringens* associated diarrheal diseases.

3.2 Introduction

Clostridium perfringens is a gram positive, sporulating anaerobic bacterium responsible for a wide spectrum of diseases in animals and humans [1]. The bacterium is widespread in environmental matrices such as soil and water and is commonly found in the intestines of animals. *Clostridium perfringens* does not exhibit adherence and invasive properties towards healthy intestinal mucosa [2]. Alteration in the physiological equilibrium of the intestine and resident Microbiolflora due to antibiotic therapy, contaminated feed or improperly fermented silage allows colonization of toxigenic clostridia leading to enterotoxemia and hemorrhagic enteritis [3, 4]. The pathogenicity of the organism is associated with several toxins.

Members of the species *C. perfringens* can be subtyped into five toxin types (A, B, C, D and E) based on the production of four major toxins: alpha, beta, epsilon and iota [2]. Alpha-toxin (α -toxin), the main lethal toxin of *C. perfringens*, is a multifunctional phospholipase, produced in varying amounts by almost all *C. perfringens* isolates. It causes hydrolysis of membrane phospholipids in different cells, resulting in lysis [5, 6]. The beta-toxin (β -toxin) is a highly trypsin-sensitive protein. It causes mucosal necrosis resulting in central nervous symptoms observed in domestic animals [7]. Epsilon-toxin (ϵ -toxin) is a potent toxin responsible for lethal enterotoxaemia in livestock [2]. The iota-toxin (ι -toxin) consists of two proteins, one active component (Ia) and a binding protein (Ib). The Iota toxin is known to increase vascular permeability and is dermonecrotic and lethal to mice at higher doses [8, 9].

Along with the major toxins, enterotoxin and beta2 toxin (β_2 -toxin), play the major role in several severe diseases [2, 8, 9]. The beta2 toxin was identified recently [8, 9] and has been associated with enteric diseases in domestic animals, especially piglets [10, 11, 12] and horses

[3]. Fisher et al., [13] suggested a synergistic role of beta2 along with enterotoxin of *C. perfringens* in etiology of antibiotic associated diarrhea (AAD) and sporadic diarrhea (SD). Enterotoxin (CPE) is the most studied of the *C. perfringens* toxins [14]. It interacts with epithelial cell tight junction proteins, causing diarrhea and intestinal cramping due to leakage of water and ions [1].

Most veterinary diagnostic laboratories use bacteriological techniques for isolation and identification of *C. perfringens*. Detection of toxinotypes of *C. perfringens* is done by conventional PCR [15]. Real-time PCR technology allows for simultaneous detection and amplification of specific gene targets, thereby providing immediate identification of pathogenic bacteria. Additionally, real-time PCR provides higher sensitivity over conventional PCR and is less time consuming as post-PCR processing is avoided. We report *C. perfringens* toxin typing by real-time PCR assay using fluorogenic probes. The optimized assay was found not only to be specific and sensitive, but capable of analyzing cattle feces harboring *C. perfringens* toxin producing genes.

3.3 Materials and Methods

3.3.1 Selection of the primers and probes

Primers and the Taqman probes for the Real Time PCR were designed using Premier Biosoft Beacon Design Software (Palo Alto, CA, USA) (Table 3.1).

The FAM (6-carboxy-fluorescein) was used as fluorescent reporter dye and conjugated to 5' ends of probes to detect amplification products specific for alpha toxin gene (*cpa*), epsilon toxin gene (*etx*), and iota toxin gene (*ia*), respectively. The HEX (6-carboxy-2', 4, 4', 5', 7, 7'-hexachlorofluorescein) was used as fluorescent reporter dye and conjugated to 5' end of probes

to detect amplification products specific for beta2 toxin gene (*cpb2*), beta toxin gene (*cpb*), and enterotoxin gene (*cpe*), respectively. The quencher dye TAMRA (6-carboxytetramethyl-rhodamine) was attached at the 3' end of all six probes. Primers and probes were synthesized by Sigma Genosys (St. Louis, MO, USA) and Operon Biotechnologies (Huntsville, AL, USA). Specificity of the probes and primers at the species level was verified by performing a BLAST search of GenBank (National Center for Biotechnology Information, MD, USA).

3.3.2 Preparation of Bacterial DNA

Five ATCC (Manassas, VA, USA) reference strains (Table 3.2) of *C. perfringens* that encode for α -, β -, ϵ -, ι -, and enterotoxin were used in the study. A *C. perfringens* strain that encoded for β 2 toxin was obtained from Dr. Brenda Love at the Pennsylvania Animal Diagnostic Laboratory. DNA from the six isolates was extracted from pure cultures grown in Reinforced Clostridial Medium (Oxoid, USA) broth for 20-24 h at 35°C in anaerobic conditions using a routine phenol-chloroform extraction method [17]. The concentration of the eluted DNA was determined by measuring the optical density at 260 nm using a spectrophotometer (Beckmann DU7400, CA, USA). Pure culture template DNA was stored at -20°C prior to PCR. The isolated DNA served as a template for standardization of the multiplex real- time PCR assay.

3.3.3 Isolation of the Bacterial DNA from Feces

Fecal samples were collected from the 307 lactating cattle from 7 dairy herds in central Pennsylvania. Fecal samples were collected per rectum using a sterile disposable rectal sleeve. Approximately 2-10 grams of feces was transferred from the sleeve to a sterile labeled 50 ml screw cap centrifuge tube. The tubes were transported to the laboratory over ice and processed

the same day. All fecal samples were examined for the presence of *C. perfringens* toxin genes (α , β , β_2 , ϵ , ι , and *cpe*) using Real time PCR assay. All the fecal samples collected were subjected to a 4 hour pre-enrichment in the Reinforced Clostridial Medium (RCM) by inoculating approximately 1 gram of feces into 9 ml of the pre-enrichment broth and incubated at 37⁰C for 4 hours. After incubation, 10 ml aliquots of 4 hour pre-enrichment broth culture were taken for DNA purification using QIAamp DNA Stool Mini Kit™ according to the manufacturer's instructions (Qiagen, CA, USA). The eluent from the spin column containing DNA was used immediately in PCR assay or stored at -70 °C for later use.

3.3.4 Standardization of optimal real-time multiplex PCR conditions

A duplex PCR reaction was standardized in three reaction tubes comprising the following *C.perfringens* toxins:

Reaction 1: *C.perfringens* alpha + beta2 toxin

Reaction 2: *C.perfringens* beta + epsilon toxin

Reaction 3: *C.perfringens* iota + enterotoxin

The 24 μ l duplex PCR reaction mix consisted of 5.5 μ l ddH₂O, 12.5 μ l of 2x Brilliant Multiplex QPCR Master Mix™ (Stratagene, CA, USA), 1 μ l of forward and reverse primers (10nmol) of respective toxin genes, and 1 μ l (100nmol) of Taqman hybridization probe for each toxin gene.

One Microbiolliter of target DNA template was added to each reaction and subjected to real-time PCR using the Stratagene MX 3000P instrument (CA, USA). Cycling conditions comprised of initial 10 min incubation to activate the DNA polymerase, followed by 40 cycles of denaturation at 95⁰C for 30 s and annealing and extension at 55⁰C for 1 min. Fluorescence was measured at 515 and 556 nm wavelength during the annealing step.

The primer/probe set was optimized for annealing temperature, primer concentration, probe concentration, primer ratios, template concentration, and cycling conditions (two step cycles). Optimal PCR conditions were defined as the lowest concentration of the DNA that produced a positive result. Positive reactions were assigned a cycle threshold (C_t) value equal to the cycle number where the fluorescence observed in the experimental tube was first detectable above background. A negative result was assigned when no amplification occurred or the (C_t) value was higher than 36 cycles. The linear dynamic range of the real-time PCR assay was assessed using triplicates of serial logarithmic dilutions of DNA encoding for *C.perfringens* toxins α , β , β_2 , ϵ , ι and enterotoxin in PCR-grade water.

3.3.5 Determination of sensitivity of multiplex assay

To determine the sensitivity of the Real time PCR Multiplex assay, 10-fold serial dilutions of known concentrations of template DNA of alpha, beta, beta2, iota, epsilon and enterotoxin were assayed and standard curves were established.

3.3.6 Determination of multiplex assay specificity

DNA used to assess the specificity of the assay was prepared from a range of organisms that had been identified in the clinical laboratory, published in peer reviewed journals or were derived from commercial stocks (ATCC, Manassas, VA, USA). The bacteria included in the specificity assay are listed in Table 2. A fluorescent signal 10-fold higher than the standard deviation of the mean baseline emission was indicative of a positive detection.

3.4 Results

3.4.1 Detection sensitivity of real-time multiplex PCR assay

Genomic DNA was prepared from an overnight culture of *C. perfringens* strains, was 10 fold serially diluted and used as template to determine the sensitivity of real time PCR and to construct standard curves by plotting fluorescence values (dR) versus threshold cycle (C_t) produced for each of the six target genes (Figure 3.1). Standard curves showed a linear relationship between the fluorescence values and the C_t (PCR cycle at which the fluorescent intensity raises above the threshold). The six primers and probes sets produced similar amplification yields and fluorogenic detection of target genes. The detection limits of the PCR assay for target sequences *cpa*, *cpb2*, *etx*, *cpb*, *ia*, and *cpe* were 30, 70, 10, 10, 20, and 5 pg of DNA, respectively. The cycle threshold varied between 24 to 29 cycles for all the six target genes. The efficiency of amplification and correlation (R^2) of the data collected was measured after each PCR assay. The amplification efficiency for all the six Taqman probes was 100%. The regression coefficient obtained for the six gene targets was in the range of 0.982 to 1.00. The slopes of the curves for genes *cpa*, *cpb*, *cpb2*, *ia*, *etx*, *cpe* were -3.15, -3.49, -3.24, -3.197, -3.45 and -3.45 respectively (Figure 3.2). The slopes observed were within the linear range of the standard curve.

3.4.2 Specificity of the real-time multiplex PCR assay

The assay's specificity was demonstrated by its ability to exclude all non *C. perfringens* and non-clostridial species. The specificity of the primers and probes was tested and optimized against strains of *C. perfringens*, *C. difficile*, *C. novyi*, *C. glycolicum*, *C. septicum*, *C. sordelli* and other non-clostridial organisms as listed in Table 2, all of which were found to be negative.

3.4.3 Detection of *C. perfringens* toxin producing strains in feces of dairy cattle

A total of 307 fecal samples from 7 dairy herds in central Pennsylvania were examined to determine the prevalence and distribution of *C. perfringens* toxin producing strains in feces of lactating cattle.

The number of farms and cows that shed *C. perfringens* and their toxin type are shown in Table 3.3. PCR assay revealed that *cpa*, *cpb*, *ia*, *etx*, *cpb2* and *cpe* toxin genes were detected in 68 (28.2%), 6 (2.5%), 6 (2.5%), 4 (1.6%), 164 (68%) and 11 (4.6%) samples, respectively. Toxin gene typing of *C. perfringens* isolates from 241 PCR positive fecal samples revealed the presence of toxin type A (93%) being widely prevalent, followed by type B, C, E (2.5%) and D (1.6%). PCR reactions which showed no fluorescence or values similar to negative control were considered negative for the gene target. The cycle threshold for PCR positive samples ranged from 24-36 cycles, thereby indicating a wide variation in the bacterial load of toxigenic *C. perfringens* in feces of lactating cattle.

3.5 Discussion and Conclusions

With the advent of highly efficient detection chemistries, sensitive instrumentation, and optimized assays the number of DNA molecules of a particular sequence can be accurately determined with unique accuracy and sensitivity. Molecular methods with amplification and detection of target nucleic acids have generally been found to have superior sensitivity and specificity and have the potential to produce rapid results [18]. Bacteriology and animal based typing assays are being replaced by conventional PCR based confirmation techniques to identify the five toxinotypes of *C. perfringens* (A-E) [2].

To date, PCR assays have been developed for the specific detection of *C. perfringens* toxins in feces samples, all of them require post-PCR manipulations for analysis of amplification products by gel electrophoresis. Real-time PCR has advantage over conventional PCR in that it is the most quantitative and reliable tool to determine bacterial concentrations in environment and clinical samples.

Herein, we describe a simple and rapid culture independent real-time multi-duplex PCR for detection of *C. perfringens* toxin producing strains in fecal samples of lactating dairy cattle using Taqman[®] probes. At present, there has been limited efforts in the use of real time quantitative PCR to quantitate and identify *C. perfringens* in dairy cattle. Wang, et al., [19] employed a method using nonspecific DNA binding dye SYBR green to estimate *C. perfringens* DNA using the 16S rDNA primer pair. A quantitative real-time PCR assay utilizing fluorogenic, hydrolysis type probe was developed by Wise, et al., [16] to detect and quantify the levels of *C. perfringens* in the fowl gastrointestinal contents. The assay could detect approximately 50fg of *C. perfringens* DNA.

As reported in previous literature [20], most of the enteric pathogens, including *Clostridia*, are present in very low numbers in environmental samples like feces. Further, feces contain many inhibitory substances that affect the amplification efficiencies in a PCR reaction. Accurate determination of the threshold cycle (C_t) depends upon the efficient performance of the PCR amplification, and detection of the reporter fluorophores [21]. To improve the detection of such very low levels of *Clostridium* in feces, the samples were cultured in an appropriate pre-enrichment broth. In the present study, a pre-enrichment broth comprising of Reinforced Clostridial Media along with Neomycin [22] was used to enrich the fecal samples. The broth inhibited the growth of nonclostridial organisms and allowed for concentration and purification

of the target bacteria from the crude fecal samples. The QIAamp DNA stool Mini kit was found to be suitable for isolation of DNA from the pre-enrichment broth. The DNA extraction and purification procedure described using the kit provided steps for removing inhibitors such as polyphenolic compounds that could adversely affect the sensitivity of the assay. The use of a pre-enrichment broth and DNA stool mini kit prior to PCR amplification improved the sensitivity of the assay by reducing the C_t value from 34 cycle's (prior to pre-enrichment) upto 29 cycles (post pre-enrichment).

In the present study, we have demonstrated the ability of our real time multiplex PCR assay to distinguish *C. perfringens* toxin genes *cpa*, *cpb*, *cpb2*, *ia*, *etx*, *cpe* from other randomly selected non clostridial enteric organisms and other *C. perfringens*, *C. sordelli*, *C. difficile*, *C. septicum*, *C. glycolicum*, and *C. novyi* organisms. The specificity was confirmed by detection of the toxin genes only in *C. perfringens* without nonspecific detection in other enteric bacteria. The beta2 probe and primers showed late amplification (C_t value >35) when tested on *C. novyi* and *C. septicum* DNA. Since the amplification was observed late after cycle number 35, it was considered as negative. The sensitivities of the FAM and HEX labeled probes in specifically detecting and quantifying the six toxin genes were determined by plotting the DNA concentrations of the six strains used for the assay. The minimum detection limit ranged from 5 pg to 70 pg of DNA for the six toxin genes. The PCR assay was validated using fecal samples from seven dairy herds around Pennsylvania. Efforts were made to maximize and equalize the efficiency of amplification among each of the two primer probe sets. With the primers and probes designed in our study it was possible to perform multiplex PCR for simultaneous detection of six toxin genes of *C. perfringens* in a duplex PCR reaction. In this study, the

fluorescent signal obtained was directly correlated to target cell densities found in the fecal samples.

Of the 241 PCR positive fecal samples tested by the assay, 164 (68%) were positive for *cpb2* gene followed by 68 (28.2%) being positive for *cpa* gene. Previous studies have reported isolation of *C. perfringens* harboring beta2 toxin gene from healthy animals [23]. Bueschel et al., [24] screened 3270 field isolates of *C. perfringens* from various animal species and observed that 37.2% of PCR positive *C. perfringens* isolates harbored the *cpb2* toxin gene. They also reported 35.1% of Type A genotype which also harbored *cpa* gene carrying *cpb2* gene. These findings clearly suggest that α - toxin and β 2- toxin gene harboring *C. perfringens* genotype are widely distributed among animal species. Gibert et al., [9] suggested the presence of *Clostridium perfringens cpb2* toxin gene on a mobile genetic element, owing to its distribution in all toxin types except toxin type E. Schotte, et al., [23] suggested a synergistic action of alpha and beta2 toxin, resulting in an increase of necrotic and hemorrhagic lesions in the small intestinal loops infected with beta2 toxigenic *C. perfringens* type A. The presence of *C. perfringens* strains harboring *cpb2* toxin gene in healthy animals may not be considered risk by itself, but can be considered an emerging threat to animal health if the physiological equilibrium of the intestine and resident Microbiolflora is disturbed due to change in feed, antibiotic therapy [3] or supplementation of trypsin inactivating food components like soybean flour [4]. Beta, iota, epsilon and enterotoxin genes were detected in 6 (2.5%), 6 (2.5%), 4 (1.6%), and 11 (4.5%) isolates respectively.

The real-time multiplex PCR and detection of target toxin genes in fecal samples described here allows analysis of large number of fecal samples in a single assay. The assay

developed in this study may have widespread applications in future monitoring programs for *C. perfringens* toxin producing strains in animal agriculture, silage, feed and disease outbreaks.

3.6 ACKNOWLEDGMENTS

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Table 3.1 Primer and Probe Sequences for Real Time PCR Assay.

Target (nucleotide sequence Genbank accession number)	Oligonucleotide	Sequence (5'→3')
<i>cpa</i> L43545	cpa F	TGCACTATTTTGGAGATATAGATAC
	cpa R	CTGCTGTGTTTATTTTATACTGTTC
	cpa Pr	FAM-TCCTGCTAATGTTACTGCCGTTGA-TAMRA
<i>cpb2</i> L77965	cpb2 F	TAACACCATCATTTAGAACTCAAG
	cpb2 R	CTATCAGAATATGTTTGTGGATAAAC
	cpb2 Pr	HEX-TGCTTGTGCTAGTTCATCATCCCA-TAMRA
<i>cpb</i> X83275	cpb F	ATTCATTAGTTATAGTTAGTTCAC
	cpb R	TTATAGTAGTAGTTTTGCCTATATC
	cpb Pr	HEX-AACGGATGCCTATTATCACCAACT-TAMRA
<i>etx</i> M95206	etx F	TTAACTAATGATACTCAACAAGAAC
	etx R	GTTTCATTAAAAGGAACAGTAAAC
	etx Pr	FAM-TGCTTGTATCGAAGTTCCCACAGT-TAMRA
<i>ia</i> X73562	ia F	CAAGATGGATTTAAGGATGTTTC
	ia R	TTTTGGTAATTTCAAATGTATAAGTAG
	ia Pr	FAM-TTTCATCGCCATTACCTGGTTCAT-TAMRA
<i>cpe</i> X81849	cpe F	AACTATAGGAGAACAAAATACAATAG
	cpe R	TGCATAAACCTTATAATATACATATTC
	cpe Pr	HEX-TCTGTATCTACAACCTGCTGGTCCA-TAMRA

Table 3.2 Specificity of the real-time multiplex PCR assay.

Reference Strains	Toxins					
	<i>cpa</i>	<i>cpb</i>	<i>cpb2</i>	<i>etx</i>	<i>ia</i>	<i>cpe</i>
<i>C. perfringens</i> ATCC 13124	+	-	-	-	-	-
<i>C. perfringens</i> ATCC 51880	+	+	-	-	-	-
<i>C. perfringens</i> ATCC 27324	-	-	-	-	+	+
<i>C. perfringens</i> ATCC 3626	+	-	-	+	-	-
<i>C. perfringens</i> ATCC 12916	+	-	-	-	-	+
<i>C. perfringens</i> beta2, Dr.Love, PADLS	+	-	+	-	-	-
<i>C. difficile</i> , Dr.Love, PADLS	-	-	-	-	-	-
<i>C. novyi</i> , Dr. Love, PADLS	-	-	-	-	-	-
<i>C. sordelli</i> , Dr. Love, PADLS	-	-	-	-	-	-
<i>C. septicum</i> , Dr. Love, PADLS	-	-	-	-	-	-
<i>C. glycolicum</i> , Dr. Love, PADLS	-	-	-	-	-	-
<i>E. coli</i> ATCC 43895	-	-	-	-	-	-
<i>Salmonella typhi</i> ATCC 14028	-	-	-	-	-	-
<i>Listeria monocytogenes</i> J187 lab strain	-	-	-	-	-	-
<i>Staphylococcus aureus</i> ATCC 29213	-	-	-	-	-	-

Table 3.3 Prevalence and distribution of *Clostridium perfringens* toxintype in feces.

Toxin Type	Toxins	Cows	
		Positive / Total	%
A	$\alpha + \beta 2$	51/241	21
	$\alpha + \beta 2 + \text{cpe}$	3/241	1
	$\beta 2 + \text{cpe}$	7/241	3
	α	10/241	4
	$\beta 2$	154/241	64
B, C	$\alpha + \beta$	1/241	0.4
	$\beta + \beta 2$	4/241	1.6
	$\beta + \text{cpe}$	1/241	0.4
D	$\alpha + \varepsilon$	1/241	0.4
	$\varepsilon + \beta 2$	3/241	1
E	$\text{í} + \alpha$	2/241	0.8
	í	4/241	1.6

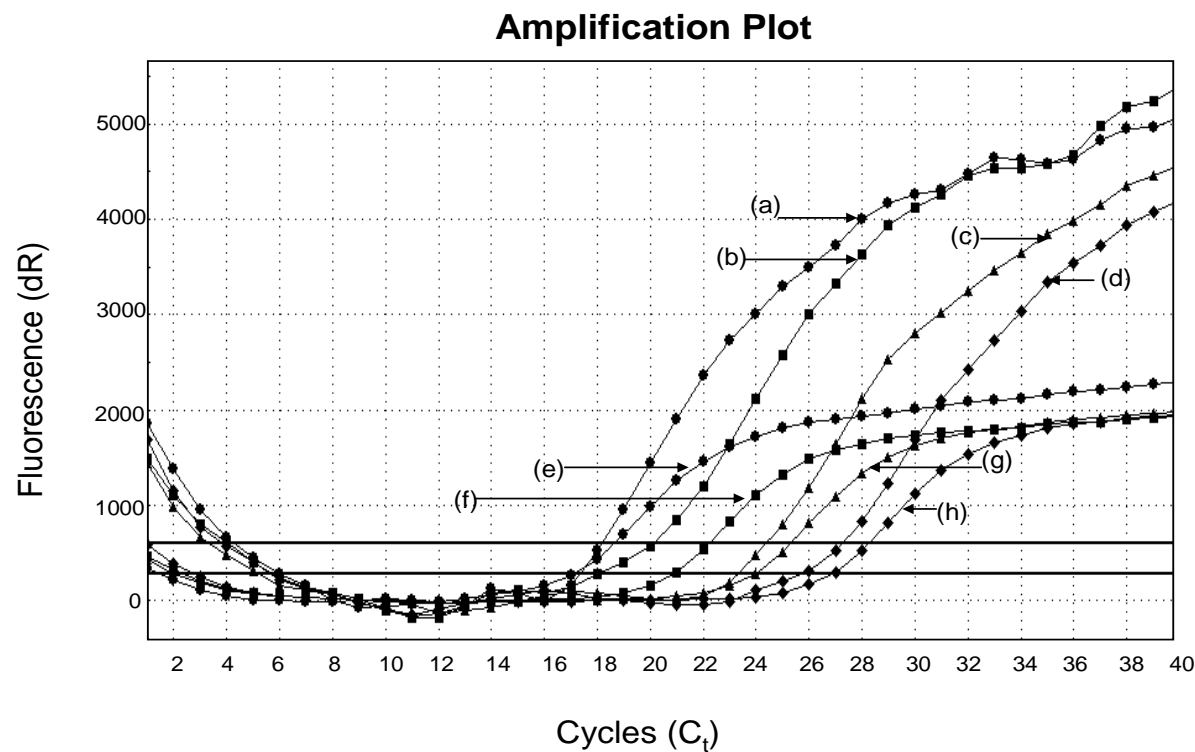


Figure 3.1 Amplification curves of Multiplex Real time PCR for detection *cpa* and *cpb2* gene of *C. perfringens*;
 (a) 3×10^4 pg of *cpa* DNA, (b) 3×10^3 pg of *cpa* DNA, (c) 3×10^2 pg of *cpa* DNA, (d) 3×10^1 pg of *cpa* DNA, (e) 7×10^4 pg of *cpb2* DNA, (f) 7×10^3 pg of *cpb2* DNA, (g) 7×10^2 pg of *cpb2* DNA, (h) 7×10^1 pg of *cpb2* DNA.

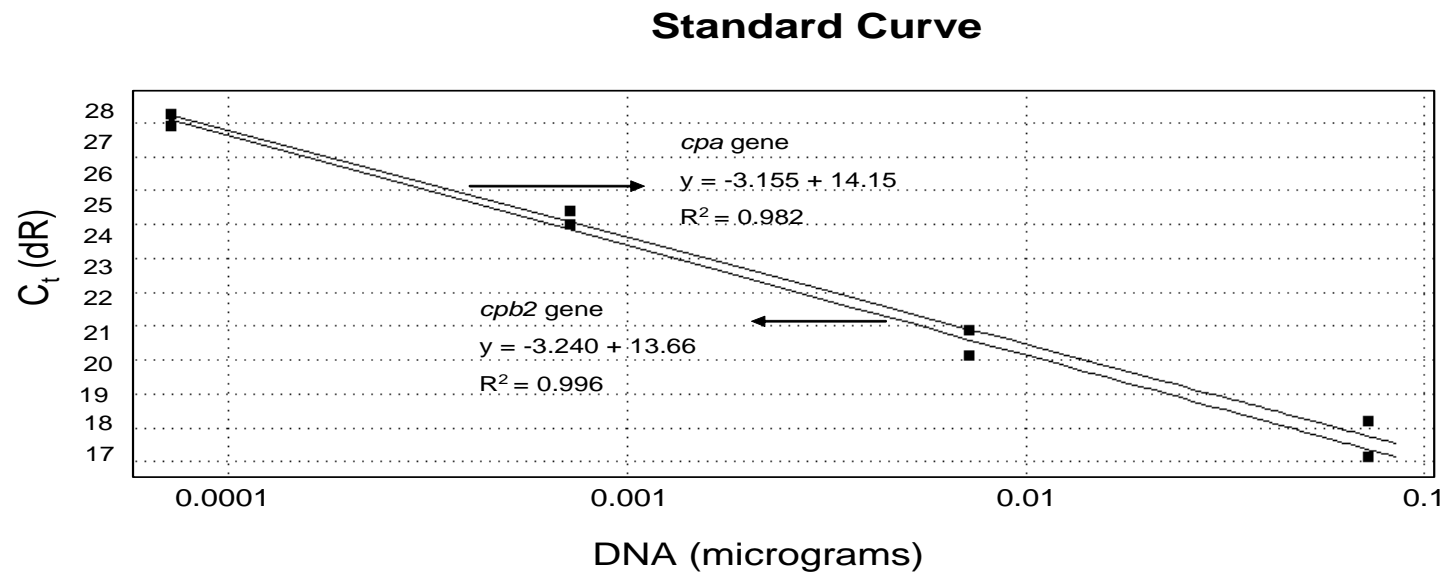


Figure 3.2 Linear regression of cycle threshold versus DNA concentrations for a serial logarithmic dilutions of *Clostridium perfringens* *cpa* gene and *cpb2* gene.

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CHAPTER 4

**Expression, crystallization and preliminary X-ray diffraction studies of recombinant
Clostridium perfringens beta2 toxin.**

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4.1 Abstract

Clostridium perfringens is a Gram positive, sporulating anaerobic bacterium responsible for a wide spectrum of diseases in animals, birds and humans. The virulence of *C. perfringens* is associated with the production of several enterotoxins and exotoxins. Beta2 toxin is a 28kD exotoxin produced by *C. perfringens*. It is implicated in necrotic enteritis in animals and humans, a disease that is characterized by a sudden acute onset with lethal hemorrhagic mucosal ulceration. The recombinant expression, purification and crystallization of the beta2 toxin using the batch under oil technique are reported here. Native X-ray diffraction data to 2.9 Å resolution was obtained at the synchrotron beam line at F2 station at Cornell High Energy Synchrotron Source (CHESS), Cornell on an ADSC Quantum-210 CCD detector. The crystals belong to space group R3, with the hexagonal axis setting of the unit cell parameters being $a = b = 103.71$ Å and $c = 193.48$ Å, $\alpha = \beta = 90^\circ$ $\gamma = 120^\circ$ with a dimer in the asymmetric unit. Self rotation function shows that the two molecules are related by a non-crystallographic two fold axis with polar angles, $\omega = 90.0^\circ$ and $\phi = 210.3^\circ$.

4.2 Introduction

Clostridium perfringens is an anaerobic, gram positive, spore-forming bacteria with an arsenal of virulence factors associated with a wide variety of diseases affecting most domestic animal species and humans (Songer, 1996; Niilo, 1986).

Clostridium perfringens is widely spread in the environment and frequently inhabits the intestines of animals. *Clostridium perfringens* does not exhibit adherence and invasive properties towards healthy intestinal mucosa (Songer, 1996). Alteration in the physiological equilibrium of the intestine and resident Microbiolflora due to antibiotic therapy, management related stress, ingestion of soil and feces contaminated feed or improperly fermented silage or haylage allows colonization of toxigenic clostridia leading to enterotoxemia and hemorrhagic enteritis (Herholz *et al.*, 1999; Niilo, 1986).

Clostridium perfringens bacterium is shown to produce at least 15 different toxins, 5 of which (alpha-, beta-, epsilon-, iota-, and enterotoxin) are responsible for tissue lesions and host death (Daube, 1992; Songer, 1996; Petit *et al.*, 1999). The toxins iota, alpha, beta, and epsilon have been used for classification of *C. perfringens* into five isotypes or toxin-types (A, B, C, D, and E) where each type carries a different combination of the toxin genes (Al-Khaldi *et al.*, 2004). The alpha-toxin gene (*cpa*) had been found in all 5 toxin-types, while the beta-toxin (*cpb*) is found only in types B and C. The epsilon toxin (*etx*) can be found in types B and D. The iota toxin (*iA*) has been shown to be present in type E only. In addition to the above mentioned major toxins, there are other toxins which play an important role in many human and animal diseases. *Clostridium perfringens* toxin-type A strains can produce an enterotoxin (*cpe*) and have been mostly associated with outbreaks of food poisoning.

Recently, a variant of the beta toxin known as beta2 (*cpb2*) has been associated with enteric diseases in a wide range of animals including swine, cattle, poultry, sheep, horses, and dogs (Klaasen *et al.*, 1999; Garmory *et al.*, 2000; Waters *et al.*, 2003). Since its recognition as a separate toxin producing strain, beta2-toxigenic *C. perfringens* has also been isolated from various avian and aquatic species (Boujon *et al.*, 2005). Fisher *et al.* (2005) suggested a potential role of beta2-toxin as an accessory toxin with enterotoxin in *C. perfringens* antibiotic associated diarrhea and sporadic diarrhea.

During a bacterial invasion bacterial toxins exhibit certain inhibitory mechanisms on the host system. They act by inhibiting protein synthesis or by disrupting cellular membranes. By forming membrane pores the toxins can deliver toxic components to specific cellular sites (Tilley *et al.*, 2006). These pore forming toxins are classified according to whether they form α -helical channels or β -barrels. *Clostridium perfringens* is implicated for necrotic and hemorrhagic enteritis and enterotoxemia in different species of animals. The pathogen colonizes the intestinal mucosa, multiplies and causes damage to the target cells (Manteca *et al.*, 2001).

We have initiated a study aimed at determining the three dimensional structure of beta2 toxin to understand its function from a structural perspective. In this study we describe the purification of beta2 toxin from a recombinant bacterial system, the crystallization of the full length molecule and preliminary crystal characterization.

4.3 Methods and Results

4.3.1 PCR amplification of *beta2* toxin gene

Chromosomal DNA of *C. perfringens* was used to amplify beta2 toxin gene using specific primers. The primers beta2-Forward (beta2-F) and beta2-Reverse (beta2-R) span the beta2 ORF but exclude the signal peptide. The beta2-F and beta2-R primers together with DNA from the field strain of *C. perfringens* were used to amplify 704bp fragment. The sequence of beta2-toxin primers is as follows: Forward primer: 5'-CGAATTCCAAAGAAATCGACGCTTAT-3'; Reverse primer: 5'-CCGCTCGAGTGCACAATACCCTTCACC-3'. The PCR reaction contained 0.2mM dNTP, 1.5mM MgCl₂, 0.1μmol each forward and reverse primers, 5μl 10X PCR buffer, 2.5 U Platinum pfx DNA polymerase, appropriate template DNA and autoclaved distilled water to make a total volume of 50μl. The PCR was performed under standard conditions i.e. denaturation at 94°C for 15 seconds, annealing at 57°C for 30 seconds and elongation at 68°C for 1 minute, for 35 cycles, in a Peltier Thermo Cycler (MJ Research, MA, USA). The amplified beta2-toxin gene was isolated by agar gel electrophoresis and purified using Quantum Prep Freeze 'N Squeeze DNA Gel Extraction Spin Column (Bio Rad, Hercules, CA).

4.3.2 Cloning and Expression of *beta2* toxin gene into a pGEX-4T

A 704 bp PCR product was digested with *XhoI* and *EcoRI*. Following digestion, this fragment was sub cloned into pGEX-4T vector which was linearized using the same restriction enzymes. Transformation was carried out using 5μl of ligation mix (pGEX-4T + digested *beta2* toxin gene). DH5α strain of competent *E-coli* cells were transformed via electroporation using a BioRad Genepulser at 2400 V, 25 μF and 200 Ω for 0.70msecs. Transformants containing the complete plasmid were identified by plasmid isolation and

digestion using *XhoI* and *EcoRI* restriction enzymes. The transformed cells were cultivated on Luria-Bertani medium containing 100µg/ml of ampicillin.

4.3.3 Overexpression of pGEX-4T

pGEX-4T/DH5α cells were inoculated in 200 ml of Luria Bertani medium supplemented with ampicillin 100µg/ml. The cells were incubated overnight in shaker flasks at 37°C. Subsequently, this seed culture was used to inoculate 4 L of Luria Bertani medium supplemented with ampicillin (100 µg/ml). Cells were grown at 37°C and *beta2* expression was induced by addition of 1 mM isopropylthio-β-D-galactopyranoside at a density (OD₆₀₀) of 0.3. Upon reaching an OD₆₀₀ of 0.8, cells were harvested by centrifugation at 10,000g for 30 min., the resulting cell pellet was stored at -20°C.

4.3.4 Purification of the recombinant Beta2 toxin

The resulting pellet was resuspended in 200ml lysis buffer at pH 7.3 containing 1X Phosphate buffered saline (PBS). The cell suspension was lysed using ultrasonic treatment, three 20s pulses, with 1 minute intervals, and then treated with 1% protease inhibitor mixture (2mM phenylmethyl sulfonyl fluor, 25mM iodoacetamide, 5ug/ml aprotinin, 10ug/ml leupeptin, 10ug/ml pepstatin A). The sonicated cells were then centrifuged for 1 hour at 14000 rpm at 4°C. The beta2 protein present in the supernatant was separated from the pellet following centrifugation. The supernatant containing the soluble proteins was subjected to chromatography on glutathione – Sepharose - 4B column (Amersham Biosciences, NJ, USA) according to the manufacturer's protocol.

4.3.5 Glutathione Sepharose affinity chromatography

A 2 ml Glutathione Sepharose bed volume was prepared. Through the column 200 ml of soluble proteins sonicate was applied. The supernatant was loaded at a flow rate of 0.2ml/min. The column was washed with 20 ml 1X PBS for a total of three washes. The GST- beta2 fusion protein was eluted from the column at room temperature using 2 ml one column volume Glutathione elution buffer (10 mM reduced Glutathione in 50 mM Tris-HCl, pH 8.0). The elution buffer was incubated in the column for 15 minutes and then elute was collected in a separate tube. The elution was repeated three times to remove all the bound GST- beta2 fusion protein.

4.3.6 Thrombin cleavage of GST- beta2 fusion protein

The affinity purified beta2 fusion protein has a GST tag attached to it. The purified protein was treated with a site specific protease, Human Thrombin to cleave the GST tag and obtain a purified recombinant protein. Thrombin at the concentration of 10 units/mg of fusion protein was added and the reaction was incubated at room temperature for 12-16 hours. The cleaved protein was passed through the GST affinity column and a purified beta2 protein was collected.

4.3.7 SDS-PAGE

The SDS-PAGE analysis of the purified recombinant beta2 protein was performed using 12% polyacrylamide gels according to the method of Laemmli using Bio-Rad equipment (Bio Rad, Hercules, CA). The proteins were visualized by staining with

Coomassie brilliant blue (Figure 4.1). The concentration of the recombinant beta2 protein was determined using BCA Protein Assay kit (Pierce, IL, USA).

4.4 Dynamic Light Scattering

Dynamic Light scattering measurements were made using a Viscotek 802 optical system equipped with a 60mW diode laser at a wavelength of 830nm. Ten light scattering runs of 10 seconds each indicated that the sample was monodisperse and had a single mass distribution at a radius of 3.2 nm, with an approximate molecular weight of 51 kDa. The predicted molecular weight suggested a dimer in solution.

4.5 Crystallization of *Clostridium perfringens* beta2- toxin

The initial search for crystallization condition was carried out at the Hauptman Woodward Institute (HWI) in Buffalo, New York. A purified *C. perfringens* beta2- toxin (800 µl, 10 mg/ml, in 10mM Tris buffer) was sent to HWI for screening of preliminary crystallization conditions. Small crystals were observed in some of the conditions comprising PEG3350 and 0.2M Potassium sulfate, 0.2 M Sodium sulfate, 0.2M Lithium sulfate, 0.2M Magnesium acetate or 0.2M ammonium sulfate. These promising conditions and further X-ray diffraction screening were pursued at the Pennsylvania State University macromolecular crystallographic facility. The crystallization experiments were performed using the batch under oil method with 96-well Microbiol batch plates from Hampton Research. Custom grids were designed with the leads from the initial screening. Controls were setup for each condition to confirm that the crystals seen were indeed those

of protein. A dye test (Hampton Izit) was used to further confirm that the crystals grown were of protein.

Each well in the Microbiol batch plate contained 4 μ l of crystallization buffer (0.2M lithium sulfate, 28-36% PEG 3350) and 4 μ l of protein solution (concentration 10 mg/ml) layered under 20 μ l mineral oil. Triangular prism or rhombohedron shaped crystals appeared in conditions comprised of 32-36% PEG 3350 after one week (Figure 4.2) and in conditions comprised of 28 to 30% PEG3350 in about 2 weeks.

These crystals were well defined with the long axis of the rhombus measuring about 200 Microbiolns in size. However, the crystals were extremely fragile and shattered easily when picked up with a nylon loop. An additive screen obtained from Hampton Research Inc. was used to address this problem. It was observed that approximately 12% 1, 1, 1,3,3,3 hexa-fluoro 2-propanol when used as an additive made the crystals robust for handling.

4.6 X-ray Screening, Data Collection and Processing

Oil above the crystallization drop was carefully removed prior to crystal mounting. Single crystals were picked by a nylon loop (0.2 Microbioln) and frozen in a stream of cold nitrogen (-180°C). Since the crystals were grown in a buffer containing high percentage of PEG 3350 (28-36%), no other cryoprotectant was necessary. The initial crystal screening for X-ray diffraction showed spots up to 4 Å. Our attempts to improve diffraction by gradual dehydration by increasing PEG concentration up to 38% and 40% were not effective. Soaking the crystals in 5-10% glycerol before freezing improved diffraction up to 3.07 Å. Diffraction up to 2.9 Å was seen when a cryo-

annealing technique was employed where the frozen crystal was thawed for about 10 seconds and frozen again. Longer thawing and refreezing however deteriorated the diffraction. X-ray diffraction data to 2.9 Å resolution were collected at the synchrotron beam line at F2 station at CHESS, Cornell on an ADSC Quantum-210 CCD detector (Figure 4.3). The overall R-merge was high at 12.9% and the R-merge value in the highest resolution bin was 57.8 % due to the overall data quality and weak diffraction at high resolution. The preliminary X-ray crystallographic information is listed in Table 4.1. The data was integrated using Denzo and scaled and merged using the Scalepack program (Otwinowski, 1993).

4.7 Non Crystallographic Symmetry (NCS)

A self rotation function calculation was carried out using the program POLARRFN (CCP4, 1994). The axis of non crystallographic two fold symmetry relating the two monomers were clearly seen in the κ 180° section (Figure 4.4). Native data from 3.5 to 8 Å and a Patterson radius of 28 Å was found to be most suitable after trials with other possible values for these parameters. The highest peak after the origin peaks were on the kappa 180° section and corresponded to the NCS 2 fold. There were no additional peaks seen at other kappa sections ruling out any other possibility. The polar angles defining the NCS two fold are $\omega = 90.0^\circ$ and $\phi = 210.3^\circ$. Through crystallographic symmetry the NCS two fold is close to being parallel along the y-axis. However processing the X-ray data in R32 leads to very high merging R-values and a high number of reflections being rejected.

Table 4.1**Data Collection statistics**

Unit Cell Parameters (Å)	$a = 108.13, b = 108.13, c = 195.52$
Unit Cell Parameters (°)	$\alpha = 90, \beta = 90, \gamma = 120$
Space group	R3
Matthews Co-efficient (Å ³ /Da)	3.93
# of molecules per asymmetric unit	2
Solvent content (%)	68.69
Resolution of native data (Å)	2.9
# of reflections observed	108011
# of unique reflections	19279
Linear merging R-factor (%)	12.9
Polar angle ω° defining NCS (°)	90.0
Polar angle ϕ° defining NCS (°)	210.3

4.8 Conclusions

Clostridium perfringens beta2 toxin was successfully cloned expressed and purified using Glutathione sepharose affinity chromatography and crystallized using the batch under oil technique. Preliminary crystallographic analysis showed the crystal obtained belongs to primitive rhombohedral space group *R3*, with unit cell parameters in the hexagonal setting as, $a = 108.13, b = 108.13, c = 195.52$ Å. As no homologous protein structures are available in the Protein Data Bank, it is not possible to use molecular replacement for structure solution. Attempts are underway to solve the phase problem using the multiple isomorphous replacement method. We are also in the process of cloning and purifying a selenomethionine derivative of the protein in order to solve the structure using a multiwavelength anomalous dispersion method. Structure determination

of beta2 will provide a molecular picture of the residues responsible for its antigenicity, membrane association and provide clues in our efforts to develop a better dairy vaccine.

4.9 Acknowledgements

We would like to thank the staff at Hauptman-Woodward medical research institute for the initial robotic screening. Special thanks are given to staff especially Irina Kriksunov, at F2 station for help with data collection at CHESS.

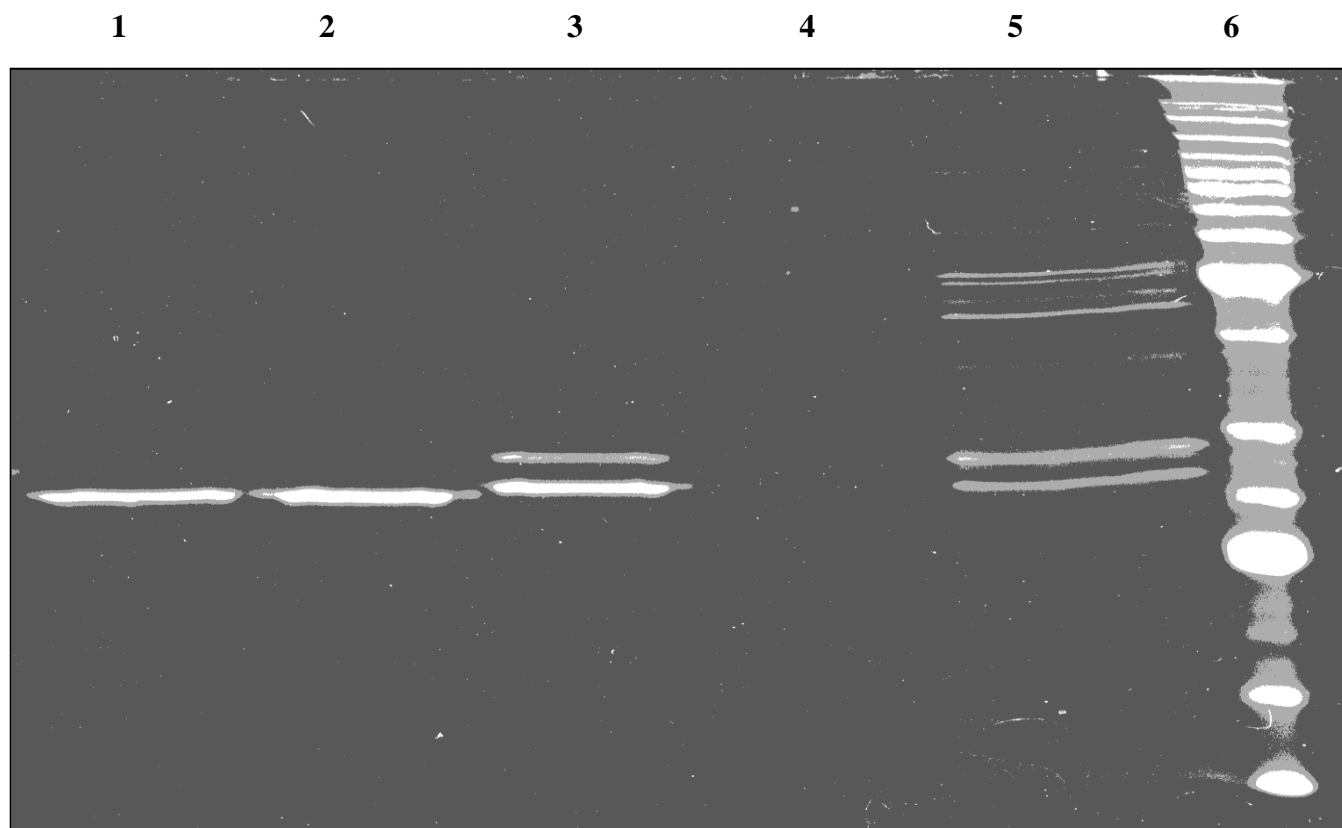


Figure 4.1. SDS-PAGE analysis of the cleaved beta2 toxin showed a specific single polypeptide band with a molecular weight of 28 kDa. Legend: Lane 1&2 – Purified recombinant beta2 toxin, Lane 3 – Beta2 toxin + GST tag, Lane 4 – GST column flow through, Lane 5 – BL21 cell lysate, Lane 6 – Molecular weight marker.

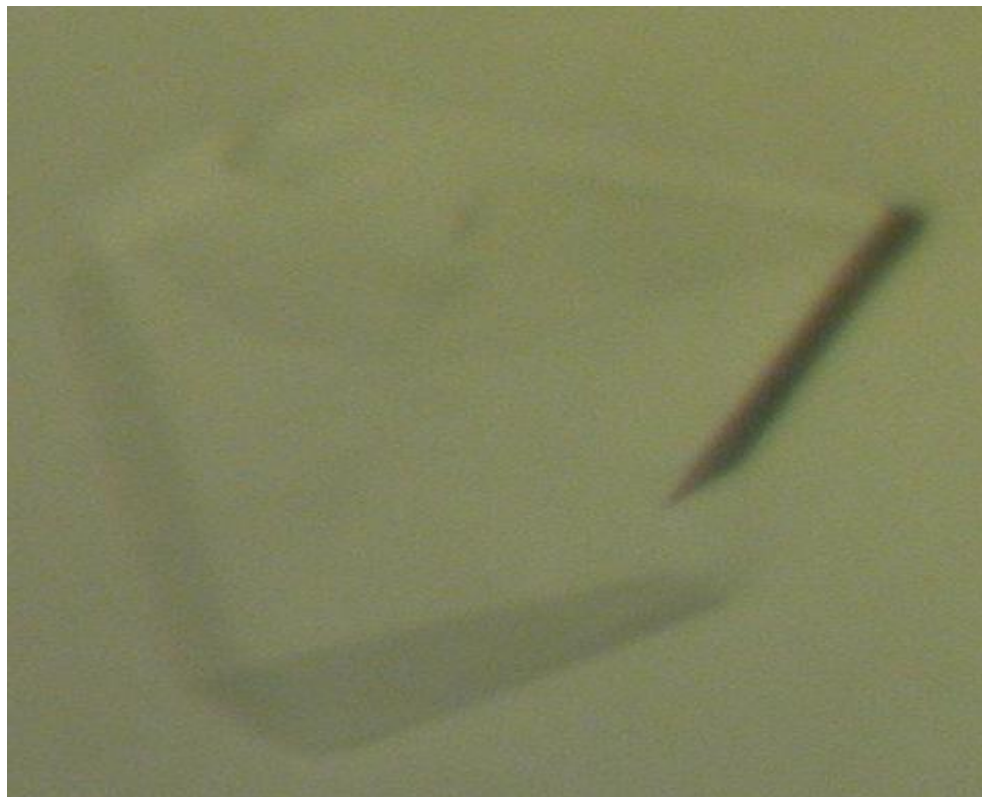


Figure 4.2 Triangular prism shaped crystal of beta2 toxin measuring about 200 Microbiolns.

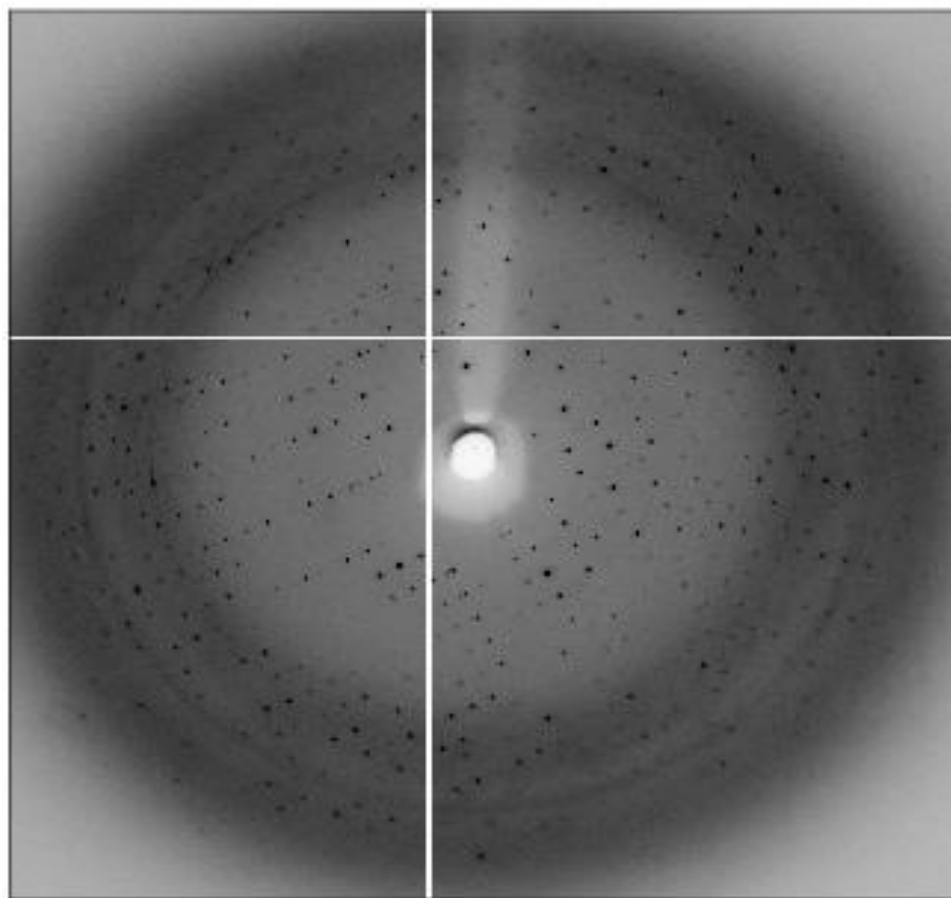


Figure 4.3 X-ray diffraction image from a native beta2 toxin crystal showing diffraction spots upto 2.9 Å resolution.

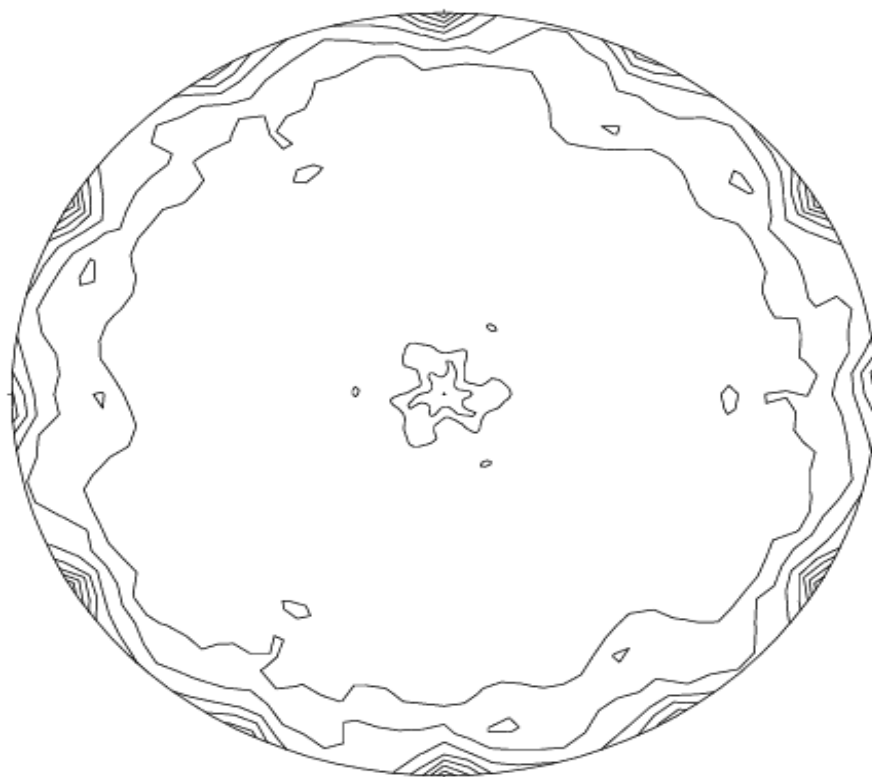


Figure 4.4 Stereographic projection of κ 180° section of the self rotation function. The peaks around the outer edge are positions of noncrystallographic 2 folds. Polar angle ω varies from $0, 180^\circ$ at the center to $90, 270^\circ$ at edge; φ varies from 0 to 360° around the circle. The polar angles defining the NCS two fold are $\omega = 90.0^\circ$ and $\varphi = 210.3^\circ$.

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CHAPTER 5

Short Communication

Cloning, Expression, Purification and study of Cytopathic effects of *Clostridium perfringens* beta2 toxin.

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Veterinary Microbiology (In Preparation)

5.1 Abstract

Clostridium perfringens strains are divided into five major toxinotypes (A-E), according to the type of toxins they produce. *Clostridium perfringens* beta2 toxin is produced by all toxin types. The human adenocarcinoma cell line *CaCo2* has been reported to be susceptible to *C. perfringens* beta2 toxin. In this study, the beta2 toxin induced cellular swelling, membrane blebbing, and leakage of cytoplasm from the affected *CaCo2* cells. The cytopathic effects of the recombinant beta2 toxin were observed at a higher concentration indicating the cytotoxic effects of beta2 toxin alone is observed at concentrations > 10 ug/ml. The damage to the cell membrane of cells exposed to beta2 toxin was assessed by measuring the release of lactate dehydrogenase (LDH) from the cells. In vitro release of LDH from cells provided an accurate measure of cell membrane integrity and cell viability. Monoclonal antibodies raised against *C. perfringens* beta2 toxin neutralized the cytopathic effects of the recombinant beta2 toxin. Cytopathic effect of the recombinant toxin supports our hypothesis that the toxin may act in a similar way as other enterically active *C. perfringens* toxins by functioning as a pore forming toxin.

5.2 Introduction

Several studies have indicated the association of *C. perfringens* beta2 toxin with enteric diseases such as necrotic enteritis, and enterotoxemia in domestic and wild animal species (2, 8). The pathologies exhibited by the affected animals strongly suggests *C. perfringens* beta2 toxin as a marker of virulence.

The toxins of *C. perfringens* are responsible for causing damage to the gastrointestinal tract of mammals. With the exception of iota toxin, all other toxins of *C. perfringens* interact with the cell membrane. The prominent cellular changes resulting due to action of toxins that interact with the cell membrane is membrane disruption or pore formation (3). It has been also reported that both beta and beta2 toxin share similar biological activity as they both exhibit similar action on intestinal cells and have identical mouse lethality (6). Garmory et al, (5) revealed that the beta2 toxin sequence has no significant amino acid sequence homology with *C. perfringens* beta toxin or any other known toxin. Gibert and his co workers (6) expressed and purified recombinant beta2 toxin using pAT19 vector and maintained the cloned plasmid pMRP268 into *C. perfringens* 667-76. The beta2 toxin gene from *C. perfringens* has been cloned and sequenced revealing an open reading frame of 265 amino acids, including a 30 amino acid signal sequence absent in mature protein (6).

In spite of the importance of beta2 toxin in veterinary medicine the biological activity of the protein is not completely understood. This study reports the cytopathic effects of the recombinant beta2 toxin on human adenocarcinoma cell line *CaCo2*. This paper also reports the production and evaluation of the monoclonal antibodies to *C. perfringens* beta2 toxin.

5.3 Materials and Methods

5.3.1 Cloning, expression, and purification of recombinant *C. perfringens* beta2 toxin

Cloning, expression and purification of recombinant *C. perfringens* beta2 toxin was done as described in Chapter 4.

5.3.2 *Clostridium perfringens* beta2 Monoclonal Antibody

Monoclonal antibody against *Clostridium perfringens* beta2 toxin was raised in Balb/cByJ female mice at the Hybridoma and Cell Culture Laboratory at the Department of Veterinary and Biomedical Sciences at Pennsylvania State University. Briefly, Sp2/0-Ag14 strain myeloma cells were dislodged from the 150 cm flask. The cells were spun at 400xG for 8 minutes. The supernatant was decanted and the cells were resuspended in 40 ml DMEM-0. The process was repeated twice and the cells were counted in hemocytometer. The volume of cell suspension was calibrated up to 15 million viable cells. Female Balb/cByJ mice were immunized with approximately 2 mg of purified recombinant beta2 toxin and the serum antibody titer were determined over 2-3 collections. The immunized mice were euthanized and their splenocytes were harvested according to previously established protocol. Approximately, 30 million viable splenocytes were harvested and counted by hemocytometer. In a new 50 ml centrifuge tube, the volumes of myeloma and spleen cells were combined and fused using Polyethylene glycol.

The fused cells were plated into a 96 well culture plate and the cells were maintained in HAT medium. ELISA screening of the selected hybridoma cells for anti-fibrinogen antibody-producing hybridomas was carried out and approximately 5

candidate hybridoma cells lines were selected and were grown to confluency in culture flask to upscale the production of monoclonal antibodies against *C. perfringens* beta2 toxin.

5.3.3 Cell culture and cytotoxicity assay

The *CaCo2* cells were obtained from American Type Culture Collection (ATCC Manassas, VA, USA) and grown to confluence in Eagle's Minimum Essential Medium (MEM) supplemented with 20% fetal bovine serum, 0.1mM non-essential amino acids, 1.5% sodium bicarbonate, 2mM L-Glutamine and 1mM sodium pyruvate at 37°C with 5% CO₂. For cytotoxicity assays, cells were seeded in 24-well culture plates (200 µl of ~ 10⁵ cells/per well) and cultured for 12 h at 37°C in a 5% CO₂ atmosphere. Beta2 toxin was added to each well and incubated for an additional 12 h and 24 h. The cells were exposed to different concentrations (10, 20 and 40 µg/ml of toxin in PBS) of toxin to assess the effect of the toxin on the cultured cells. Cell lysis buffer was added to positive control wells as per manufacturers instructions. The cells exposed to beta2 toxin were stained with DRAQ5TM dye (Biostatus, Leicestershire, UK) that stained the nucleus red, while the cytoplasm was stained green using Cell TrackerTM dye (Molecular Probes, OR, USA). Cells were visualized by fluorescence Microbiolscopy using Olympus BX61Epi-Fluorescence Microbiolscope and Hamamatsu Orca-ER camera (Olympus, PA, USA). The images were processed using SlideBook 4.1 software (Olympus, PA, USA). The release of lactate dehydrogenase (LDH) from *CaCo2* cells exposed to beta2 toxin and PBS (control) was determined with a Microbiolplate LDH assay kit (Promega, WI, USA).

5.3.4 Neutralization of Cytotoxicity of Beta2 toxin by the Monoclonal Antibody

CaCo2 cells ($\sim 10^5$ cells /ml) were seeded in a 24-well culture plate (Corning, NY, USA), and grown to confluence as described previously. In a separate 24-well plate, 100 μ l aliquots of beta2 monoclonal antibody (diluted 1:10 to 1:10000 in PBS) was added to appropriate wells. Into each of these wells, 50 μ l of a beta2 toxin (10, 20, and 40 μ g/ml) diluted in PBS, was added. Cell control wells contained PBS only, while toxin control wells contained toxin but no monoclonal antibody. This cell culture plate was then incubated at 37°C for 1 h. Aliquots (50 μ l) of monoclonal antibody only, beta2 toxin only and mixture of monoclonal antibody and beta2 toxin were transferred to wells containing adherent *CaCo2* cells. The cells were incubated at 37°C for 2 h and each of the well was refreshed with 100 μ l of growth media. The cells were incubated further 2-2.5h at 37°C with 5% CO₂. At the end of incubation, the cells were treated with the nucleus and cytoplasmic stain.

5.4 Results

5.4.1 Beta2 toxin cytotoxicity

The cytotoxicity of *C. perfringens* recombinant beta2 toxin was determined using a *CaCo2* cell culture model followed by staining the cells with nucleic acid and cytoplasmic dye. The *CaCo2* cells treated with 10 μ g/ml of recombinant beta2 toxin showed a marked swelling and cell rounding. Subsequently, one or several large blebs surrounded each cell. Following membrane disruption leakage of the cytoplasm was observed. When the cells were incubated with the toxin at concentrations from 10 μ g/ml

to 40µg/ml, the cytopathic effects increased in a dose dependent manner under the conditions (Figure 5.1). The differences between control cells and toxin treated cells were prominent.

Lactate dehydrogenase is a cytosolic enzyme that is rapidly released into the culture medium following loss of membrane integrity resulting from either apoptosis or necrosis. LDH activity, therefore, can be used as an indicator of cell membrane integrity and serves as a general means to assess cytotoxicity. The cytotoxicity caused by beta2 toxin is reportedly attributed to the formation of large pores by the toxin in the target cell plasma membrane and thereby causing necrosis of the affected cells. The toxin treated cells showed significant increase in the leakage of the lactate dehydrogenase (LDH) as measured by the LDH assay (Figure 5.2). The results from our study indicated that, the LDH release by the lysed cells by higher concentration of the beta2 toxin (40µg/ml) was approximately double as compared to the LDH release by 10µg/ml of beta2 toxin. The higher concentration of beta2 toxin caused complete lysis of the treated cells as indicated by the LDH release comparable to that of the positive control used as indicated by manufacturers instructions (Figure 5.2).

5.4.2 Neutralization of beta2 toxin cytotoxicity

The monoclonal antibody against beta2 toxin inhibited beta2 toxin mediated cytotoxicity in a dose dependant manner. The negative control did not inhibit cytotoxicity at any dose (Figure 5.3).

At a concentration of 10µg/ml of toxin, the monoclonal antibody could neutralize the cytotoxic effects at the dilutions 1:10 to 1:1000 except at 1:10000. As expected, when

the dilutions of the neutralizing antibody increased the cytopathic effects mediated by the beta2 toxin became more prominent. It was observed that at concentration of 40µg/ml of beta2 toxin, the monoclonal antibody failed to neutralize the cytotoxic effect of the toxin.

5.5 Discussion

The objective of this study was to analyze the cytotoxic activity of the recombinant *C. perfringens* beta2 toxin and evaluate the neutralizing activity of beta2 monoclonal antibody. The results of our study showed that following a 24 h exposure of *CaCO2* cells to recombinant beta2 toxin resulted in membrane blebbing, cell swelling and disruption of the plasma membrane leading to leakage of cytoplasm. In view of these results, it can be suggested that the morphological changes in the toxin treated cells are induced through pores formed by the toxin in the plasma membrane. The release of LDH confirms that the recombinant beta2 toxin causes disruption of cell membrane resulting in leakage of cytoplasm. Our results augment previously reported studies that indicated beta2 toxin to be a pore forming toxin (4). The cytotoxic effects indicate a marked influx of water into the cells, and also adversely affect the membrane ion permeability. These effects could lead to functional disturbances such as absorption capacity of the intestinal epithelial cells (7). Our study suggests that *C. perfringens* beta2 toxin could act on the intestinal epithelial cells forming large pores leading to necrosis of the affected cells.

A high concentration of beta2 toxin (>10 µg/ml) was required to induce cytotoxic effects. This perhaps suggests the potential weak toxicity of the beta2 toxin. Based on our results and the reported literature (9), it could be concluded that *C. perfringens* beta2 toxin primarily acts as an accessory toxin and not as a primary toxin in enteric diseases of

clostridial origin. The toxin acts on the intestinal epithelial cells causing membrane pores and necrosis, thereby allowing the major toxins of *C. perfringens* such as alpha and beta toxin to initiate a cascade of pathologic events leading to necrotic enteritis and enterotoxemias. Based on the cytotoxicity it could be concluded that the pathogenesis of beta2 toxin is similar to other enterically active toxins like clostridial enterotoxin (10) and *Staphylococcus aureus* alpha toxin (1).

The monoclonal antibody raised against the *C. perfringens* beta2 toxin neutralized the cytotoxic effects of the beta2 toxin on *CaCo2* cells. The neutralization titers were determined by the lowest mAb concentration that resulted in neutralizing the cytotoxicity to at least 90% of that of control cells containing no toxin. It was observed that neutralization of the cytotoxicity was dose dependant. The neutralization was also effective when the monoclonal antibody was able to bind to the beta2 toxin prior to the addition of the toxin to the cells. Based on the results of our study it can be concluded that beta2 mAb effectively neutralized the lethal effects of the *C. perfringens* beta2 toxin.

In summary, the findings of this study show that the recombinant toxin was immunologically active and was used to raise monoclonal antibody against beta2. The recombinant beta2 toxin could exhibit a potent cytopathic effect on the *CaCo2* cell monolayer. Further studies are needed to analyze and compare the cytopathic effects of recombinant beta2 toxin on few other cell lines such as MDCK, Vero cells with *CaCo2*. The monoclonal antibody showed neutralizing effects on cytotoxicity exhibited by the toxin. The monoclonal antibody could be further utilized for other diagnostic purposes in investigating the role of beta2 toxin in clostridial enteric diseases.

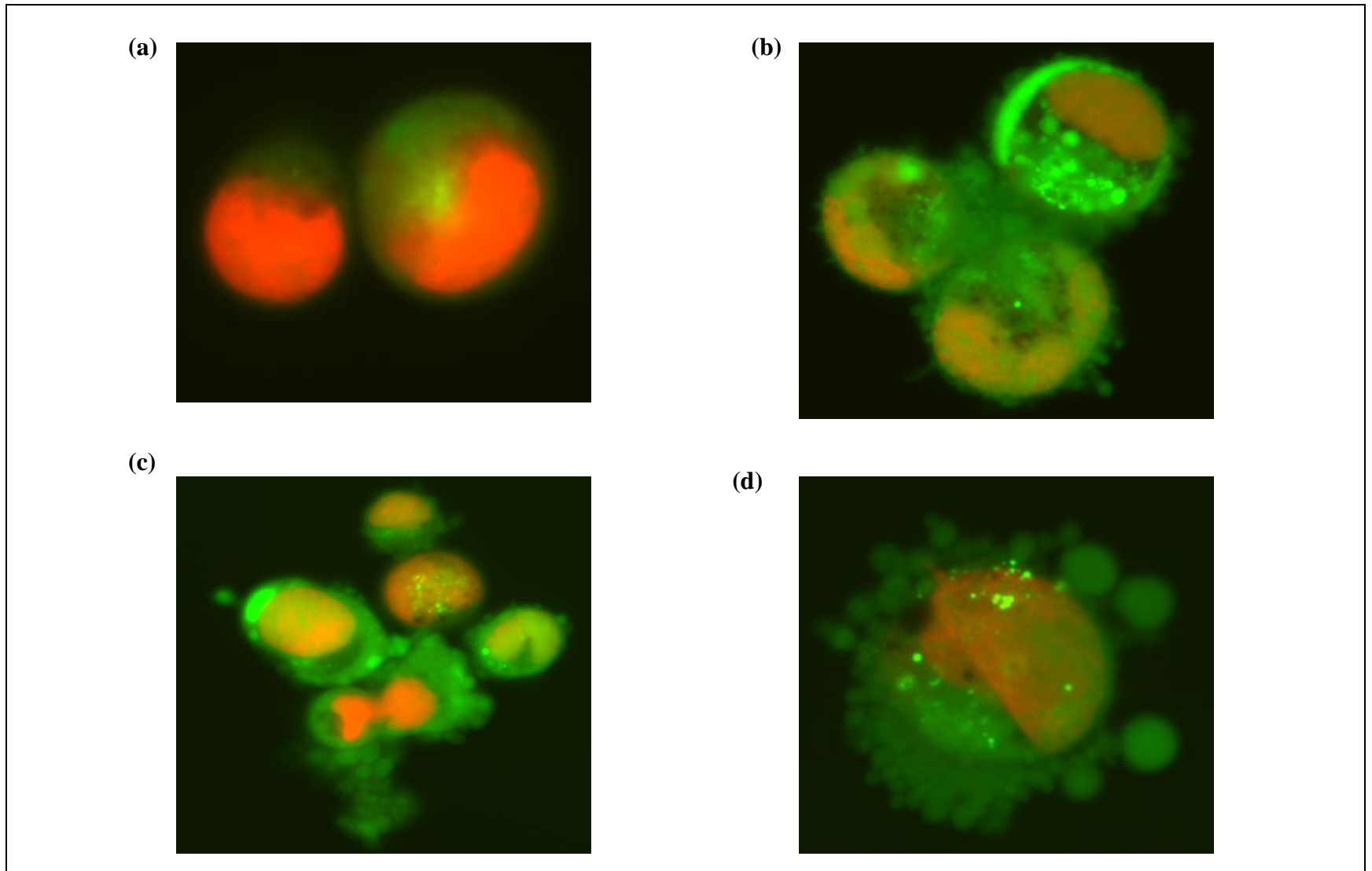


Figure 5.1 Cytopathic effects of recombinant *C. perfringens* beta2 toxin on CaCo2 cells. (a) Control Cells, (b) 10 µg/ml of beta2 toxin, (c) 20 µg/ml of beta2 toxin, (d) 40 µg/ml of beta2 toxin. The cells were stained with nuclei stain (Red) DRAQ5TM, and cytoplasmic stain (green) Cell TrackerTM.

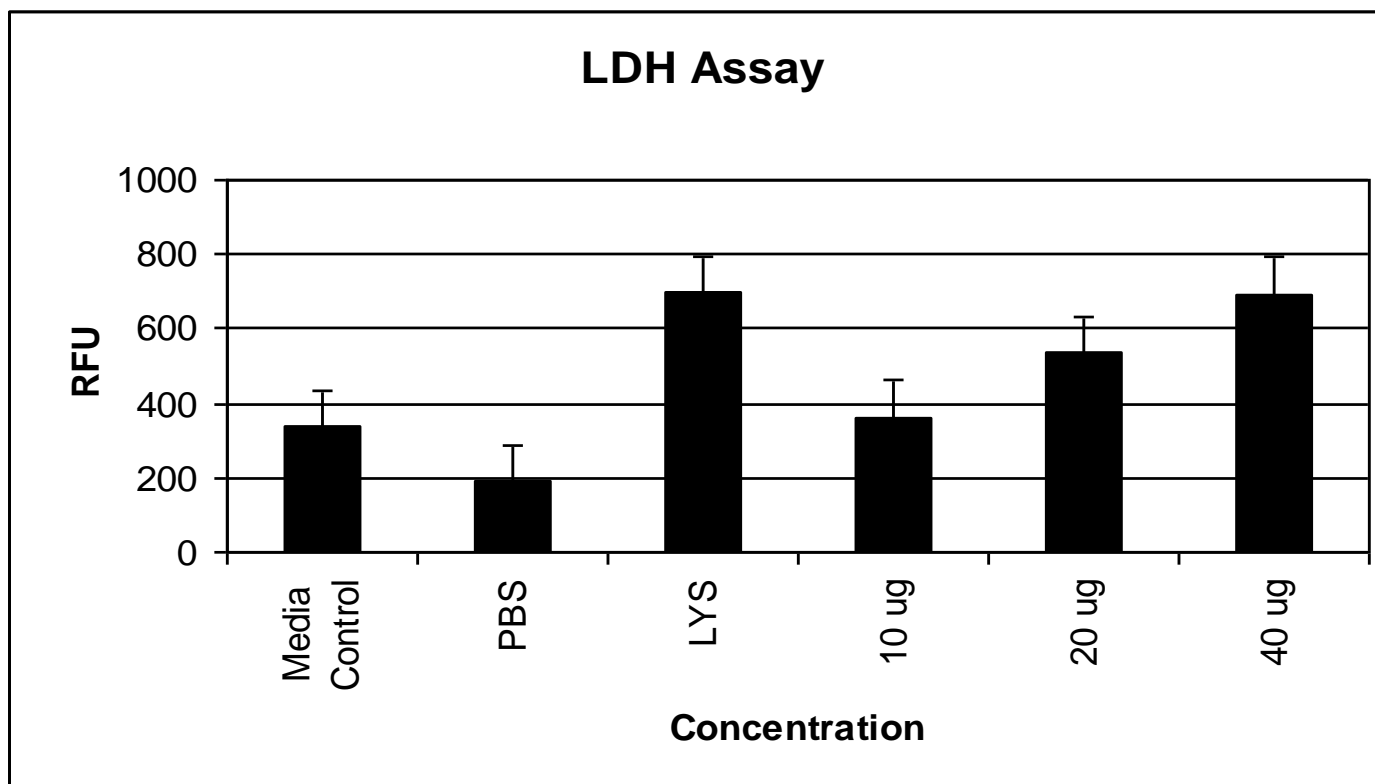


Figure 5.2 Lactate Dehydrogenase (LDH) as measured by LDH assay. Legends: Media Control- Media no cells (Negative control), PBS- Phosphate buffer LYS- Lysis solution (Positive control), 10-40 $\mu\text{g/ml}$ - beta2

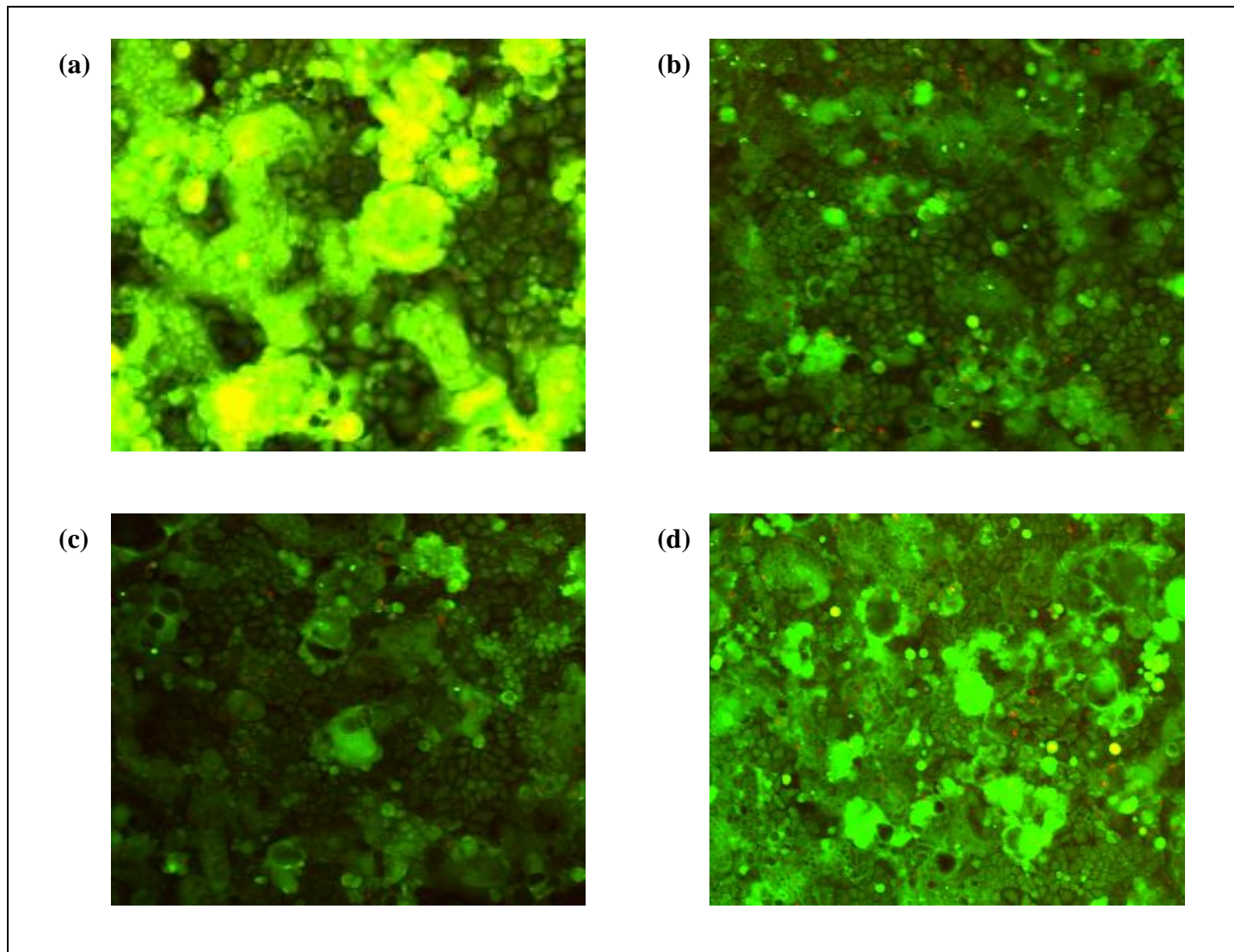


Figure 5.3 Neutralization of cytopathic effects of recombinant *C. perfringens* beta2 toxin on CaCo2 cell monolayer. (a) 10 µg/ml of beta2 toxin without beta2 MAb, (b) 10 µg/ml of beta2 toxin + 1:1000 beta2 MAb, (c) 20 µg/ml of beta2 toxin + 1:100 beta2 MAb, (d) 40 µg/ml of beta2 toxin + 1:10 beta2 MAb.

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CHAPTER 6

Evaluation of a Synthetic Lipopeptide Vaccine against *Clostridium perfringens* beta2 toxin.

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6.1 Abstract

The immunogenic effects of a synthetic lipopeptide vaccine comprised of two immunodominant epitopes of *Clostridium perfringens* beta2 toxin were investigated in mice. Immunization of ICR mice with two different protective epitopes of beta2 toxin fused with synthetic lipid moiety S-[2, 3-bis (palmitoyltoxy) propyl] cysteine (Pam2Cys) confirmed the effectiveness of a novel self-adjuvanting vaccine delivery system. Mice were immunized parenterally with a lipopeptide formulation, which contained the N-terminal region of beta2 sequence from amino acid position 62 - 75 and a second sequence covering amino acid 104 -114. Immune response of the vaccine was determined upon exposure via intraperitoneal and subcutaneous routes. The synthetic lipopeptide generated a vigorous immunoglobulin G (IgG) response, with higher IgG1 than IgG2a titers in subcutaneously vaccinated animals as compared to animals immunized through the intraperitoneal route. The high antibody titer of IgG1 and IgG2a was indicative of a mixed Th1/Th2 response. The spleen cells from subcutaneously vaccinated mice showed higher proliferation as compared to the intraperitoneally vaccinated mice. The spleen cells from immunized mice produced high amounts of IFN- γ , but not IL-2, IL-10 or TNF- α , after in vitro stimulation with recombinant beta2 toxin. This observation suggests the induction of a T helper 1 (Th1) response. The lipopeptide vaccine induced a protective response in immunized animals against beta2 toxin challenge. Results of our study suggest that subcutaneously injected synthetic lipopeptide vaccine induces both humoral and cellular response and could be a useful candidate for the development of peptide vaccines against *C. perfringens* beta2 toxin.

6.2 Introduction

Clostridium perfringens is considered to be the most important clostridial enteric pathogen of domestic animals (Songer, 1996; Niilo, 1986). The association of beta2 toxin gene (*cpb2*) positive strains of *C. perfringens* type A with enteric diseases in domestic animals including dairy cattle, piglets, horses, dogs, and more recently in an African elephant with ulcerative enteritis has been widely reported in the literature (Klaasen *et al.*, 1999; Garmory *et al.*, 2000; Waters, *et al.*, 2003; Bueschel, *et al.*, 2003). In humans, Fisher *et al.*, (2005) suggested a role of beta2 toxin along with enterotoxin of *C. perfringens* in etiology of antibiotic-associated diarrhea and sporadic diarrhea. Alterations in normal, healthy Microbiolbial ecology of the gut due to the effects of environmental perturbations (diet, antibiotics, or pathogens) could permit the colonization of toxigenic clostridia leading to enterotoxemia and hemorrhagic enteritis (Niilo, 1986; Herholz *et al.*, 1999).

Several commercially available vaccines are used for the prevention of *C.perfringens* enterotoxemia (Hauer *et al.*, 2006). All of the available vaccines are based on formaldehyde-treated bacterial culture filtrates or whole cell cultures and confer a moderate to high degree of protection in immunized animals (Oyston *et al.*, 1998). There is growing interest in synthetic immunogenic peptides as modulators of the immune responses. The use of synthetic peptides as vaccine candidates has been considered a goal in vaccinology because they are safe, affordable, easy to store and handle, and ideally suited for specific targeting which is a drawback of conventional vaccines (Jackson *et al.*, 2002).

Lipids and lipopeptides have been successful in adjuvanting weak immunogens. Reports have demonstrated that in a lipopeptide construct, a lipid moiety with known adjuvanticity can be covalently linked to a peptide to generate a fully synthetic self-adjuvanting and potentially safe vaccine (BenMohamed *et al.*, 1997; BenMohamed *et al.*, 2000; Nardin *et al.*, 1998; Wiesmuller *et al.*,

1989; Deres *et al.*, 1989). Pam2Cys, a naturally found component of *Mycoplasma* activating lipopeptide 2 (MALP2) was demonstrated to have superior Toll Like receptors 2 (TLR2) stimulation properties and subsequent superior virus specific CTL response by Lau *et al.*, (2006). Romero *et al.*, (2004) demonstrated the ability of synthetic analog of MALP2 to induce granulocyte-macrophage colony stimulating factor (GM-CSF) in vivo.

The ability to synthesize a chemically synthetic lipopeptide vaccine using immunostimulatory lipids provides an opportunity to manipulate the final structure of the vaccine to give the best or appropriate immune response. It has been demonstrated that when attached to an appropriate carrier, synthetic peptides corresponding to some bacterial toxins such as diphtheria and cholera toxin were able to induce antibodies that could neutralize the biological activity of the native toxin (Harari *et al.*, 1988).

The aim of this study was to evaluate the potential of lipopeptides containing two different protective epitopes of beta2 toxin fused with synthetic lipid moiety Pam2Cys to induce beta2 toxin specific immune response.

6.3 Materials and Methods

6.3.1 Animals

Female ICR mice were purchased from Harlan Sprague-Dawley (Indianapolis, IN). The animals were 5-6 weeks of age, maintained in a specific pathogen free environment in the animal facilities at Pennsylvania State University, and were provided with food and water ad libitum. All experiments were approved by the Office of Research Protection's Institutional Animal Care and Use Committee at The Pennsylvania State University.

6.3.2 Design and synthesis of peptides and lipopeptides

The two epitopes of the beta2 toxin were theoretically selected based on their 1) structural features, 2) amino acids essential for binding the groove of MHC-II molecules, 3) predicted surface probability, and 4) hydrophilicity as described by Hopp and Woods (1981). These determinations were done with the recombinant beta2 peptide sequence derived from ADL strain of *C. perfringens* (unpublished data). The first epitope was located near the N-terminal region from amino acid position 62 - 75 and the second epitope covered amino acid 104 -114. Synthetic peptides representing residues 62-75 (N-S-Q-K-S-D-N-K-E-I-F-N-V-K) and 104-114 (R-T-K-V-E-N-E-G-K-Y-I) from the recombinant beta2 toxin were synthesized using Fmoc chemistry (Partidos *et al.*, 1997). The synthetic peptides were fused to Pam2Cys through a lysine linker. The identity of the defined lipopeptides was determined by electrospray mass spectrometry and calculated mass patterns of lipopeptide mixtures were confirmed at the Proteomics facility at Penn State University. The lipopeptide vaccine was synthesized to order by Invitrogen (Carlsbad, CA). The quality of the synthesized vaccine was determined using RP-HPLC. The yield of the vaccine was 3.4 mg at 95% purity by HPLC.

6.3.3 Immunization protocol

Three groups of four mice each were used for the study. One group of mice was used as a control, while two other groups were used for subcutaneous and intraperitoneal vaccination of the lipopeptide vaccine, respectively. The mice in the control group received normal saline intraperitoneally and subcutaneously. The mice were immunized on days 0, 14, and 28. The synthetic lipopeptide was dissolved in normal saline and used at 50 µg/dose concentration (500 µl/animal).

6.3.4 Sample collection

Serum samples were collected on days 0, 14, 28 and 42, post immunization and stored at -20°C for subsequent determination of beta2 toxin specific antibodies. The immunized mice were challenged intraperitoneally with culture supernatant of a known strain of beta2 toxin (400 µg/animal) producing *C. perfringens* seven days after last booster dose of the vaccine. The challenged mice were observed for 4-5 days for development of any pathological signs. The mice were sacrificed on day 42 and the final sampling was performed. On necropsy, any abnormal signs were noted. The spleens from the sacrificed mice were collected and splenocytes were purified for analysis of cellular immune response.

6.3.5 Detection of beta2 toxin specific antibodies in serum by ELISA

The serum samples of the mice were analyzed by an indirect ELISA kit to quantify the class and IgG subtypes of *C. perfringens* beta2 toxin specific antibodies. The ELISA test was performed as per the manufacturer's instructions (QED Bioscience, San Diego, CA). Briefly, 96 well ELISA plates were coated with recombinant *C. perfringens* beta2 toxin (5 µg/ml) or capture goat antimouse IgG1, IgG2a, IgG2b, IgG3, IgM, IgA and kappa light chain antibodies (5 µg/ml) overnight at room temperature. Serially diluted sera samples and immunoglobulin standards were incubated for 30 min at room temperature with gentle agitation. The plate was washed three times with wash buffer, followed by 30 min incubation with HRP-conjugated goat anti-mouse class or IgG subtype antibodies. After the final wash step, the plate was incubated with a substrate solution. The concentration of the beta2 toxin specific antibodies was calculated for the IgG subtypes, IgA, IgM and Kappa light chain classes by using the standards developed with the appropriate class or subtype specific HRP-conjugated antibodies.

6.3.6 T cell proliferation assay

The spleens isolated from the mice were pooled and stored in 30 ml of ice cold 1% PBS + 2% Fetal Bovine Serum (FBS). The pooled spleens were homogenized to get a single cell suspension. The cells were spun down at 250xg for 10 min at 4⁰C and resuspended in ACK lysis buffer to lyse the red blood cells. The cells were again spun down, washed with ice cold 1% PBS + 2% Fetal Bovine Serum (FBS), and resuspended in 1 ml of complete medium. The cell concentration was adjusted to 2x10⁶ cells/ml in complete medium. The cells were seeded at 100 µl/well in flat bottom 96 well Microbioltiter plate. The plate was incubated for 96 h with three concentrations (5 µg, 10 µg, 20 µg) (total concentration) of recombinant beta2 toxin. Medium alone was used as a negative control, whereas phorbol myristate acetate (PMA, 100ng/ml) with ionomycin (500 nM) was used as a positive control. Each concentration was tested in triplicate. During the final 16 h of culture, 0.5 µCi [³H] thymidine was added to each well. Cells were harvested on paper filters by using a cell harvester. The amount of incorporated [³H] thymidine into the DNA of proliferating cells was determined by a γ-scintillation counter (Wallac-PE, Waltham, MA). The results are expressed as the arithmetic mean of [³H] thymidine uptake in cpm.

6.3.7 Multiplex cytokine assay

Culture supernatants from the proliferating cells were collected at the end of 96 h of proliferation and stored at -70⁰C. A bead based multiplex cytokine assay was performed to detect the cytokines in the supernatants from in vitro stimulated spleen cells. A mouse Th1/Th2 4-Plex kit (Lincoplex, St.Charles, MO) was used according to the manufacturer's protocol with a Bio-Plex system. The cytokines determined in the Th1 type of immune response were IL-2, TNF-α, IFN-γ, while the cytokine determined for Th2 immune response was IL-10. The cytokines were quantified (pg/ml) by using appropriate standards.

6.4 Results

6.4.1 Design and assembly of the synthetic lipopeptide vaccine

The purified recombinant beta2 toxin was submitted to the Mass Spectrometry Facility at Penn State University for determination of the amino acid sequence. Immunodominant epitopes from the amino acid sequence of *Clostridium perfringens* beta2 toxin were identified based on the regions containing high amount of surface residues and hydrophilicity as described by Hopp and Woods (1981 & 1983). The lipopeptide construct comprised of two peptides (Peptide I: amino acid position 62-75, Peptide II: amino acid position 104-114) and the Pam2Cys was synthesized using Fmoc chemistry (Figure 6.1) from a commercial source (Invitrogen); the purity of the construct was confirmed to be more than 95%.

6.4.2 Prime/boost regime of the synthetic lipopeptide vaccine results in beta2 specific humoral response in intraperitoneal and subcutaneously vaccinated animals

The animals were immunized with synthetic lipopeptide vaccine (50 µg/dose) either by intraperitoneal or subcutaneous routes on day 0, and boosted with the same dose on days 14 and 28. The immunization protocol followed was expected to elicit an efficient cellular and humoral immune response against *C. perfringens* beta2 toxin.

The capacity of synthetic lipopeptide vaccine to stimulate efficient humoral immune responses was assessed by determining the serum titers of beta2 toxin specific antibodies in vaccinated mice. The concentration of IgG and its subclasses were observed at low levels until day 28 after the first vaccination, whereas the second dose of vaccination boosted the response with a peak concentration observed on day 42. A stronger IgG1 immune response occurred in mice vaccinated subcutaneously as compared to those vaccinated intraperitoneally was observed at the end of 42 days (Figure 6.2a). The

IgG2a response in subcutaneously vaccinated mice was higher compared to that of intraperitoneally vaccinated mice (Figure 6.2b). The concentration of IgM antibody was observed to be slightly higher in mice vaccinated through a subcutaneous route than those with intraperitoneal route. There was no significant IgA antibody response in either group of mice. All sera were tested in triplicate at 1/100 dilution.

6.4.3 Elicitation of beta2 toxin specific cellular immune response

To evaluate and compare the efficacy of the synthetic lipopeptide vaccine at inducing cellular immune responses in animals immunized through intraperitoneal and subcutaneous route, lymphoproliferative assays were performed. Weak responses were observed in the animals that received the synthetic lipopeptide vaccine intraperitoneally (Figure 6.3a). A strong proliferative response was observed in mice vaccinated through subcutaneous route for all the three concentrations of the recombinant beta2 toxin (Figure 6.3b).

The T cell response was further characterized by evaluating beta2 toxin specific Th1 and Th2 cytokines (IFN- γ , IL-2, TNF- α and IL-10). The cytokines were analyzed in the supernatants of the proliferating cells at the end of 96h for respective immunization routes.

In mice receiving prime/boost vaccination through intraperitoneal route, the splenocytes predominantly secreted IFN- γ in response to three different concentrations of the beta2 toxin (Figure 6.4a). There were no significant levels of IL-2 or TNF- α in response to beta2 toxin observed in mice immunized through intraperitoneal route (Figure 6.4b and d). An appreciable IL-10 response was observed in mice that received the lipopeptide vaccine intraperitoneally (Figure 6.4c).

In subcutaneously immunized mice, there was predominant IFN- γ secretion by the proliferating splenocytes (Figure 6.5a). The IFN- γ response was dose dependent and increased with

increasing dose of the beta2 toxin. There was a moderate IL-2 response (Figure 6.5b), and no significant TNF- α response to beta2 toxin observed in mice immunized through subcutaneous route (Figure 6.5d). When compared to mice immunized intraperitoneally, there was weak IL-10 response in the subcutaneously immunized mice (Figure 6.5c).

6.4.4 Protective immunity against *C. perfringens* beta2 toxin challenge

The mice were challenged intraperitoneally with double the LD₅₀ dose (400 μ g/animal) of *C. perfringens* beta2 toxin and observed for signs of intoxication. The unimmunized mice showed signs of lethargy, reduced appetite and rough coat at the end of 24-48 hours. On day 42 after first immunization the mice were sacrificed and observed for any pathological changes. The unimmunized mice showed signs of enlarged liver, accumulation of blood colored fluid in abdominal cavity and moderately enlarged spleen. Animals that were immunized with the lipopeptide vaccine showed no obvious signs of pathology on necropsy. The sera from the subcutaneously immunized mice could also immunodetect native and recombinant *C. perfringens* beta2 toxin on a western blot assay (Figure 6.6).

6.5 Discussion

Beta2 toxin is a 28kD exotoxin implicated in necrotic enteritis in animals and humans, a disease that is characterized by a sudden acute onset with hemorrhagic ulceration of intestines. The beta2 toxin gene is transcribed during the vegetative growth, especially during the exponential growth phase. The beta2 toxin gene is regulated by the VirR/VirS two component regulatory system (Ba-Thein *et al.*, 1996). Our laboratory has successfully cloned, expressed and crystallized a recombinant beta2 toxin (Gurjar *et al.*, 2007). The recombinant toxin showed significant cytotoxic effects on a CaCo2 cell line and was shown to be reactive with monoclonal antibodies raised against the toxin (unpublished data).

Synthetic peptide vaccines offer an advantage over proteins, as they can be constructed with unusual geometries to enhance the immunogenicity and circumvent the toxicity of native proteins (van der Burg *et al.*, 2006). Zeng *et al.*, (2002) showed that chemically well defined vaccines provide an opportunity to incorporate immunostimulatory lipids like Pam2Cys at various positions in the immunogens. This allowed manipulation of the final structure of the lipopeptide vaccine to generate appropriate immune response (Figure 6.1). Pam2Cys is a synthetic analog to the lipid moiety of macrophage activating lipopeptide 2 isolated from *Mycoplasma* (Sacht *et al.*, 1998; Muhlradt *et al.*, 1997; 1998). The lipid Pam2Cys is a known potent stimulator of macrophages through interaction with the Toll like receptors present on the macrophages (Muhlradt *et al.*, 1997; 1998; Metzger *et al.*, 1995). This stimulation transmits signals for cellular maturation and migration of macrophages and produces inflammatory molecules required for pathogen clearance (Akira *et al.*, 2001). Water solubility and potency of the lipopeptide immunogen were improved by incorporating two ester bound palmitic acids and two serine residues between the lipid moieties (Figure 6.1) (Zeng *et al.*, 2002).

The lipopeptide conjugate described above was used to immunize two groups of four mice each. The first group was immunized intraperitoneally, while the second group was immunized subcutaneously. The dose of the lipopeptide vaccine (50 µg/dose) used for immunization was based on a previous report (Boersma *et al.*, 1995).

Serum antibody response was used to assess the efficacy of the beta2 synthetic vaccine. A high IgG1 antibody concentration was observed which was indicative of Th2 immune response. Similar observations were reported by Coffman and colleagues (Coffman *et al.*, 1988). IgG2a antibody concentration was higher in mice vaccinated subcutaneously than intraperitoneally which was suggestive of Th1 immune response (Mosmann *et al.*, 1989). The serum antibody concentration of both IgG1 and IgG2a suggests that a mixed Th1/Th2 response was elicited. A study conducted by

Zeng *et al.*, (2002) analyzed the efficacy of a synthetic lipopeptides as immunocontraceptive vaccines reported similar results. The induction of IgG1 and IgG2a isotype of antibodies could have been influenced by factors like adjuvant used, route of administration and location of antigen (Guan *et al.*, 1998). The immunized mice were protected against the pathological effects of the *C. perfringens* beta2 toxin. Finally, we could conclude that our lipopeptide vaccine could protect the vaccinated mice against *C. perfringens* beta2 toxin through circulating anti-beta2 antibodies. Synthetic lipopeptides have been reported as potent activators of murine and human macrophages, monocytes and polyclonal stimulators of murine B cells (Hope *et al.*, 2003).

The IgA and IgM antibody concentrations in the subcutaneously and intraperitoneally vaccinated mice were not different than the control group of mice. It has been reported that the immune response generated by clostridial vaccines was predominantly IgG1 (Reynolds *et al.*, 1990). In our study, the lipopeptide vaccine was constructed to generate systemic humoral response (IgG1 and IgG2a) and was not directed to stimulate mucosal immune response (IgA and IgM).

The T cell mediated immune response was measured by harvesting splenocytes from mice at the end of the vaccination trial. Results of the study showed that spleen cells from mice immunized subcutaneously with the lipopeptide vaccine exhibited a potent proliferative response when compared to mice that were immunized intraperitoneally. Mice immunized intraperitoneally failed to produce any proliferative response. There was no significant dose response (Figure 6.3a and b). The route of administration is important in the efficiency of immune response. The subcutaneous route was the most effective route, which may be due to the local inflammatory response that occurred after the immunization. The local immune response would result in an increase in the concentration of cytokines.

The type of Th response stimulated after immunization was further characterized by measuring four cytokines including IFN- γ , IL-2, IL-10, and TNF- α . The cytokines were measured in the supernatants collected from in vitro stimulated cells. IFN- γ was the most prominent cytokine in all the immunized mice through both the routes of administration. The high IFN- γ secretion pattern is indicative of dominant Th1 response (Saikh *et al.*, 1998). This Th1 type response was in accordance with the beta2 toxin specific IgG2a antibody isotype found in the same group of animals. IFN- γ stimulated innate cell-mediated immunity through the activation of macrophages which might be critical for driving the type of immune response observed (Saikh *et al.*, 1998). A predominant IFN- γ response was also observed by Saikh *et al.*, (1998), who demonstrated that a polypeptide and a DNA vaccine conferred protection against tetanus toxin. IL-2 could not be detected in vivo probably due to insufficient secretion by the cells.

IL-10 cytokine was moderately produced in both the groups of animals. The production of the cytokine IL-10, although lower than IFN- γ , is suggestive of a Th2 mediated response. This Th2 response was also in accordance with the beta2 specific IgG1 isotype of antibody production. The production of IL-10 cytokine and IgG1 antibody supported the presence of Th2 specific epitope in the lipopeptide construct. The lack of production of TNF- α in the supernatants of the proliferating splenocytes strongly suggests that the cytokine and antibody production was antigen specific and not being secreted by innate immune cells in response to toxin exposure.

The findings of our study indicated that humoral and cellular responses in a subcutaneously vaccinated group were considerably higher as compared to mice vaccinated intraperitoneally. The difference in these responses (subcutaneous vs. intraperitoneal) could be due to bioavailability of the lipopeptide or due to the regional distribution of specific receptors on the target cells as similar explanation was suggested by Hauge *et al.*, (2007). Eggert *et al.*, (1999) demonstrated the ability of

the dendritic cells to preferentially accumulate to greater extent in the T cell areas of the draining lymph nodes in subcutaneously immunized animals as compared to intraperitoneally immunized animals. This difference could explain the variation in the immune response through both the routes.

In conclusion, the overall findings of our study show that the synthetic lipopeptide vaccine against the *C. perfringens* beta2 toxin induces a beta2 specific immune response. The lipopeptide vaccine could serve a model for designing synthetic vaccines against various toxins and antigens of *C. perfringens* and related species. Future studies are required to evaluate the ability of the vaccine to provide intestinal immunity, and to evaluate whether the vaccine could protect against all beta2 toxin variants. The current lipopeptide vaccine can be further optimized to yield an earlier and a comprehensive immune response and could be compared with a recombinant beta2 toxin administered with a standard Freud's adjuvant.

6.6 ACKNOWLEDGEMENTS

We thank Ms. Nisebita Sahu of Dr. Avery August laboratory for her assistance in carrying out analysis of cellular immune response. We are also very grateful to Mrs. Shailaja Hegde for her assistance in animal studies. The present study was funded by College of Agriculture Seed Grant 2004 (Jayarao) and a Pennsylvania Department of Agriculture research grant, 2005 (Jayarao, ME# 444241).

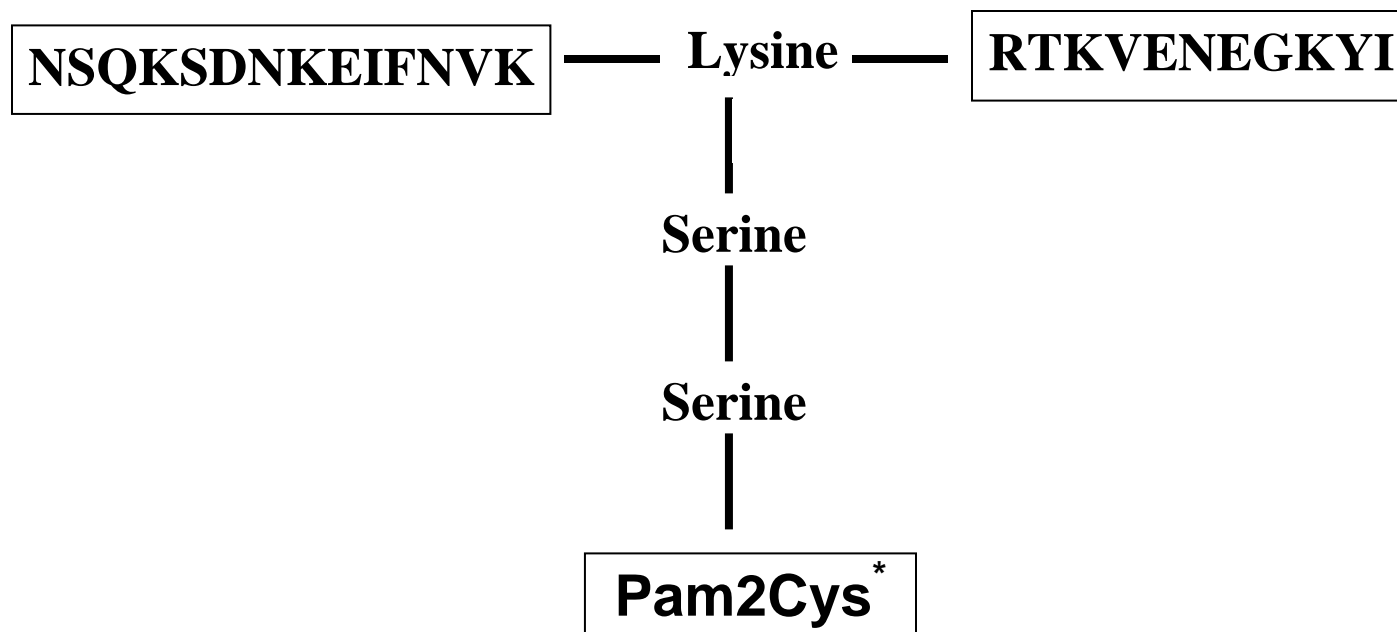
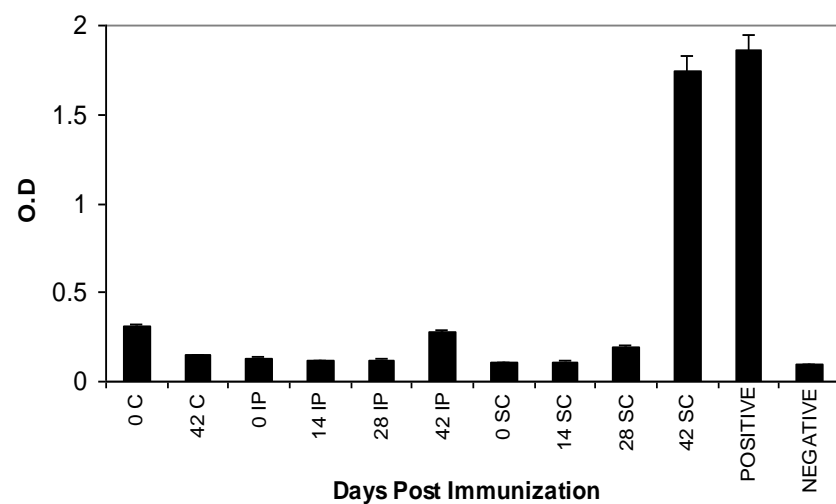


Figure 6.1 Lipopeptide construct including peptide 1 (NSQKSDNKEIFNVK), peptide 2 (RTKVENEGKYI), and Pam2Cys.

(a)



(b)

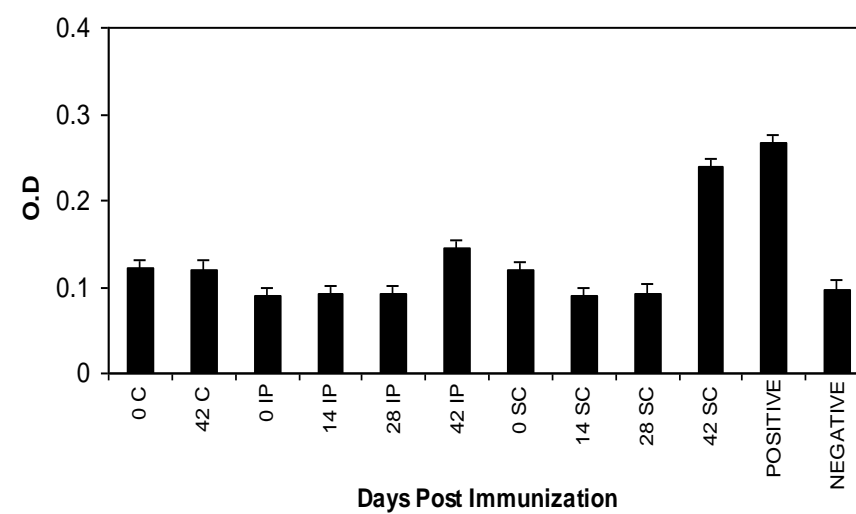
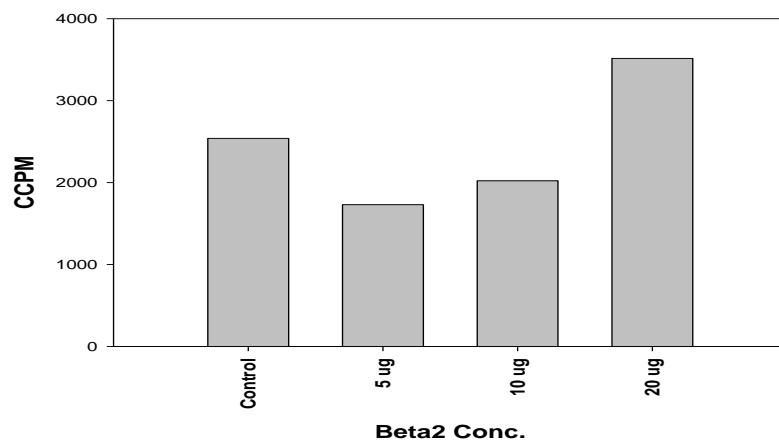


Figure 6.2 (a) The IgG1 Antibody Isotype response after vaccination. (b) The IgG2a Antibody Isotype response after vaccination. Legends: C - Control animals, IP – Intraperitoneal vaccinated animals, SC – Subcutaneous vaccinated animals, Positive control - Provided in ELISA Kit, Negative control – PBS.

(a)



(b)

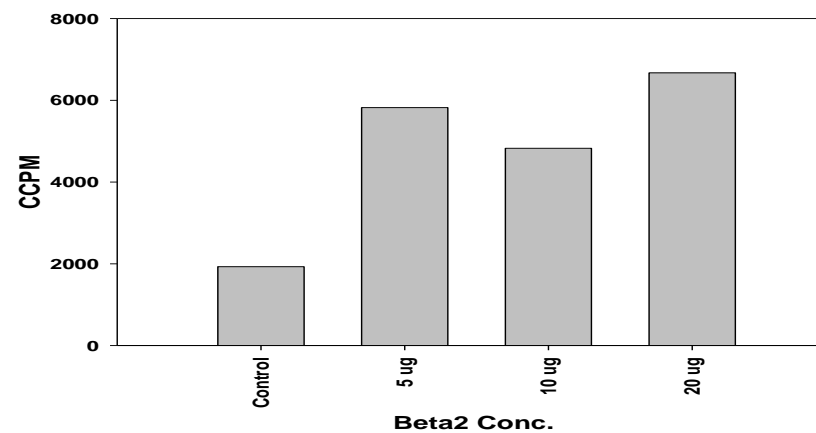
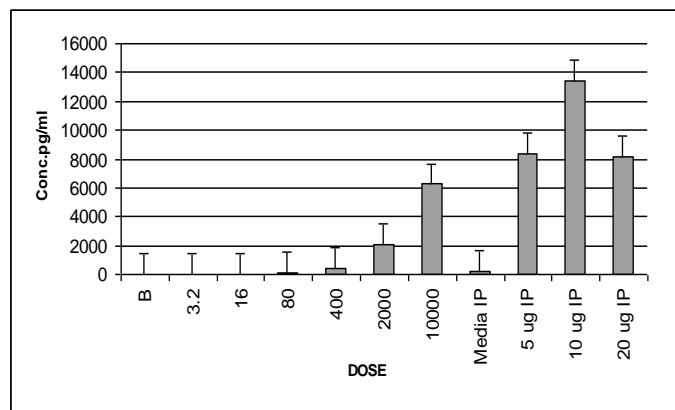
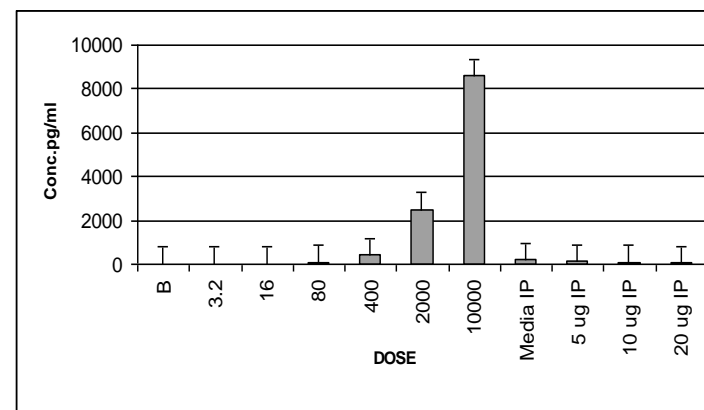


Figure 6.3 T-cell proliferation against various beta2 toxin concentrations in vaccinated animals. **(a)** Intraperitoneally vaccinated animals. **(b)** Subcutaneously vaccinated animals. (CCPM: corrected counts per minute)

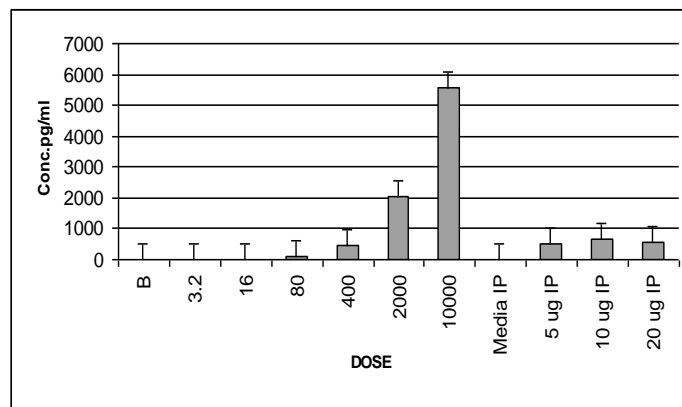
(a)



(b)



(c)



(d)

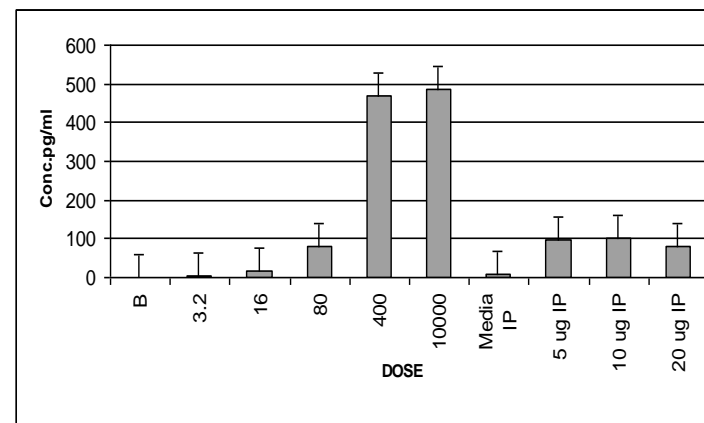
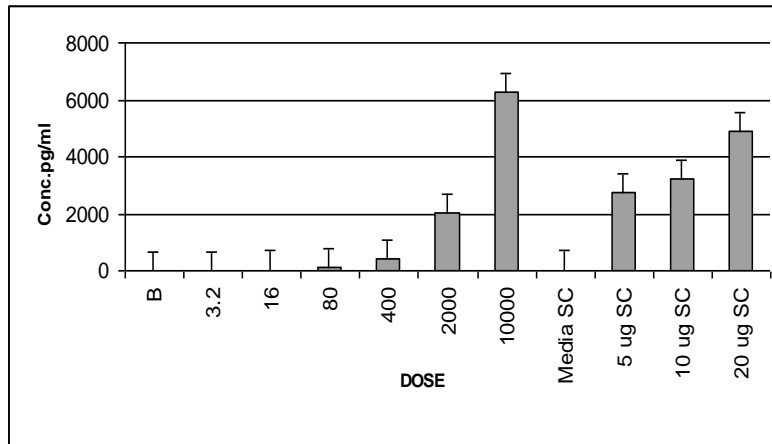
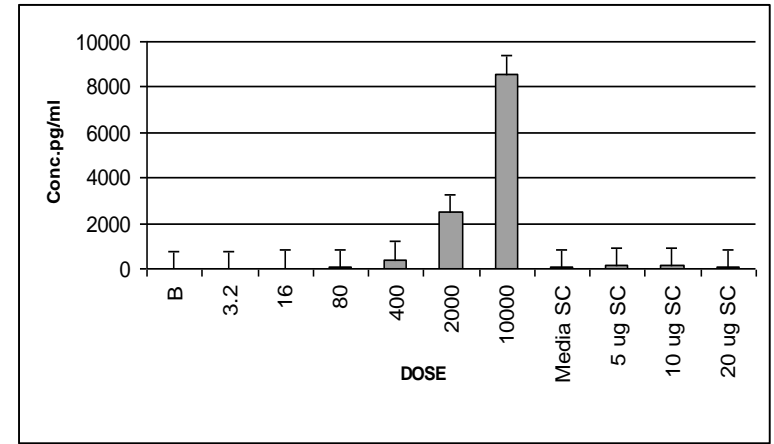


Figure 6.4 Cytokine response in Intraperitoneally vaccinated mice. (a) INF- γ secretion profile, (b) IL-2 secretion profile, (c) IL-10 secretion profile, (d) TNF- α secretion profile. Legends: B- Blank, 3.2-10,000 pg/ml of Standards, 5ug/ml- 20ug/ml- Beta2 Toxin Concentration Used for assay.

(a)



(b)



(c)

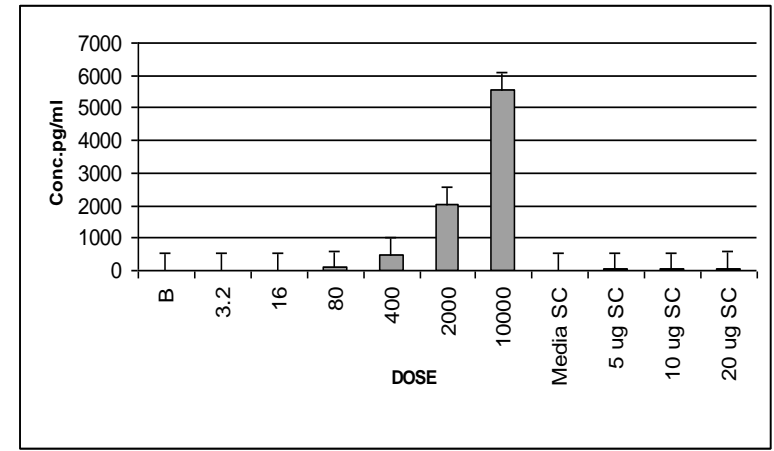
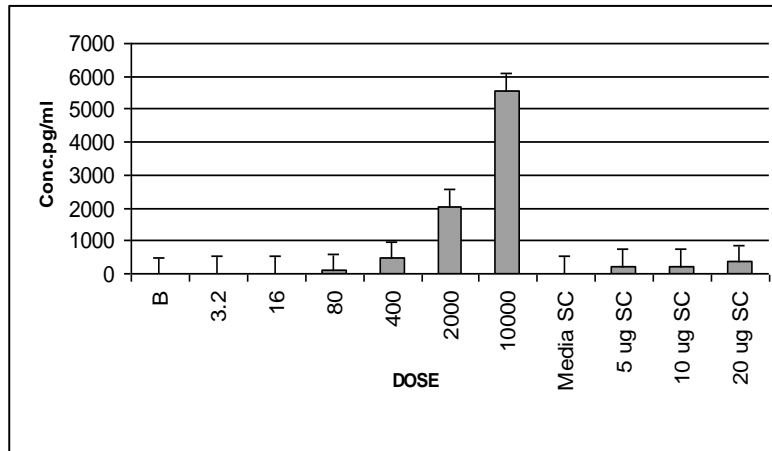


Figure 6.5 Cytokine response in Subcutaneously vaccinated mice. (a) INF- γ secretion profile, (b) IL-2 secretion profile, (c) IL-10 secretion profile, (d) TNF- α secretion profile. Legends: B- Blank, 3.2-10,000 pg/ml of Standards, 5ug/ml- 20ug/ml- Beta2 Toxin Concentration Used for assay.

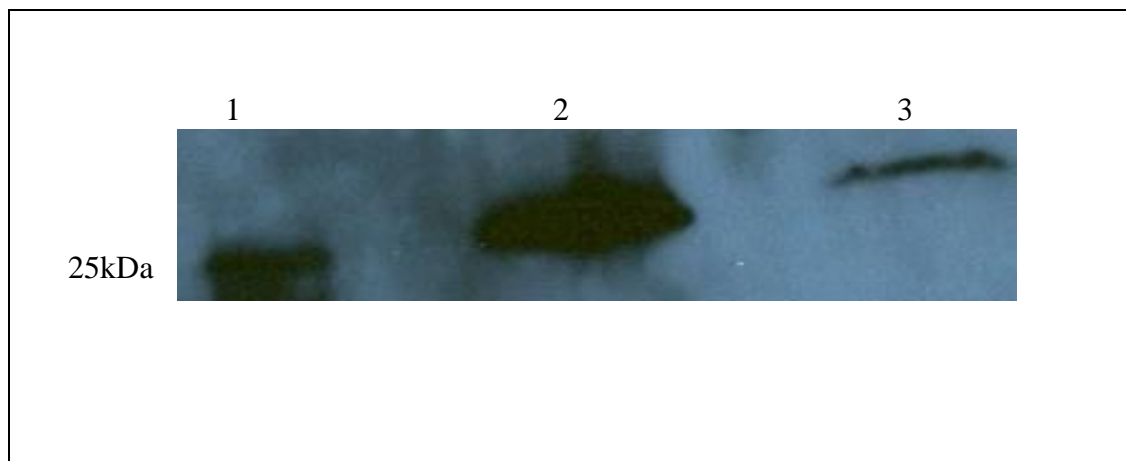


Figure 6.6 Western Blot assay showing immunodetection of recombinant (Lane 2) and native (Lane 3) Beta2 toxin by sera of subcutaneously immunized mice. MW: Molecular Weight marker (Lane 1).

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CHAPTER 7

SUMMARY AND CONCLUSION

7.1 Summary and Conclusion

The results reported in this dissertation reveal a high prevalence of *C. perfringens* beta2 toxin producing strains in the feces of lactating dairy cattle. Based on the toxinotyping results it was inferred that *C. perfringens* toxinotype A was widely distributed among the seven dairy herds screened for *C. perfringens* using real time PCR assay. The real-time multiplex PCR and detection of target toxin genes in fecal samples described in the study allows analysis of large number of fecal samples in a single assay. The application of the real time assay would permit rapid and accurate diagnosis of *C. perfringens* toxin types in a diagnostic laboratory setting and in conducting epidemiological investigations.

We were successful in using a well defined GST fusion system for expression of recombinant *C. perfringens* beta2 toxin. The overall technique described for expression of beta2 toxin can serve a model for cloning, expression and purification of other clostridial toxins. The purified recombinant beta2 toxin was cytotoxic to *CaCo2* cell monolayer at a concentration $>10 \mu\text{g/ml}$. Cytopathic effects like membrane blebbing, membrane disruption and cytoplasm leakage was apparent on Microbiolscopy. Cell detachment from the monolayer reflective of damage to the cell matrix contact was also observed under the Microbiolscope. These results suggest that the *C. perfringens* beta2 toxin could act like a pore forming toxin and has the potential to damage the intestinal epithelial cells. The recombinant beta2 toxin was immunologically active and was used to raise monoclonal antibody against beta2 toxin. The monoclonal antibody was shown to neutralize the cytopathic effects of the beta2 toxin on *CaCo2* cells. These monoclonal

antibodies could provide valuable diagnostic and therapeutic tools for veterinary practitioners, diagnosticians and researchers.

An attempt was made to determine the crystal structure of beta2 toxin with the objective of identifying the antigenic amino acid residues of beta2 toxin. Triangular prism or rhombohedron shaped crystals measuring 200 μ in size appeared in buffer conditions comprised of 32-36% PEG 3350 in one week. The crystals diffracted up to 2.9Å under cryo-annealing technique. The efforts made to optimize the crystallization technique for beta2 toxin could considerably enhance future studies that focus on solving the entire structure of the toxin. Based on our experience in doing this part of the study, it is felt that selenomethionine derivative crystallization and heavy atom soak studies will provide information for solving the entire crystal structure. Further structure determination of beta2 will provide an insight into the mechanism by which the toxin is able to interact with cell membrane.

To date, there are no commercially available immunoprophylactic products that offer protection against beta2 toxin. Also, concerns governing undesirable post vaccination effects have prompted veterinary biologics industry to seek a new paradigm for preparation and delivery of immunoprophylactic products. In this study we took the approach to designing and evaluating a safe chemically well defined vaccine against *C. perfringens* beta2 toxin. We have successfully developed and evaluated a synthetic lipopeptide vaccine against beta2 toxin. Two immunodominant epitopes from beta2 toxin were identified and synthesized. The epitopes were chemically linked to a known potent macrophage stimulator Pam2Cys (synthetic lipid moiety). The lipopeptide conjugate successfully elicited a strong protective immune response in subcutaneously vaccinated

animals. The synthetic lipopeptide induced a mixed Th1 and Th2 immune response as determined by the humoral and cellular immune response analysis. In conclusion, the results of this study indicate that lipopeptide vaccine could serve a model for designing synthetic vaccines against various toxins and antigens of *C. perfringens* and related species.

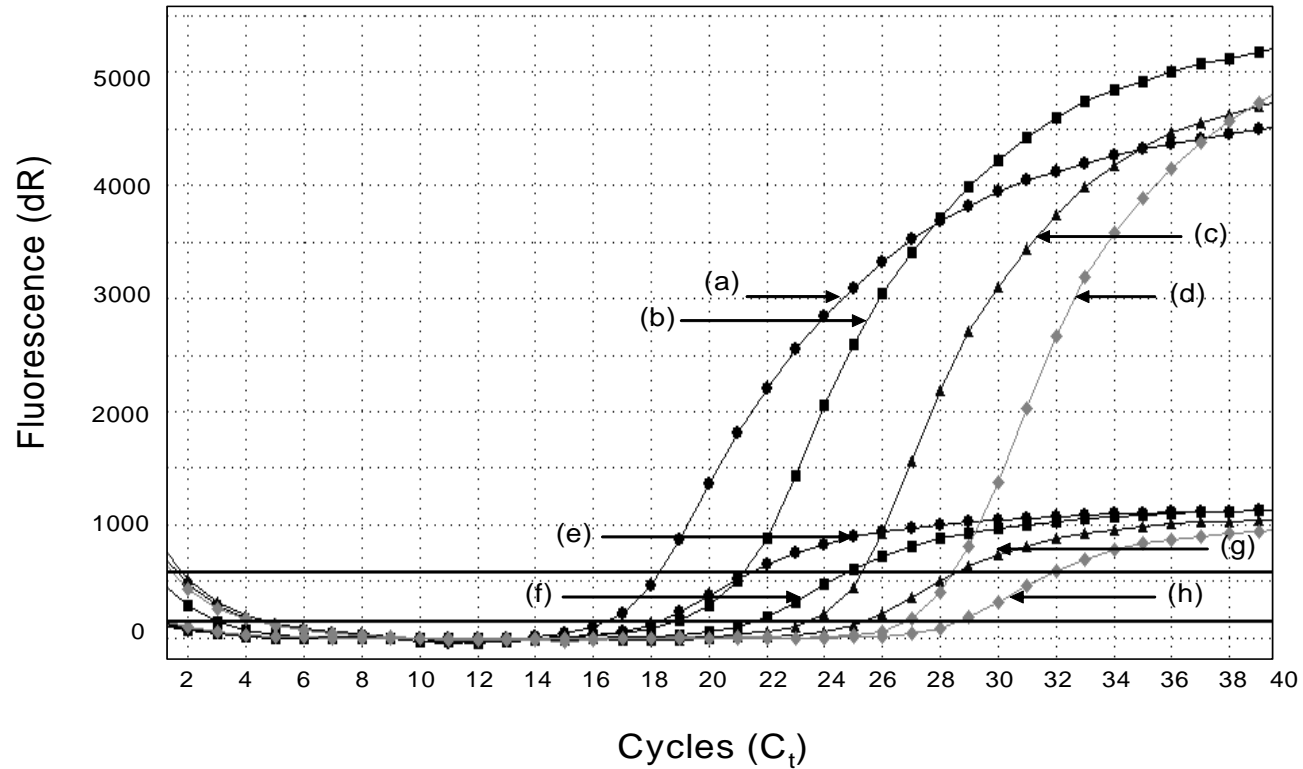
In summary, this dissertation demonstrates the applicability of using a real time PCR assay for rapidly toxintyping *C. perfringens* isolates. Our study has successfully demonstrated the designing and standardization of TaqMan probe based real time PCR assay for detection and toxintyping of *C. perfringens* from feces of lactating cattle. In addition, this data suggests a wide prevalence of *C. perfringens* alpha and beta2 toxin producing toxintypes in Pennsylvania dairy herds. Furthermore, this study strongly supports the premise that the *C. perfringens* beta2 toxin is a pore forming enteric toxin. The study demonstrates the successful crystallization of recombinant beta2 toxin. Although it was not possible to address all aspects to successfully resolve the structure of the beta2 toxin, this study provides a platform for continued investigation of the crystallographic analysis of beta2 toxin. This study also provides the “proof of concept” of a synthetic lipopeptide vaccine in immunoprophylactic measures to control clostridial enteric diseases. A complete understanding of the structural and biological mechanism of beta2 toxin activity will provide the necessary information for therapeutic approaches that prevent the beta2 toxicity.

APPENDICES

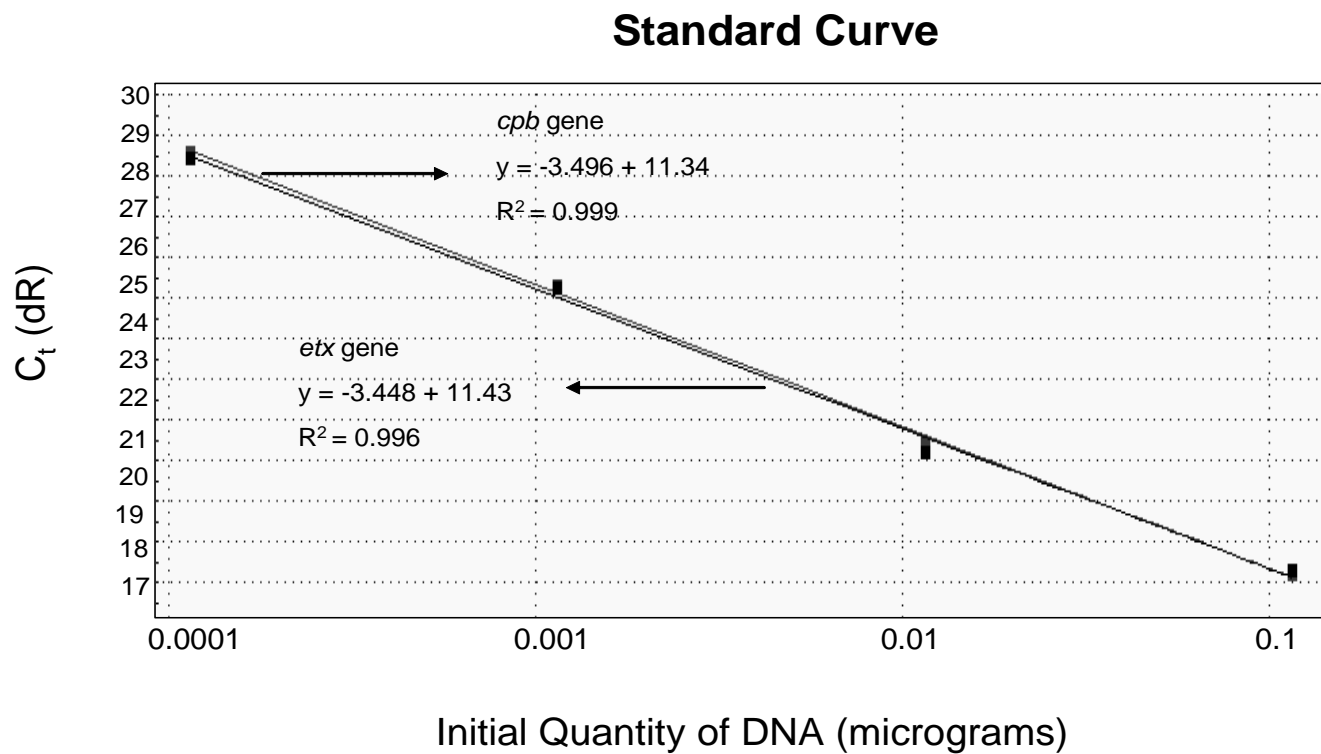
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Appendix A

Amplification Plot

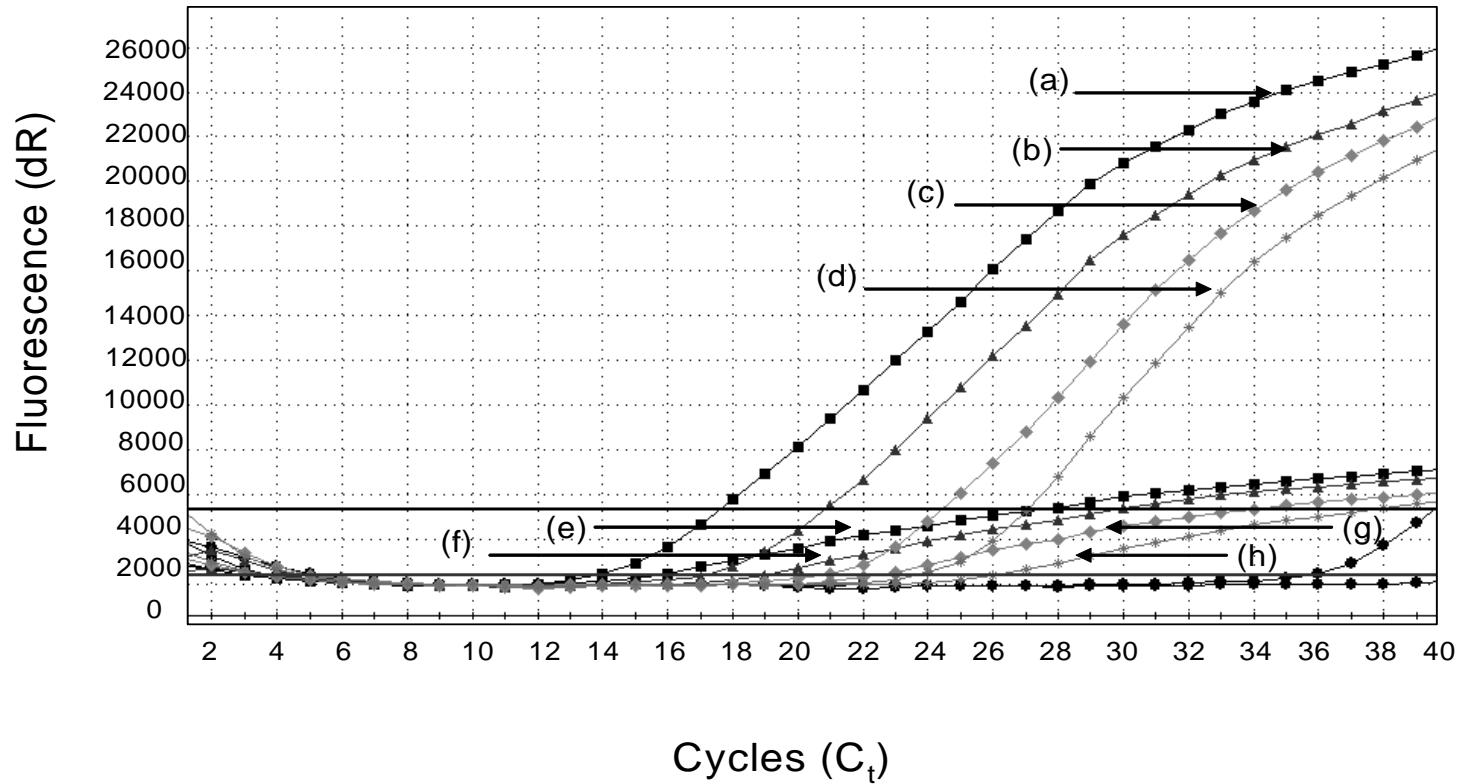


Amplification curves of Multiplex Real time PCR for detection *cpb* and *etx* gene of *C. perfringens*; (a) 1×10^4 pg of *cpb* DNA, (b) 1×10^3 pg of *cpb* DNA, (c) 1×10^2 pg of *cpb* DNA, (d) 1×10^1 pg of *cpb* DNA, (e) 1×10^4 pg of *etx* DNA, (f) 1×10^3 pg of *etx* DNA, (g) 1×10^2 pg of *etx* DNA, (h) 1×10^1 pg of *etx* DNA.

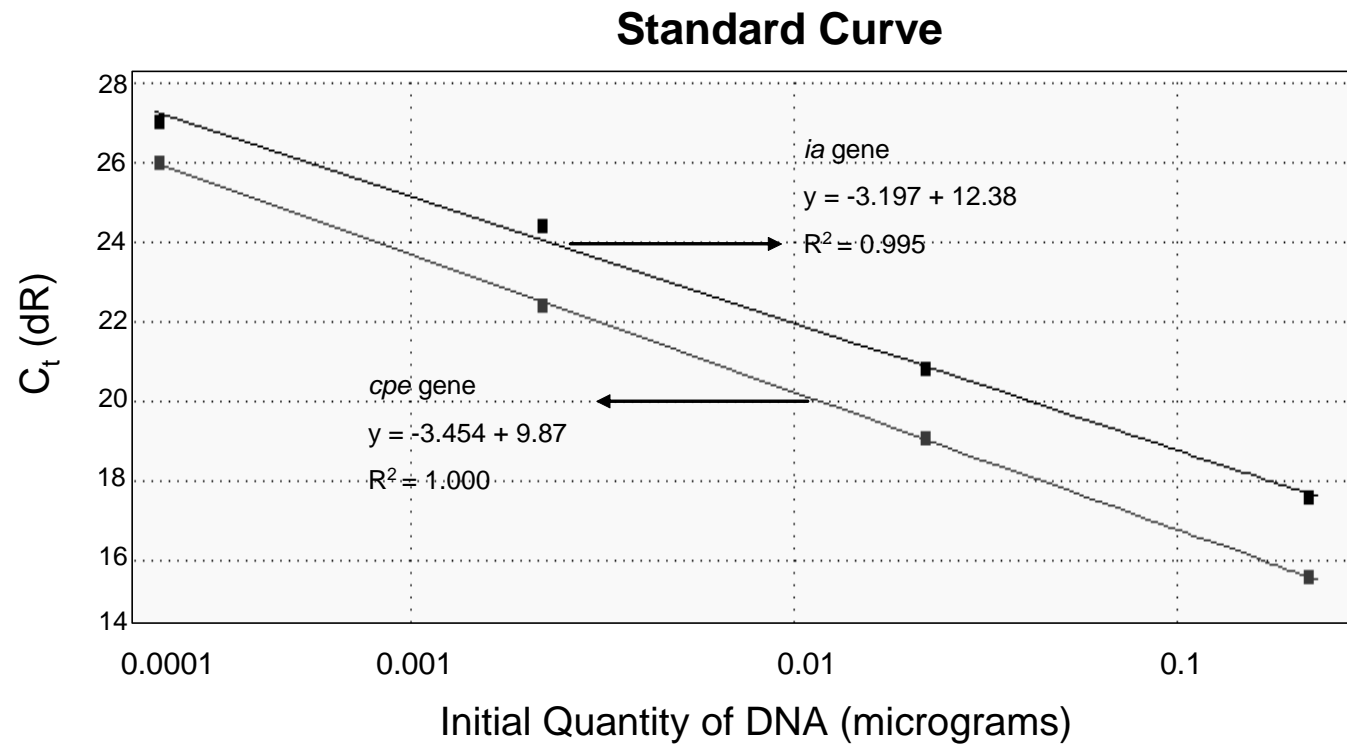


Linear regression of cycle threshold versus DNA concentrations for serial logarithmic dilutions of *Clostridium perfringens* *cpb* gene and *etx* gene.

Amplification Plot



Amplification curves of Multiplex Real time PCR for detection *ia* and *cpe* gene of *C. perfringens*; (a) 2×10^4 pg of *ia* DNA, (b) 2×10^3 pg of *ia* DNA, (c) 2×10^2 pg of *ia* DNA, (d) 2×10^1 pg of *ia* DNA, (e) 0.5×10^4 pg of *cpe* DNA, (f) 0.5×10^3 pg of *cpe* DNA, (g) 0.5×10^2 pg of *cpe* DNA, (h) 0.5×10^1 pg of *cpe* DNA.



Linear regression of cycle threshold versus DNA concentrations for serial logarithmic dilutions of *Clostridium perfringens* *ia* gene and *cpe* gene.

Appendix B

AAA GAA ATC GAC GCT TAT AGA AAG GTA ATG GAG ATT TAT CTT AAT
GCT TTA AAA AAC TAC GAT ATT AAT ACA GTT GTA AAC ATT TCA GAA
GAT GAA AGA GTA AAT AAT GTT GAA CAG TAT AGA GAA ATG TTA GAA
GAT TTT AAA TAT GAT CCT AAC CAA CAA CTG AAA TCT TTT GAA ATA
CTT AAT TCA CAA AAG AGC GAT AAT AAA GAA ATA TTT AAT GTA AAA
ACT GAA TTT TTA AAT GGT GCA ATT TAT GAT ATG GAA TTT ACT GTA TCA
TCT AAA GAT GGA AAA TTA ATA GTA TCT GAT ATG GAA AGA ACA AAA
ATT GAG AAT GAA GGA AAA TAT ATT TTA ACA CCA TCA TTT AGA ACT
CAA GTT TGT ACA TGG GAT GAT GAA CTA GCA CAA GCA ATT GGG GGA
GTT TAT CCA CAA ACA TAT TCT GAT AGA TTT ACA TAT TAT GCA GAT
AAT ATA TTA TTA AAC TTC AGA CAA TAT GCA ACT TCA GGT TCA AGA
GAT TTA AAA GTA GAA TAT AGT GTT GTA GAT CAT TGG ATG TGG AAA
GAT GAT GTT AAA GCT TCT CAA ATG GTA TAT GGT CAA AAT CCT GAT
TCT GCT AGA CAA ATA AGA TTA TAT ATA GAA AAA GGA CAA TCT TTC
TAT AAA TAT AGA ATA AGA ATT AAA AAC TTT ACA CCT GCA TCA ATT
AGA GTA TTT GGT GAA NGG TAT TGT GCA CTC NAG CGG CCG CAT CGT
GAC TGA CTG ACG ATC TGC CTC GCG CGT TTC GGT GAT GAC GGT GAA
AAC

***Clostridium perfringens* beta2 toxin gene GeneBank Accession Number EU260099.**

Crystallographic Conditions	Results
1.5M Lithium Bromide + 0.1M Tris 5 M Lithium Chloride + 0.1M MES	Crystals of 50μ in size formed after 3 weeks but confirmed as salt crystals
1.1M Lithium Sulfate + 0.1M Tris pH 8,8.5,9.0 24 Well Plate	Crystals of 50μ in size formed after 3 weeks but confirmed as salt crystals
1.1M Lithium Sulfate + 0.1M Tris pH 8,8.5,9.0 96 Well Microbiol plate	Crystals of 50μ-100μ in size formed after 1 weeks but confirmed as salt crystals
0.2M Mg.acetate + 30-36% PEG, 0.2M Mg.sulfate + 30-36% PEG, 0.2M Li.sulfate + 30-36% PEG, 0.2M Am.sulfate + 30-36% PEG	Very good Crystals of 50μ-100μ in size seen in 2 weeks.
0.2 M Li.sulfate + 30-36% PEG3350	Crystals of size 150-200μ Observed in 2 weeks
0.2 M Li.sulfate + 30-36% PEG3350 + 12% of 1, 1, 1,3,3,3 hexa-fluoro 2-propanol screen	Very good three dimensional crystals with size 200 μ observed in 2 weeks

Preliminary Screening Conditions for Crystallization of *C. perfringens* beta2 toxin

Full Image

Actions

Plate Number 13 Well Number 1153

1536 Well Plate Specifications

Plate Number: X000006634

Well Number: 0050

Date (mm-dd-yy): 04-05-2006

Time (hh:mm): 10:10

Composite Information

Cocktail #: 6_C1153

Commercial Code:

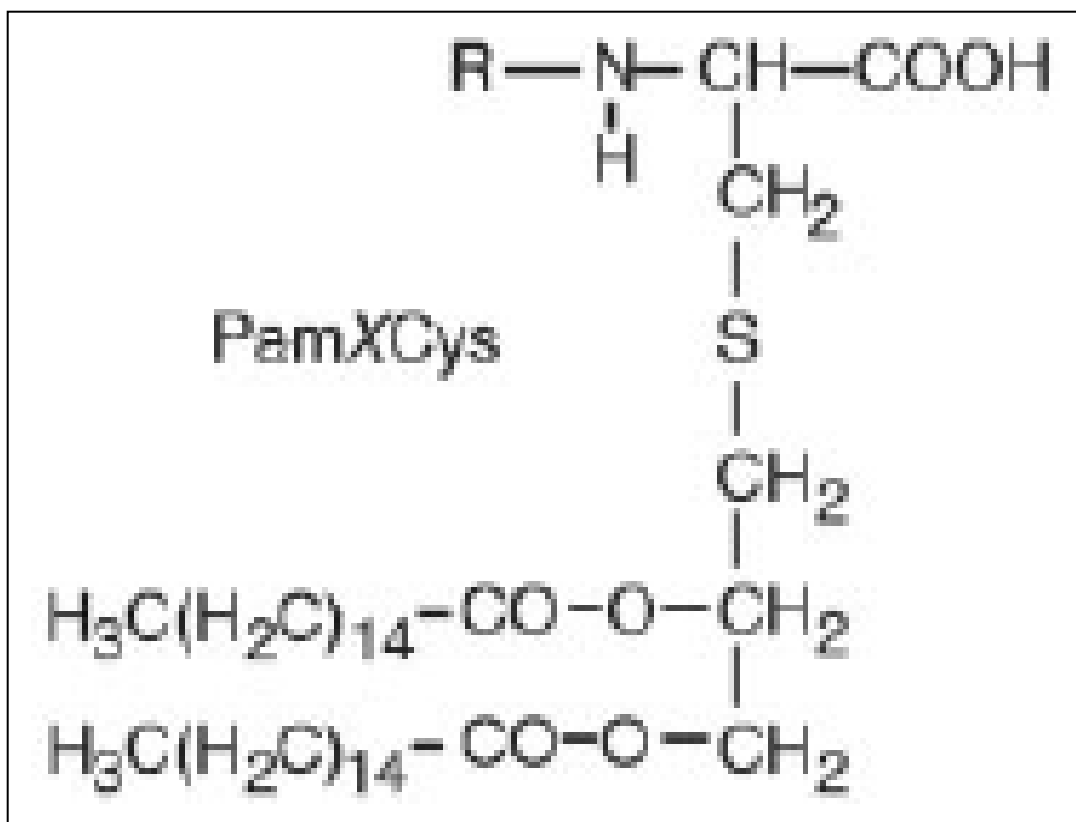
HR PEG/Ion Screen 25

Chemical Information:

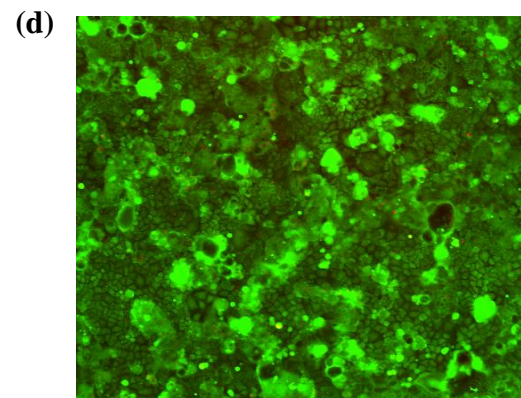
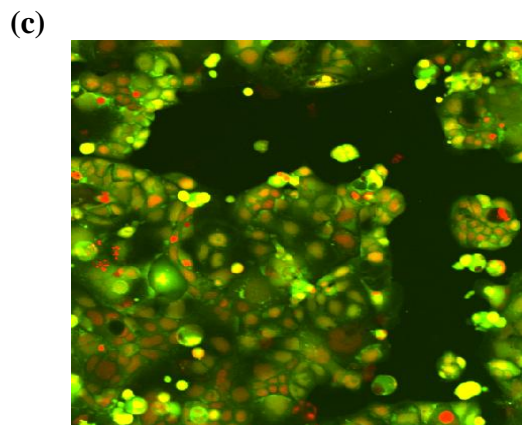
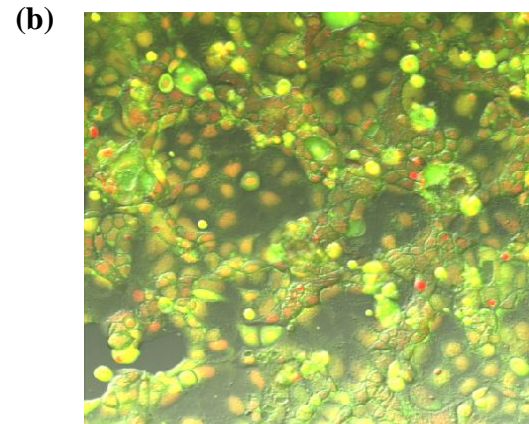
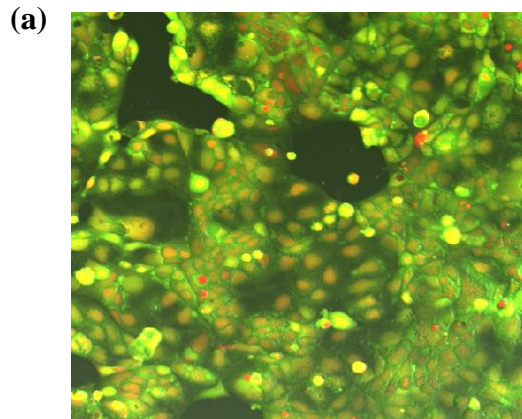
0.2M magnesium acetate tetrahydrate ()

20% (w/v) PEG 3350

Clostridium perfringens beta2 toxin needle shaped crystals in preliminary screening conditions at Hauptmann Woodward Institute.



Chemical Structure of Dipalmitoyl-S-glyceryl cysteine (Pam2Cys). R =H.



Cytopathic effects of recombinant *C. perfringens* beta2 toxin on *CaCo2* cells monolayer. (a) Control Cells, (b) 10 µg/ml of beta2 toxin, (c) 20 µg/ml of beta2 toxin, (d) 40 µg/ml of beta2 toxin.

VITA

Abhijit Arun Gurjar

Education

Ph.D. (Pathobiology, 2004-Present), Department of Veterinary and Biomedical Sciences. The Pennsylvania State University, University Park.

M.V.Sc. in Food Hygiene & Veterinary Public Health (2001). Bombay Veterinary College, Mumbai, India.

B.V.Sc & A.H (1999). Bombay Veterinary College, Mumbai, India.

Awards and Honors

Awarded **Biological Sciences Divisional Winner Plaque** at the College of Agricultural Sciences/Gamma Sigma Delta Annual Graduate and Undergraduate Research Expo March 21-22, 2007, at Penn State University.

Awarded **1st overall Prize winner** at the 10th Annual Environmental Chemistry Student Symposium April 13 - 14, 2007, organized by Penn State University Center for Environmental Chemistry and Geochemistry.

Publications

Purification, crystallization and preliminary crystallographic analysis of *Clostridium perfringens* beta2 toxin. Acta Cryst. (2007). **F63**, 484-487.

Real-Time Multiplex PCR assays for Rapid Detection and Toxintyping of *Clostridium perfringens* Toxin Producing Strains in Feces of Dairy Cattle. Molecular and Cellular Probes. (2008). Apr **22 (2)**, 90-95.

Expression, Cloning, Purification and study of cytopathic effects of *Clostridium perfringens* beta2 toxin. (In preparation).

Evaluation of Synthetic Lipopeptide Vaccine against *Clostridium perfringens* beta2 toxin. (In preparation).