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IN *VITRO* ASSAY DEVELOPMENT AS AN ALTERNATIVE TO THE USE OF LABORATORY ANIMALS FOR MEASURING *CLOSTRIDIUM PERFRINGENS* TYPE C TOXOID

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by

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IN *VITRO* ASSAY DEVELOPMENT AS AN ALTERNATIVE TO THE USE OF LABORATORY ANIMALS FOR MEASURING *CLOSTRIDIUM PERFRINGENS* TYPE C TOXOID

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Clostridium perfringens type C produces beta toxin, which is a primary virulence determinate that can cause necrotic enteritis in neonatal animals. Vaccines directed against *C. perfringens* toxins have shown to be efficacious in preventing disease. However, the production of a commercial vaccine requires not only an efficacious antigen, but also testing methods for quantifying antigens and potency on final product. This research was undertaken to fulfill a need for the reduction in small animal usage to produce and deliver vaccines. The current testing methods are performed in *vivo* according to the Code of Federal Regulations number 9 (9CFR) United States Department of Agriculture (USDA) and European Pharmacopeia (EP) monograph guidelines. With limited in *vitro* alternatives, the aim of this work was to develop an enzyme-linked immunosorbent assay (ELISA) to be able to measure *C. perfringens* type C beta toxoid, independent of laboratory animals. To accomplish this, investigational polyclonal and monoclonal antibody candidates were screened for their specificity to beta toxin and toxoid through western blot and ELISA. Additionally, through a neutralization assay, the capability to neutralize beta toxin was demonstrated. Through this process, one polyclonal antibody and one monoclonal antibody were selected and an in-process

ELISA for quantifying beta toxoid was developed. By using the ELISA to quantify beta toxoid, the current practice of testing in animals could be reduced.

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CHAPTER I

INTRODUCTION

Clostridium perfringens, a Gram positive anaerobic rod shaped bacterium, is a major cause of enteric disease in both humans and livestock. One such enteric disease is necrotic enteritis, a severe disease found in animals that can have a significant negative financial impact to agricultural markets (Keyburn et al., 2010; Smedley et al., 2004). In addition to animal diseases, *C. perfringens* causes food poisoning and gas gangrene in humans. There are five toxinotypes, A thru E, of *C. perfringens* that are classified based on the type of toxins each produces. *C. perfringens* type C produces alpha and beta toxin, with beta toxin being the primary virulence factor of necrotic enteritis in neonates.

C. perfringens can be found in many environments, such as soil, waste water, food, feces and in both healthy human and animal intestines as normal flora. Vaccination of animals can help prevent outbreaks of disease in livestock. These vaccines use inactivated whole bacteria, recombinant proteins and/or purified toxoids. One obstacle to producing more efficacious vaccines is complying with required potency testing as mandated by the United States Department of Agriculture (USDA) and European Pharmacopoeia (EP). Currently, the in *vivo* methods available for potency testing cause pain, suffering and death in laboratory animals. Development of an in *vitro* assay to measure vaccine potency would minimize animal use and support commercialization of effective vaccines. This is long overdue as *C. perfringens* species have been known since the turn of the century and scientists have been searching for effective vaccines since the early 1900s.

1.1 HISTORY OF *CLOSTRIDIUM PERFRINGENS*

In 1892, William Welch and George Nuttall discovered *Bacillus aerogenes capsulatus* during the postmortem examination of a 38 year old man at John Hopkins University. The use of cultivation media and dyes (*i.e.,* methylene-blue, gentian-violet and fuchsine) permitted Welch and Nuttall to observe the bacterium's morphological features by fixing and staining the bacteria for microscopy. They observed that *Bacillus aerogenes capsulatus* was a rod shaped bacilli that was at times encapsulated (Welch and Nuttall, 1892). In addition, Welch and Nuttall observed the bacilli grew anaerobically as small colonies were present in the bottom two-thirds (oxygen depleted region) of an agar tube, while the upper third (microaerobic) had no growth. Based on these observations, they arrived at the name *Bacillus aerogenes capsulatus* (Welch and Nuttall, 1892). Over time the bacterium became known as *Bacillus welchii* and *Clostridium welchii,* before being renamed as *Clostridium perfringens* (Dembek, 2008).

1.2 MORPHOLOGY AND CLASSIFICATION OF CLOSTRIDIUM

Clostridium is a genus in the class Clostridia and the phylum Firmicutes. This genus consists of more than 100 species; however, the four species most commonly associated with human and animal disease are *C. perfringens, C. tetani, C. botulinum* and *C. difficile. Clostridium* spp. are non-motile, Gram positive rods that grow anaerobically and form spores (Uzal et al., 2016; Vasanthakumari, 2007). A single bacterium of *C. perfringens* averages 0.6-2 µm in diameter by 1.3-6 µm in length and can be found in pairs, single rods and small clusters (Uzal et al., 2016; Vasanthakumari, 2007). After 24 hours of growth under anaerobic conditions, *C. perfringens* colonies are smooth, circular,

semi-translucent and approximately 2-5 mm in diameter (Uzal et al., 2016). Bacteria categorized as anaerobes are incapable of growing in the presence of oxygen and can be further classified as either strict anaerobes or aerotolerant anaerobes. Strict anaerobes, do not live when exposed to oxygen, while aerotolerant anaerobes grow anaerobically but can survive being exposed to oxygen (Uzal et al., 2016). *C. perfringens* has the ability to survive under-micro-aerobic conditions, thus being classified as an aerotolerant anaerobe.

C. perfringens produces four major toxins that are essential for virulence. In addition to being essential for virulence, the four major toxins of *C. perfringens* are used to classify the bacteria into five toxinotypes A, B, C, D and E (Table 1). The four toxins being: alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX) (Uzal et al., 2014). CPA causes damage to many cell types, including erythrocytes, hepatocytes and myocytes. CPA disrupts the cell surface phospholipid bilayers by cleavage of the phosphorylcholine head groups, which disrupts the cell membrane causing necrosis and cell lysis (Li et al., 2013). In livestock, cellular pore formation is a result of CPB toxin oligomerization on the cell surface via lipid rafts. This causes an efflux of potassium ions and an influx of sodium and chloride ions causing an increase in turgor pressure leading to cell lysis (Geny and Popoff, 2006). ETX is also a pore forming toxin that affects brain, lung and smooth muscle cells. A remarkable feature of ETX is the ability to selectively cause death of oligodendrocytes without killing astrocytes, microglia or neurons of the central nervous system (Linden et al., 2015). ITX is an A-B toxin consisting of the enzymatic protein Ia and the cell surface binding protein Ib. Individually, the two components are inactive but together cause enterotoxemia by increasing intestinal cell permeability and cell rounding and death through ADP-ribosylation of G-actin (Sakurai et al., 2009).

These toxins contribute to the diversity of species and tissues affected by *C. perfringens* strains.

Table 1: *C. perfringens* toxinotype classification based on the presence or absence of the four major toxin classes.

Type	Alpha (α)	Beta (β)	Epsilon (ε)	Iota (ι)
A	+	-	-	-
B	+	+	+	-
C	+	+	-	-
D	+	-	+	-
E	+	-	-	+

1.3 INFECTIONS CAUSED BY *C. PERFRINGENS*

Each *C. perfringens* toxinotype is associated with specific human and/or animal diseases highlighting the versatility of this bacterium (Smedley et al., 2004; Uzal et al., 2014). *C. perfringens* type A and type C are the two toxinotypes associated with both human and animal disease. In humans, type A strains can induce food poisoning, non-foodborne gastrointestinal disease and gas gangrene mainly due to the effect of CPA. Type C strain infections in humans cause necrotic enteritis also known as pigbel. This is an intestinal infection causing abdominal pain, vomiting and in severe cases death due to CPB toxemia (Uzal et al., 2014; Uzal and McClane, 2011). In addition to human infections, all *C. perfringens* toxinotypes can infect adult animals; however, these infections are predominantly seen in neonates that have not established normal gut flora. *C. perfringens* type A causes gas gangrene infections in cattle, sheep, goats and horses that are similar to human infections. When type C infections occur in livestock species, they cause necrotic enteritis and enterotoxemia (Uzal and McClane, 2011; Uzal et al.,

2010). Necrotic enteritis is one of the most important diseases of *C. perfringens* type C, which is found in goats, cattle, pigs and horses.

The CPB toxin produced by *C. perfringens* type C can cause a neurological disease that includes rigid paralysis, also known as opisthotonos, where the animals demonstrate backward arching of the head, neck and spine (Nagahama et al., 2015; Tweten, 2001). This neurological involvement is due to the toxin being able to move into circulation and cross the blood brain barrier. In adult sheep, the condition known as struck refers to a type C disease. The presentation of struck within sheep is described as if they have been struck by lightning.

Characteristics of necrotic enteritis are hemorrhagic mucosal ulceration or superficial mucosal necrosis of the small intestine, which is often followed by death or acute neurological signs (Smedley et al., 2004). The other toxinotypes, such as type B which produces CPA, CPB and ETX causes hemorrhagic dysentery in sheep. Enterotoxemia of sheep, goats and cattle and enteritis in dogs, cattle and pigs are caused by type D (ETX) and type E (ITX) respectively (Uzal et al., 2014). Each of the *Clostridial* toxinotypes has a specific pathogenesis; however, since this thesis focuses on the development of a CPB analytical assay, only *C. perfringens* type C will be further discussed.

1.4 PATHOGENESIS OF *C. PERFRINGENS* TYPE C

Clostridium perfringens is found in soils, feces and human and animal digestive tracts. For infections to occur in neonates, *C. perfringens* must first gain entry into the host digestive tract. The normal intestinal microbiota takes time to develop a stable

community in newborns, which makes them highly susceptible to colonization by type C strains, resulting in disease in young animals (*i.e.,* exposure 24 – 48 hours after birth) (Uzal et al., 2014; Uzal and McClane, 2011). Transmission occurs via the fecal oral route by suckling contaminated teats, ingesting colostrum during nursing from mothers who carry the bacterium, licking contaminated objects in the surroundings, or by coming into close contact with contaminated fecal material from infected animals (Niilo, 1988). Susceptibility is believed to be due to low or absent proteinase activity in the neonate, and the trypsin inhibitors in colostrum may contribute to this problem (Uzal et al., 2016). Once the development of normal flora is established in young animals, opportunistic infections by toxinotype C bacteria are greatly reduced (Tweten, 2001). While type C strain infections primarily occur in infant animals, adult animals are also susceptible to infections. This is likely due to gastrointestinal factors that affect an increase in the pH of the stomach, which favors growth, colonization and toxin production in the intestine. A diet high in foods with trypsin inhibitors such as soybeans may contribute to the susceptibility of adult animals (Uzal et al., 2016). Another factor contributing to type C strain infections are unvaccinated herds, where the mortality rate can be greater than 50% (Uzal and McClane, 2011).

Once *C. perfringens* toxinotype C bacteria reach the intestine, they adhere to the epithelium of the small intestine and secrete alpha and beta toxins causing necrosis. The damage caused by CPB allows other toxins (*i.e.,* CPA) to be introduced into the host's blood (Nagahama et al., 2015). Circulating CPA exacerbates the destruction of epithelial cells and permits invasion of bacteria. All of this causes a cascade effect, which leads to increased toxin production, cellular necrosis, hemorrhage and death (Nagahama et al.,

2015; Niilo, 1988). These observations contribute to the belief that CPB is the most important toxin for virulence (Gurtner et al., 2010; Sayeed et al., 2008; Uzal et al., 2009; Vidal et al., 2008).

1.5 EVIDENCE FOR THE INVOLVEMENT OF BETA TOXIN

A number of animal models have been used to determine the function of CPB as a virulence factor. When animals and humans were given crude beta toxoid, they were protected against strain type C infections suggesting that CPB was an essential virulence factor (Walker et al., 1979). A mouse model using intravenous injection of CPB from type C culture supernatants also demonstrated that CPB was important for virulence (Fisher et al., 2006). Lethality of CPB is inhibited by monoclonal antibodies to CPB, protecting mice from a lethal intravenous challenge; however, lethality was not attenuated in mice with a monoclonal antibody to CPA (Fisher et al., 2006; Uzal and McClane, 2011). In addition, studies using *cpb* null mutants to infect mice, rabbit and goats in an intestinal loop model demonstrated that *cpb* mutants are attenuated in virulence relative to the wild-type strains (Sayeed et al., 2008). Taken together, CPB is essential for virulence in toxinotype C infections.

1.6 CHARACTERIZATION OF BETA TOXIN

Bacterial toxins are generally classified as either endotoxins or exotoxins. Endotoxins are associated with the cell envelope of Gram negative bacteria which have a lipopolysaccharide complex associated with the outer membrane. Exotoxins are proteins that are secreted by many Gram positive and Gram negative bacteria. A subclass of

exotoxin is enterotoxins, which target the intestine and are commonly pore-forming toxins that disrupt cell membranes. The CPB exists as a monomer or oligomer and is defined as an exotoxin that forms pores (Popoff, 2014; Shatursky et al., 2000). The monomer is the toxic form and can readily convert to the nontoxic oligomer form in *vitro* (Sakurai and Nagahama, 2006); however, the oligomeric form is unable to dissociate back into the monomeric form. The 35 kDa CPB is secreted during the post-exponential growth phase and is known to be susceptible to proteolytic and heat inactivation, but not by low pH (Sakurai and Duncan, 1978; Uzal and McClane, 2011; Vidal et al., 2008). The *cpb* gene is carried on a virulence plasmid found in type C isolates and codes for a 336 amino acid protein containing a 27 amino acid leader sequence that is cleaved to form the mature beta toxin protein (Hunter et al., 1993; Nagahama et al., 2015; Sakurai et al., 2004; Uzal et al., 2010). In susceptible cell lines, such as primary porcine cells, human umbilical vein endothelial cells (HUVEC), and human leukemia cells (HL-60 cells), CPB intoxication results in swelling and cell lysis (Nagahama et al., 2015; Uzal and McClane, 2011).

1.7 MODE OF ACTION OF BETA TOXIN

The *C. perfringens* type C beta toxin is a pore forming toxin that forms multimeric complexes in HUVEC cell membranes (Steinthorsdottir et al., 2000) and cation-selective pores in phospholipid bilayers (Shatursky et al., 2000). In addition, CPB induces swelling and cell lysis in the human leukemia cell line HL-60 (Nagahama et al., 2003). One hypothesis is that CPB monomers (35 kDa) are incorporated into susceptible membranes and oligomerize into ~228 kDa hexamers or heptamers to form a

transmembrane pore (Smedley et al., 2004). More recently, it was postulated that CPB monomers recognized an unidentified receptor on the cell surface, then oligomerize to form a prepore and the prepore is inserted into the lipid bilayer to form a pore (Geny and Popoff, 2006; Popoff, 2014). Irrespective of the mechanism, CPB induced cell death is initiated by the formation of pores that cause an efflux of K^+ and Ca^{2+} with a concomitant influx of Na^+ and Cl^- ions resulting in cell lysis (Autheman et al., 2013; Nagahama et al., 2003; Uzal et al., 2014). To prevent infections in animals caused by the pore-forming CPB, prophylactic vaccination has mostly proven to be efficacious in preventing type C disease.

1.8 VACCINES

Commercially available *C. perfringens* type C vaccines for veterinary use contain chemically inactivated cultures where the toxin is now referred to as toxoid of type C strains, or in combination with types A, B or D strains. These inactivated cultures produce an antigenic response to the type C CPB despite detoxification of the antigen. The use of pure or mixed cultures concomitant with the production of extraneous proteins has created a situation where the efficacy of vaccines is highly variable, and has increased the demand for more reproducible and quantifiable antigens such as recombinant proteins.

The variability of vaccine preparations can be seen in a field trial for Per-C-porc®. In this trial, sows were given Per-C-porc® a type C toxoid vaccine, at a breeding facility that had experienced significant losses of piglets due to the *C. perfringens* type C disease. Prior to vaccination, three batches of the Per-C-porc® vaccines were tested in

accordance with the European Pharmacopoeia, the same requirements as the Code of Federal Regulations number 9 (9CFR) USDA, in which there has to be at least 10 international units (IU) of beta antitoxin per mL of rabbit serum (Springer and Selbitz, 1999). The results from the in *vivo* efficacy testing of the vaccine batches revealed that all three batches fulfilled the regulatory requirements, however the mean titer values varied greatly with a range of 17.14 to 98.23 IU beta antitoxin per mL in rabbit serum (Springer and Selbitz, 1999). The titer fluctuation was due to inconstancies in antigen production, which resulted in different yields of toxin during fermentation that affected performance in efficacy testing. The type C vaccine with the lowest IU beta antitoxin per mL rabbit serum result was used to vaccinate the sows. During this study, there was a slight decrease in piglet loss when the sows were vaccinated and their piglets were administered benzylpenicillin potassium within the first three days of birth. The need for antibiotic use shows the type C toxoid vaccines are not fully protective and antibiotics are needed to protect piglets from *C. perfringens* type C disease (Springer and Selbitz, 1999). The variability in manufacturing and the need for antibiotics highlighted the need for more efficacious vaccine strategies (Springer and Selbitz, 1999).

Other vaccine approaches include the use of recombinant proteins expressed in *Escherichia coli* and harvested from solubilized inclusion bodies are used as immunogens to vaccinate animals (Zeng et al., 2011). The vaccines had either monovalent recombinant α-toxin (CPA), a fusion protein of β2/β1 (CPB2B1), a trivalent fusion protein consisting of α/β2/β1-toxins (CPAB2B1) or a cocktail CPA plus CPB2B1. These recombinant protein vaccines were administered to mice, cows and sows. In a mouse toxin challenge model, mice were challenged at either 14 days or at 42 days post-

vaccination. Vaccine groups with CPA plus CPB2B1 or the multivalent CPAB2B1 had greater survival rates, even at day 42, than mice vaccinated with the monovalent CPA; suggesting that recombinant toxoids are viable vaccine candidates with extended efficacy (Zeng et al., 2011). Histopathological examination of the mice intestines revealed that vaccinated mice, no matter the treatment, did not have gross pathological changes in contrast to the control mice. In addition to pathology, antibody titers of serum from immunized mice, sows and cows and the colostrum from immunized sows and cows were also examined. The highest neutralizing antibody titers were generated in mice but the sera and colostrum of sows and cows also had detectable titers. Neutralizing antibodies in the colostrum implies the recombinant toxoids are able to stimulate maternal antibodies for protection of their offspring (Zeng et al., 2011). Immunization with CPB2B1 or CPA plus CPB2B1 produced greater neutralizing antibody titers than did the CPA monovalent vaccine. In addition, fusion proteins increase the level of safety and effectiveness relative to the use of whole-cell bacterial vaccines, as the numerous extraneous proteins are eliminated (Zeng et al., 2011).

In addition to the toxin cocktail mentioned above, a recombinant *C. perfringens* type C beta toxoid (rBT) produced in *E. coli* and purified from inclusion bodies was evaluated as a vaccine candidate (Milach et al., 2012). Potency testing of this vaccine was performed according to the 9CFR USDA and EP and was shown to be non-toxic through evaluation of the lethal dose (LD_{50}) for the recombinant compared to the native beta toxin. This group showed that 100 µg of rBT inoculated intravenously did not exhibit any clinical signs of toxicity, as compared to 0.4 µg per kg LD_{50} for the wild-type

beta toxin. In addition, the rBT was able to induce rabbit serum with 14 IU beta antitoxin per mL, which is superior to a vaccine using the wild-type toxins.

Vaccines using recombinant proteins/toxoids are considered to be more stable, safer and efficacious, and have the potential to be new candidates for commercial vaccines (Salvarani et al., 2013). This is illustrated by a study where pregnant sows were vaccinated twice with a bivalent vaccine consisting of *C. perfringens* alpha (rTA) and beta (rTB) recombinant toxoids (Salvarani et al., 2013). Protective antibody titers of anti-alpha toxin (~6.0 IU per mL) and anti-beta toxin (~14.5 IU per mL) were produced. Importantly, the antibody titers to alpha and beta toxins were higher than the USDA required minimums (Salvarani et al., 2013). The litters from vaccinated sows also exhibited titers of neutralizing antibodies to alpha and beta toxins by passive immunization through colostrum. The litters had an alpha antitoxin titer of ~4.2 IU per mL and a beta antitoxin titer of ~10.9 IU per mL, which also met the USDA required minimum standard (Salvarani et al., 2013). These data demonstrate the vaccine and immunization strategy elicited a maternal immune response comparable to other vaccine formulations (Matisheck and McGinley, 1986; Salvarani et al., 2013). Overall, using recombinant toxoids may be less labor intensive and safer to produce, and can potentially reduce the costs of manufacturing the vaccines.

Immunogenic, non-toxin proteins were identified using serum from *C. perfringens* infected birds. These two non-toxin, highly immunogenic proteins were pyruvate: ferredoxin oxidoreducate (PFO) and elongation factor-Tu (EF-Tu) (Jang et al., 2012). PFO is an enzyme that catalyzes the conversion of pyruvate into acetyl-CoA, while EF-Tu facilitates binding of charged tRNAs to the A-site of ribosomes. These

proteins were cloned, over-expressed in *E. coli,* and used in infection protection studies. The efficacies of these proteins were compared to two different recombinant toxins by assessing the levels of protection achieved in a *C. perfringens* disease infection model. The cloned toxins used in the challenge were the *C. perfringens* alpha toxin and NetB, a necrotic enteritis B-like toxin. All four proteins were mixed individually with a commercial oil-in-water adjuvant (Jang et al., 2012) and tested for their ability to protect chickens against infections with virulent cultures of *Eimeria maxima* and *C. perfringens*. In this model, the first vaccination occurred in chicks one day of age and the second vaccination occurred when the birds were seven days old. At 14 days of age, the birds received a live culture of *E. maxima* by oral lavage (Jang et al., 2012). Four days after administering the *E. maxima* culture, the birds were orally given live *C. perfringens* cultures and 20 days post-hatch the serum antibodies were measured. Increased antibody titers and weight gain were observed in birds vaccinated with NetB and PFO. Additionally, the NetB and PFO recombinant protein vaccines were more efficacious against the double challenge of *E. maxima* and *C. perfringens* challenge than was the alpha toxin and EF-Tu recombinant protein vaccines (Jang et al., 2012). However, alpha toxin and EF-Tu vaccinates were efficacious in comparison to the controls. This data highlights a different approach in selecting non-toxin vaccine candidates.

While much of the current research for development for *C. perfringens* type C vaccines seems to be progressing toward the use of recombinants, these will not replace current commercial vaccines in the near future; hence, in *vitro* assays to detect vaccine components are necessary to facilitate production and regulatory compliance.

1.9 CURRENT ASSAYS – IN *VIVO* AND IN *VITRO*

Prevention of *C. perfringens* type C infections relies heavily on vaccination with inactivated whole bacteria. For vaccines to be efficacious, they should be able to elicit a protective immune response in the host animal. The commercially available *C. perfringens* type C vaccines produce neutralizing antibodies against the clostridial toxins. Production needs and regulatory requirements necessitate in-process testing for antigen production. Currently, *C. perfringens* type C toxin materials are assayed using an in *vivo* test method, known as the L+ test. In this assay, the toxin product is diluted and combined with a known amount of anti-toxin antibody, which is specific to the beta toxin. The different dilutions are intravenously administered to five mice for each dilution and the dilution which is lethal to 80% of the mice determines the potency concentration. The concentration at the specific dilution is further used to calculate the amount of non-toxin antigen to formulate into final vaccine.

The current required test for final vaccine potency involves the use of mice and rabbits and is mandated in the 9CFR USDA guidelines and EP monograph (United States Department of Agriculture, 2017a) . According to the USDA guidelines, the in *vivo* test requires a minimum of eight rabbits subcutaneously administered with one half of the largest recommended dose, or less, based on the target host species. A second injection is given 20-23 days following the first administration. All surviving rabbits after 14-17 days post vaccination are bled, the serum pooled and the serum tested for antitoxin levels. As described in the 9CFR 113.111 (United States Department of Agriculture, 2017a), the antitoxin neutralizing antibody level is determined through a dilution series using an antitoxin standard. The neutralizing antibody levels are assessed against *C. perfringens*

beta toxin and are estimated in *vivo* using a mouse neutralization test (Ebert et al., 1999). To meet the prescribed standard, vaccines must have at least 10 IU of standard beta antitoxin per milliliter of rabbit serum. The use of animals in these validated assays has led to a desire to develop new assays that reduce the number of animals required.

The development of in *vitro* assays would be cost effective by greatly reducing the number of animals used each year. Efforts to reduce the number of animals required for toxin validation have led to eliminating the use of mice; however, rabbits are still required for sera generation (Ebert et al., 1999). While it is desirable to use only in *vitro* assays, these assays must be comparable to in *vivo* tests and to date only proof-of-principle studies have been performed, and these assays must be validated and accepted by the USDA and EP (Redhead et al., 2011). In summary, producing the toxoid is labor intensive and potency testing is time consuming and requires the use of animals. While work is being done to develop new vaccines against CPB, improvements in the potency testing are needed.

CHAPTER II

ABSTRACT

Clostridium perfringens is a Gram positive anaerobic rod and a major cause of enteric disease in both humans and livestock. There are five *C. perfringens* toxinotypes, (*i.e.*, A, B, C, D and E) that are classified based on the type of toxins each produces. The focus of this work is on the beta toxin produced by *C. perfringens* type C. The primary virulence determinant of *C. perfringens* type C is the beta toxin, which causes necrotic enteritis in neonatal animals. Fortunately, cattle and pig vaccines directed against *C. perfringens* toxins are efficacious. Production of a commercial vaccine requires not only an efficacious antigen, but also testing methods for quantifying antigens and potency determination of the finished vaccine. These methods are performed in *vivo* according to the Code of Federal Regulations number 9 (9CFR) United States Department of Agriculture (USDA) and European Pharmacopeia (EP) monograph guidelines. Currently, there are limited in *vitro* alternatives to assessing *C. perfringens* type C beta toxin in fermentation media or final vaccine potency. With the generation of polyclonal and monoclonal antibody reagents, an in *vitro* method could be developed to measure *C. perfringens* type C toxoid material independent of laboratory animals implementing the principles of the 3Rs (replacement, reduction and refinement).

2.1 INTRODUCTION

This work focuses on filling the need for an in *vitro* method to measure the *C. perfringens* type C toxoid, which will help reduce the use of laboratory animals and

facilitate vaccine production. An enzyme-linked immunosorbent assay (ELISA) was developed to quantitate the *C. perfringens* type C beta toxoid from cultivation media. This could improve accuracy in formulating vaccines in contrast to the in *vivo* method of estimating the amount of toxin. The necessary reagents were developed and the assay format assessed as shown in the following section. In addition, results from the developed ELISA are presented.

2.2 MATERIALS AND METHODS

2.2.1 BACTERIAL CULTIVATION CONDITIONS

C. perfringens type C was grown in a 10 liter Celligen BLU single use fermenter containing media components of soy peptone, casein hydrolysate (Tryptone), yeast extract, antifoam and dextrose. Actively growing culture was aseptically added at 20 mL/L to the fermenter and incubated at 36°C ± 2°C for approximately 3-6 hours with pH maintained between 7.0 – 7.5.

2.2.2 TOXIN AND TOXOID PREPARATION

For toxin preparation, the fermentation culture was centrifuged at 15,000g using a Sorvall RC 6+ centrifuge for 30 minutes at 4°C; supernatants were pooled and cell pellets discarded. The supernatant was passed through a Sterile MidiCap Sartopore 2 filtration unit containing a 0.45 + 0.2 μm membrane.

For toxoid preparation, the fermentation culture was chemically inactivated with formaldehyde prior to centrifugation at 15,000g using a Sorvall RC 6+ centrifuge for 30 minutes at 4°C; supernatants were pooled and cell pellets discarded. The supernatant was

passed through a Sterile MidiCap Sartopore 2 filtration unit containing a 0.45 + 0.2 μm membrane.

A control of *C. perfringens* type A toxoid culture medium was provided by the bacterial processing group at Zoetis Inc., Lincoln, NE.

2.2.3 ISOLATION OF MONOCLONAL ANTIBODIES

All in *vivo* work was conducted after ethical review and in compliance with local, national and international guidance. Specific pathogen-free BALB/c mice (17 to 20 g; 3 mice; no gender was specified) were immunized according to the Maine Biotechnology Services, Inc. (MBS) Rapid Immunization Multiple Sites (RIMMS) protocol. Mice were immunized five times within the 20 day period at multiple sites; each injection time point used 0.5mL of *C. perfringens* type C toxoid adjuvanted vaccine. At day 20, blood was collected and serum screened by ELISA to determine the specific antibody titer. Based on the screening results, the mice received an additional boost following the test bleed and a second test bleed was taken 10 days after the last boost. Based on the test bleed screen, MBS then selected one mouse for fusion that had the highest anti-CPB titer. The spleen was dissected from the mouse and the splenocytes were fused with SP2/0 myeloma cells to create hybridomas. The fused cells were cultured and supernatants were evaluated for antibody production. Hybridoma supernatants were assessed for CPB toxoid specificity by an indirect ELISA. There were 41 type C toxoid specific clones that were selected. These hybridoma positive culture supernatants were further processed through three rounds of subcloning by limiting dilution.

A mouse hybridoma secreting a beta toxin specific monoclonal antibody was obtained from the Center for Veterinary Biologics (CVB) Ames, IA courtesy of Dr. Paul Hauer. This monoclonal antibody detected the 38 kDa protein band associated with CPB toxoid on western blot. The CVB monoclonal antibody was subsequently used as a positive control on confirmatory assays such as western blot, ELISA and neutralization assay.

2.2.4 POLYCLONAL ANTIBODIES

Goat anti-*C. perfringens* Type C toxoid polyclonal antibodies were provided by from the Laboratory Sciences group at Zoetis Inc., Kalamazoo, MI. All in *vivo* work was conducted after ethical review and in compliance with local, national, and international guidance.

2.2.5 INDIRECT ELISA

Indirect ELISA was used to screen hybridoma supernatants for antibodies from MBS. To determine if the hybridoma supernatants had antibodies to *C. perfringens* type C toxoid only, a positive indirect ELISA was used with a type C antigen coating and a negative indirect ELISA performed with *C. perfringens* type A antigen coated on the assay plates. *C. perfringens* type C toxoid antigen was diluted in 0.01 M borate buffer (sodium tetraborate in purified water, pH 9.0 – 9.2) for a final concentration of 300 µg antigen per 100 µL buffer. Polystyrene flat bottom 96-well plates (Nunc MaxiSorp) were incubated overnight at 4°C with a volume of 100 µL per well of diluted antigen. The next day, plates were washed three times with 0.1 M phosphate buffered saline

containing 0.05% tween 20 (PBST). The plates were blocked by adding 200 µL/well of 1% casein sodium in PBST and then incubated at 37°C ± 2°C for 1 hour. After blocking, the plates were washed three times with PBST followed by 50 µL of each hybridoma supernatant dispensed into their respective well on the plate. The plates were covered and incubated for 60 minutes at 37°C ± 2°C. Post incubation, plates were washed three times with PBST followed by the addition of 50 µL/well of diluted peroxidase conjugated goat anti-mouse IgG heavy and light chain (Jackson ImmunoResearch). The peroxidase conjugate was diluted in 1% casein sodium in PBST at 1:3,000 dilution and then incubated for 60 minutes at 37°C ± 2°C. Following incubation, the plates were washed three times and 50 µL/well of ABTS (2, 2'-Azino-di-3-ethylbenzthiazoline-6-sulfonate) peroxidase substrate (Kirkgaard & Perry Laboratories) was added and the plates were incubated at room temperature for 15 to 20 minutes. The reaction of the conjugated enzyme with the ABTS substrate produces a greenish color. The optical density (OD) of each well is determined using values of 405 nm minus 490 nm as determined on a Vmax kinetic microplate reader (Molecular Devices) and the SoftMax Pro software 6.3. The same conditions and reagent concentrations were used to determine if the hybridoma supernatants had antibody present to *C. perfringens* type A toxoid for the negative indirect ELISA screen. Hybridomas were selected using the criteria of the well in the positive assay having an absorbance of ≥ 0.08 and the corresponding well in the negative assay having an absorbance of ≤ 0.05.

2.2.6 SDS-PAGE AND WESTERN BLOTTING

Proteins present in the *C. perfringens* type C beta toxin preparation were visualized on a 4-12% Bis-Tris SDS-PAGE gel using a GelCode Blue Safe Protein coomassie blue stain (Pierce Biotechnology). For western blot analysis, proteins from the gel were transferred to a nitrocellulose membrane using the iBlot™ 2 gel transfer device and iBlot™ 2 Transfer Stacks containing the nitrocellulose membranes (Invitrogen). Non-specific binding was blocked using 0.5% gelatin (from cold water fish skin) in PBST. This was followed by incubation at room temperature with anti-CPB antibodies. The blots were washed with 0.05% tween 20 in PBS and then incubated for 30 minutes at room temperature with peroxidase conjugated goat anti-mouse IgG H+L (Jackson ImmunoResearch). Membranes were developed with TMB (3, 3', 5, 5'– Tetramethylbenzidine) peroxidase substrate 3-component system (Kirkgaard & Perry Laboratories).

2.2.7 NEUTRALIZATION ASSAY

The neutralization assay was used to assess the ability of antibodies to neutralize the *C. perfringens* type C beta toxin. Vero cells, derived from the kidney of normal adult African green monkey, were cultivated in Eagles Modified Essential Medium (EMEM) (Gibco, Calsbad, CA) supplemented with 5% fetal bovine serum (FBS) (Atlanta Biologicals, Lawrenceville, GA) and maintained in a 37˚C humidified chamber with 5% CO_2. Vero cells were seeded in 96-well plates and grown to a confluent monolayer with a cell density of 125,000 cells per well. The antibody samples were diluted either 1:2 or 1:4 in EMEM medium. Two-fold serial dilutions were made across the dilution plate,

leaving the last two columns for positive and negative control wells (100 µL/well). *Clostridium perfringens* type C beta toxin was added to the dilution plate containing the diluted antibody samples (100 µL/well). The dilution plate was incubated for 2 hours at 37°C ± 2°C with 5% CO_2. After incubation, 200 µL of antibody/toxin material from the dilution plate was transferred to the actively growing Vero cell plate. Vero plates were then incubated for 2 days at 37°C ± 2°C with 5% CO_2. Following incubation, cells were assessed under a microscope for cytopathic effect (CPE). Wells having intact cells in a confluent monolayer indicate the antibody neutralized the beta toxin challenge. Wells lacking a monolayer indicate the antibody was unable to neutralize the beta toxin, resulting in CPE on the cells.

2.2.8 CHECKERBOARD ASSAY

A checkerboard assay method was used to determine the optimal dilutions of the capture (polyclonal) and indicator (monoclonal) antibodies for the in-process assay to measure *C. perfringens* type C toxoid. A checkerboard pattern was obtained by using a polystyrene flat bottom 96-well plate with the capture antibody dilutions down the y-axis (row A through row H) and the indicator antibody dilutions were along the x-axis (column 1 through column 12). Each plate was coated with capture antibody diluted in 0.01 M borate buffer; with the starting dilution of the capture antibody on the plate in row A being 1:250 and followed by two-fold serial dilution from row A through H and the plates were then incubated overnight at 4°C. Plates were washed the next day three times with PBST and then blocked with 1% casein sodium in PBST (200 µL/well) and incubated at 37°C ± 2°C for 1 hour. After blocking and washing the plates, *C.*

perfringens type C antigen was added to each of the wells at a constant concentration, 300 µg antigen per 200 µL per well diluted in 1% casein sodium in PBST. The plates were covered and incubated for 1 hour at 37°C ± 2°C. Post incubation period, the plates were washed three times with PBST and the indicator antibody diluted in 1% casein sodium was added to each plate. The starting dilution of the indicator antibody on the plate in column 1 was 1:250 and followed by two-fold serial dilution from columns 1 through 12. The plates were covered and incubated for 1 hour at 37°C ± 2°C. Post incubation, plates were washed three times with PBST, followed by the addition of 100 µL/well of a 1:2,000 dilution of the conjugate antibody (*i.e.,* peroxidase conjugated goat anti-mouse) diluted in 1% casein sodium in PBST and then incubated for 1 hour at 37°C ± 2°C. Following incubation, the plates were washed three times and ABTS peroxidase substrate was added (50 µL/well), and the plates were incubated at room temperature for 15 to 20 minutes. The plates were read at 405 minus 490 nm with Vmax kinetic microplate reader (Molecular Devices) and the SoftMax Pro software 6.3. The average OD were plotted for both capture and indicator antibodies to determine the optimal dilutions.

2.2.9 SANDWICH ELISA

A sandwich ELISA method was used to develop the in-process assay for measuring *C. perfringens* type C toxoid. Goat anti-*C. perfringens* type C toxoid polyclonal antibody was diluted in 0.01 M borate buffer and dispensed onto polystyrene flat bottom 96-well plates (Nunc MaxiSorp) that were incubated overnight at 4°C with diluted antibody (100 µL/well). The next day, plates were washed three times with 0.1 M

PBST and blocked with 1% casein sodium in PBST (200 µL/well) and incubated at 37°C ± 2°C for 1 hour. After blocking, plates were washed three times with PBST followed by the addition of *C. perfringens* type C toxoid samples. The plates were covered and incubated for 1 hour at 37°C ± 2°C. Post-incubation, plates were washed three times with PBST, followed by the addition of anti-*C. perfringens* type C monoclonal antibody diluted in 1% casein sodium in PBST (100 µL/well). The plates were covered and incubated for 1 hour at 37°C ± 2°C. Post-incubation, plates were washed three times with PBST, followed by the addition of 100 µL/well of diluted peroxidase conjugated goat anti-mouse IgG H+L (Jackson ImmunoResearch). The conjugate was diluted in 1% casein sodium in PBST at 1:2,000 dilution and then incubated for 1 hour at 37°C ± 2°C. Following incubation, the plates were washed three times and 50 µL/well of ABTS peroxidase substrate (Kirkgaard & Perry Laboratories) was added, and the plates incubated at room temperature for 15 to 20 minutes. The OD values at 405 nm minus 490 nm were determined using a Vmax kinetic microplate reader (Molecular Devices) and the SoftMax Pro software 6.3.

2.2.10 IN-PROCESS ELISA

The following describes the developed in-process ELISA method for measuring *C. perfringens* type C toxoid. The polyclonal capture antibody, anti-*C. perfringens* beta goat 31, was diluted in 0.01 M borate buffer and dispensed onto polystyrene flat bottom 96-well plates (Nunc MaxiSorp) that were incubated overnight at 4°C with diluted antibody (100 µL/well). The next day, plates were washed three times with 0.1 M PBST

and blocked with 1% casein sodium in PBST (200 µL/well) and incubated at 37°C ± 2°C for 1 hour.

During the blocking step, the reference, positive control and test samples are vortexed for 15 seconds (3 by 5 seconds) at maximum vortex speed. Then dilutions of reference, positive control and test articles are prepared by making a 1:54 dilution in 1% casein sodium in PBST. In a dilution plate, 150 µL of 1% casein sodium in PBST is dispensed into each well column 2 – 11 and rows C – G (Fig.1). In row B, 300 µL of 1:54 diluted reference, positive control and each test article is added to wells as labeled and shown in Figure 1. Two-fold dilutions are preformed down the plate by mixing the samples in row B and transferring 150 µL to row C. Changing pipette tips between rows and repeated through row G.

	1	2	3	4	5	6	7	8	9	10	11	12
A	PBST	PBST										PBST
B		Ref	+	T01	T02	T03	Ref	+	T01	T02	T03	
C		Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	Dil 2	
D		Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	Dil 3	
E		Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	Dil 4	
F		Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	Dil 5	
G		Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	Dil 6	
H		PBST										

Fig. 1: In-process assay 96-well plate configuration. Ref (Reference), + (Positive Control), T01-03 are test samples.

The blocked plates were washed three times with PBST and 100 µL from the dilution plates was transferred to blocked plates as follows; beginning in row G through row B, material is transferred to the respective wells. The outer wells on the plate (Fig.

1) receive 100 µL of PBST. The plates are covered and incubated at 37°C ± 2°C for 1 hour. Post incubation, plates were washed three times with PBST, followed by the addition of indicator antibody, mouse anti-*C. perfringens* type C toxoid monoclonal antibody (mAb 23) diluted 1:2,000 in 1% casein sodium in PBST (100 µL/well). The plates were covered and incubated at 37°C ± 2°C for 1 hour and after incubation the plates were washed three times with PBST. The peroxidase conjugate antibody, goat anti-mouse, was diluted at 1:2,000 in 1% casein sodium in PBST and added at 100 µL per well. The plates were covered and incubated at 37°C ± 2°C for 1 hour and after incubation the plates were washed three times with PBST. After the final washing step, 50 µL/well of ABTS peroxidase substrate was added to all wells on the plates. The plates were read at 405 nm minus 490 nm with Vmax kinetic microplate reader (Molecular Devices) and the SoftMax Pro software 6.3. with five second shake pre-read targeting a ≥ 0.8 OD for the first dilution of the reference (average of wells B2 and B7 as shown in Figure 1).

2.3 RESULTS

2.3.1 CHARACTERIZATION OF POLYCLONAL ANTIBODIES

Two polyclonal antibodies were provided by Zoetis Inc.; specifically, anti-*C. perfringens* beta goat 11 and anti-*C. perfringens* beta goat 31. The two polyclonal antibodies were characterized by western blot analysis to confirm recognition of the 38 kDa beta protein. Both goat 11 and 31 polyclonals recognized the 38 kDa protein band of *C. perfringens* type C (Fig. 2). As expected, polyclonal antibodies bound to a band at 38 kDa that corresponds to the CVB beta toxin positive control included on the western

blot. To assess the ability of the polyclonal antibodies to neutralize beta toxin, their neutralizing activity was evaluated (Table 2). Anti-*C. perfringens* beta goat 31 had slight neutralizing activity, while anti-*C. perfringens* beta goat 11 lacked neutralizing activity. Overall, neither of the polyclonal antibodies had pronounced neutralizing capabilities; however, the antibodies only need to capture the beta toxoid through epitope binding and neutralizing capability is not required. To assess their potential utility in an ELISA, the polyclonal antibodies were evaluated using an indirect ELISA (Fig. 3). The assay distinguished the differences between the antibodies where anti-*C. perfringens* beta goat 31 bound the *C. perfringens* type C toxoid material but anti-*C. perfringens* beta goat 11 did not. For this reason, the goat 31 anti- *C. perfringens* beta toxoid antibody was selected as the polyclonal antibody to be used as the capture for developing an in-process assay to measure type C material.

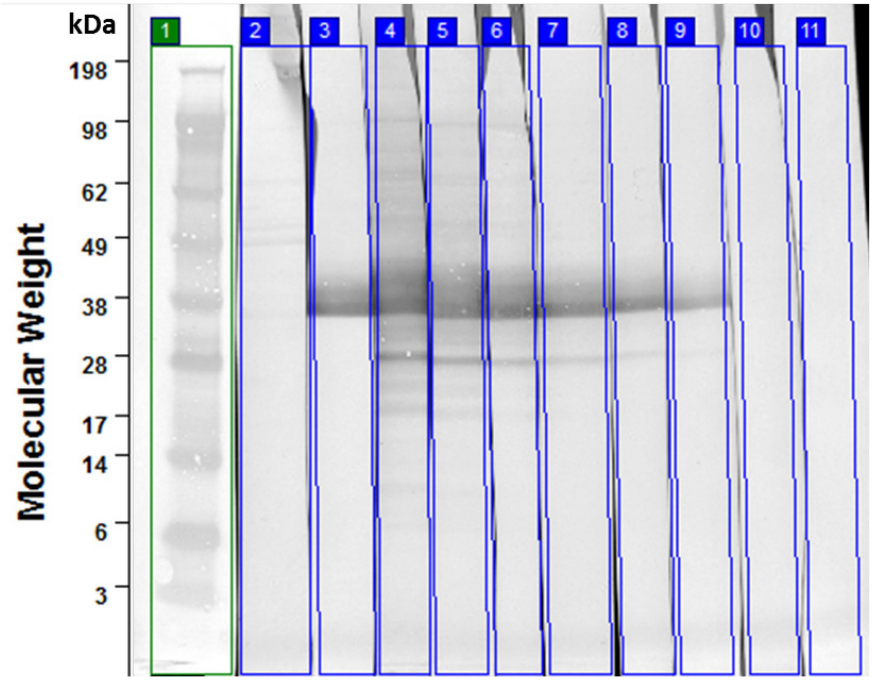


Fig. 2: Western blot analysis depicting *C. perfringens* type C beta toxin specific protein binding by polyclonal antibodies 31 and 11. Lane 1 molecular weight marker. Lane 2 negative goat serum control, 1:4,000 dilution. Lane 3 positive control monoclonal to beta from the Center for Veterinary Biologics (CVB), 1:80,000 dilution. Lane 4 through 6 are anti-*C. perfringens* beta goat 31 at 1:10,000, 1:20,000 and 1:40,000 dilutions respectfully. Lane 7 through 9 are anti-*C. perfringens* beta goat 11 at 1:10,000, 1:20,000 and 1:40,000 dilutions respectfully. Lane 10 peroxidase conjugate control for lane 3, goat anti-mouse at 1:4,000 dilution. Lane 11 peroxidase conjugate control for lane 4 – 9, donkey anti-goat at 1:4,000 dilution.

Table 2: Polyclonal antibody 31 weakly neutralizes CPB.

Sample	Average End Point Dilution (CPE)[a]
Anti-*C. perfringens* beta goat 11	<2
Anti-*C. perfringens* beta goat 31	8
Positive Control (Beta toxin Anti-sera)	256
Negative Control (Alpha toxin Anti-sera)	<2

[a]Average values of two replicates.

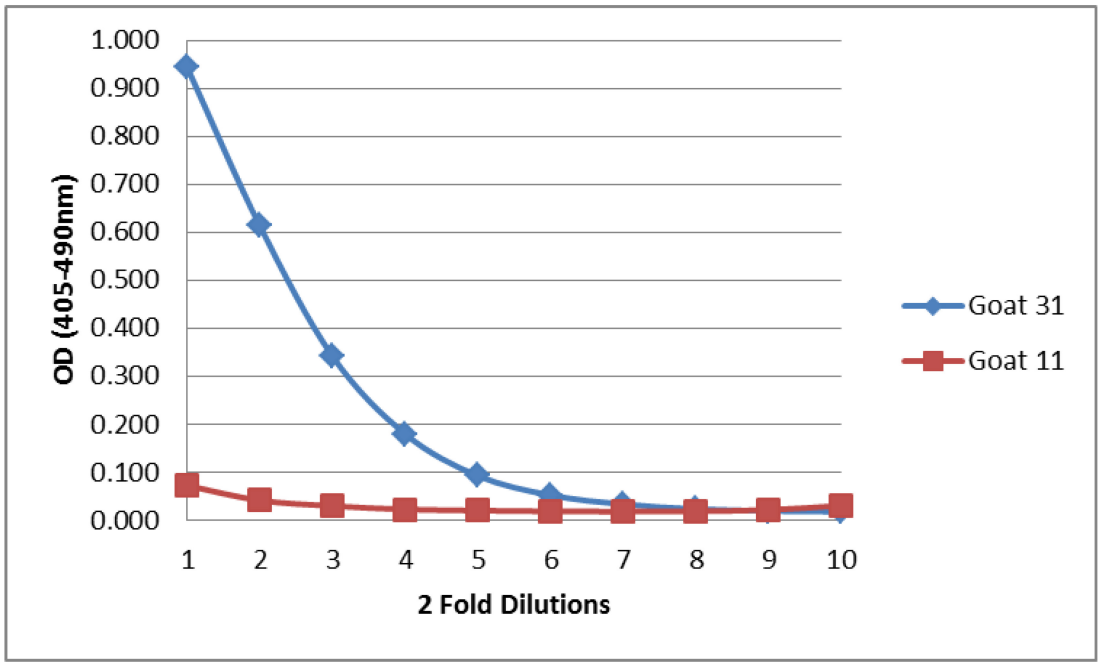


Fig. 3: Polyclonal antibody 31 produced a greater signal relative to polyclonal antibody 11 when evaluated by indirect ELISA. Data is representative of the mean OD (OD_{405} - OD_{490}) for each dilution completed in duplicate. The individual polyclonal antibodies were at an initial concentration of 1:4,000 and diluted 2-fold across the plate.

2.3.2 MONOCLONAL ANTIBODY SELECTION

Experiments were carried out to determine the most appropriate monoclonal antibody for use in the ELISA format as the indicator antibody. Forty-one hybridoma monoclonal antibodies met the selection criteria of having an OD greater than or equal to 0.08 on *C. perfringens* type C toxoid plates with less than or equal to 0.05 OD on *C. perfringens* type A toxoid plates when assessed by MBS. Fulfilling these criteria demonstrate there was minimal cross reactivity of the 41 monoclonal antibodies for CPA.

The supernatants of hybridoma monoclonal antibodies were further characterized by western blot analysis to confirm recognition of the 38 kDa beta protein. Of the 41 monoclonal antibodies screened, 11 recognized a 38 kDa protein band (Fig. 4). These 11 monoclonal antibodies were also evaluated using a sandwich ELISA (Fig. 5). Of the 11 monoclonal antibodies, two demonstrated strong reactivity throughout the dilution series. Importantly, monoclonal antibody 23 gave a strong signal even at the highest dilution, suggesting a high affinity for the antigen. An ideal monoclonal antibody for an assay has high affinity for the antigen and neutralizing activity. The neutralizing activity suggests the antibody recognizes and binds to epitopes on the antigen necessary for protection. To assess the neutralizing capability of the 11 monoclonal antibodies, neutralizing activity was evaluated (Table 3). Of the 11 hybridoma monoclonal antibodies, five had neutralizing activity, including monoclonal antibody 23.

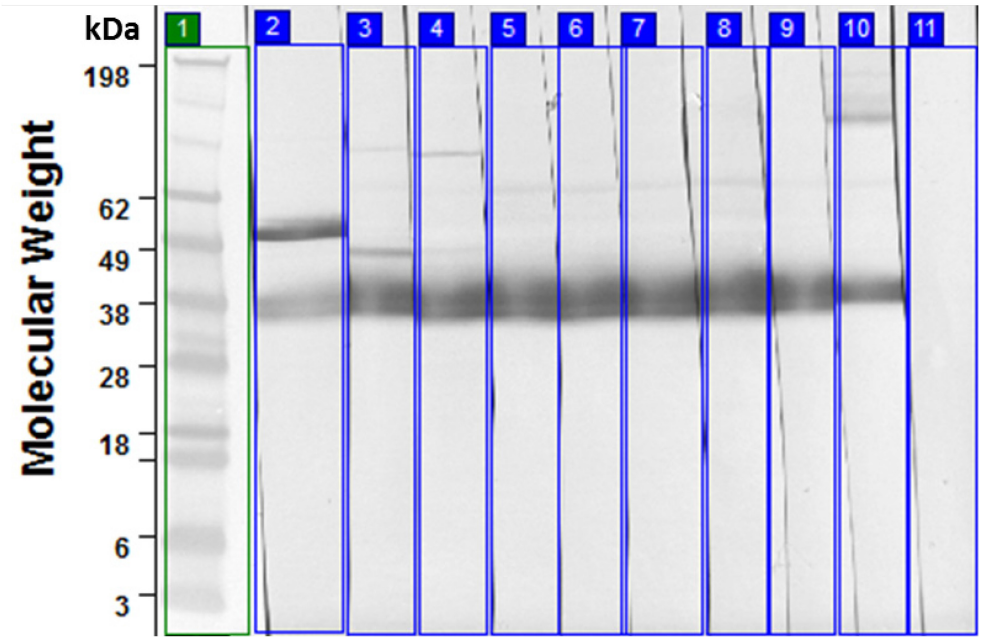


Fig. 4: Western blot analysis depicting *C. perfringens* type C beta toxin specific protein binding by different supernatants of hybridoma monoclonal antibodies. Lane 1 molecular weight marker. Eight of the 11 individual monoclonal antibodies to detect beta toxin in lanes 2 through 9 (11, 13, 22, 23, 33, 35, 37, and 40) at a 1:4 dilution. Lane 10 positive control monoclonal to beta from the CVB at 1:10,000 dilution. Lane 11 peroxidase conjugate control, goat anti-mouse at 1:4,000 dilution.

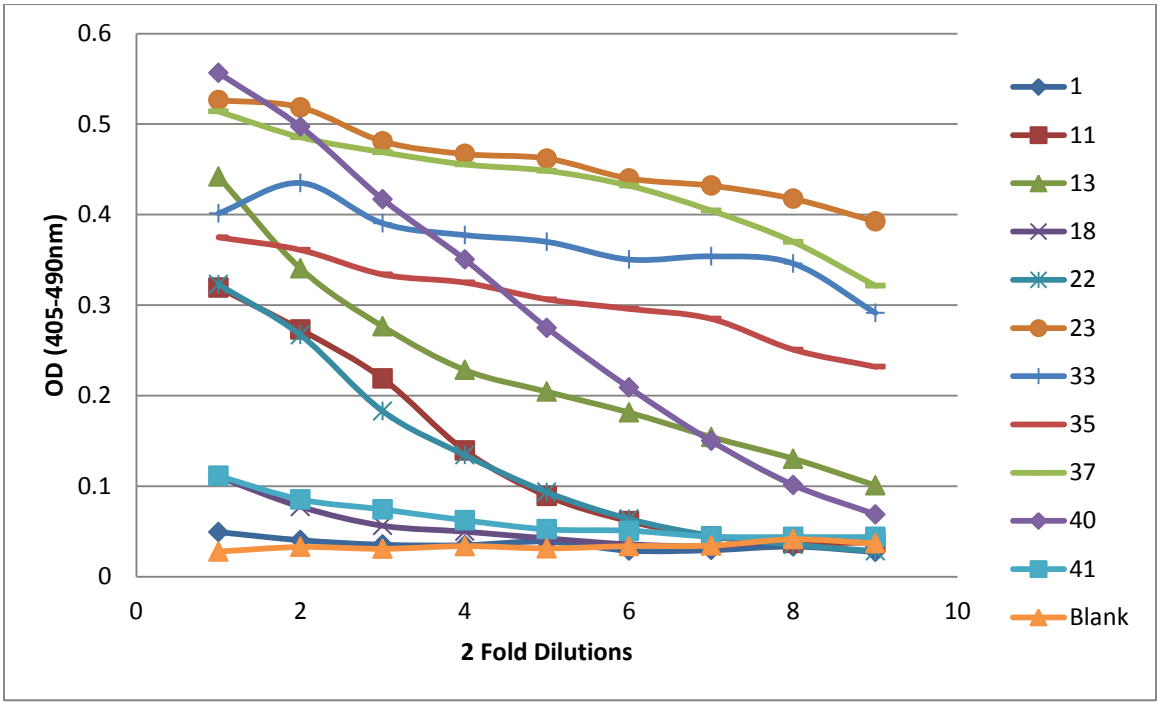


Fig. 5: Hybridoma monoclonal antibodies 23 and 37 produced the greatest signal relative to the other investigational antibodies when evaluated by sandwich ELISA. Data is representative of the mean OD (OD_{405} - OD_{490}) for each dilution completed in duplicate. The individual monoclonal antibodies were at an initial concentration of 1:2 and diluted 2-fold across the plate.

Table 3: Capability of hybridoma monoclonal candidates to neutralize CPB.

Monoclonal Number	**Average End Point Dilution (CPE)[a]**
1	<2
11	<2
13	32
18	<2
22	<2
23	128
33	64
35	64
37	128
40	<2
41	<2
CVB 10A2	<2
Fusion Media Control	<2
Positive Control (Beta toxin Anti-sera)	256
Negative Control (Alpha toxin Anti-sera)	<2

[a]Average values of two replicates.

The five hybridoma candidates which showed to neutralize the beta toxin were selected and expanded, purified, concentrated and were then reevaluated by western blot to confirm detection of the 38 kDa protein band (Fig. 6), neutralizing activity (Table 4), and absence of cross reactivity of alpha toxin by indirect ELISA (Fig. 7). Since alpha toxin is present in *C. perfringens* type C cultures, a false positive result could be obtained if the antibody also reacted to the alpha toxin. To assess the cross reactivity of the five monoclonal antibodies with neutralizing activity to beta toxin, they were analyzed using positive and negative indirect ELISA (Fig. 7). The five monoclonal antibodies demonstrated specificity for *C. perfringens* type C beta toxoid and did not cross react with type A toxoid. Taken together, these data demonstrate that monoclonal antibody 23

has specificity to the beta toxin by western blot, a high affinity as shown by ELISA, strong neutralizing activity, and no cross reactivity with *C. perfringens* type A. For these reasons, monoclonal antibody 23 was chosen for use as the indicator antibody in developing an in-process ELISA to measure *C. perfringens* type C toxoid.

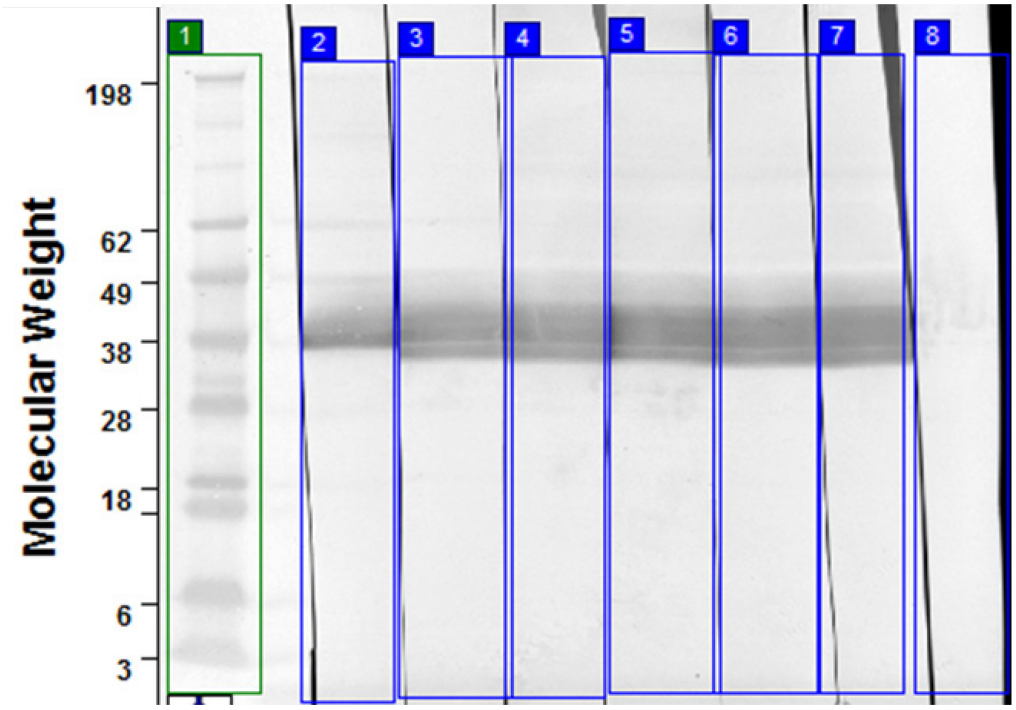


Fig. 6: Western blot analysis depicting *C. perfringens* type C beta toxin specific protein binding by monoclonal antibody candidates. Lane 1 molecular weight marker. Lane 2 positive control monoclonal to beta from the CVB at 1:20,000 dilution. Lane 3 through 7 monoclonal antibodies 13, 23, 33, 35 and 37 at a 1:10,000 dilution. Lane 8 peroxidase conjugate control, goat anti-mouse at 1:4,000 dilution.

Table 4: Monoclonal candidates to neutralize CPB.

Monoclonal Number	Average End Point Dilution (CPE)[a]
13	512
23	4096
33	1024
35	2048
37	4096
CVB 10A2	<4
Positive Control (Beta toxin Anti-sera)	256
Negative Control (Alpha toxin Anti-sera)	<4

[a]Average values of two replicates.

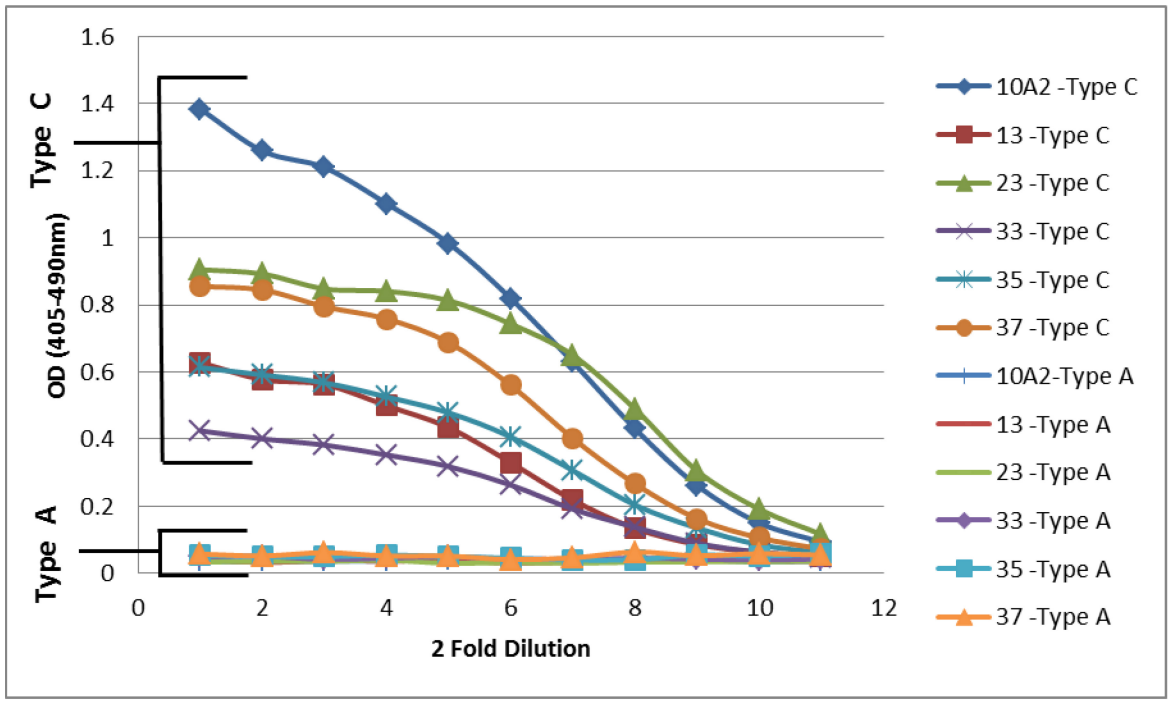
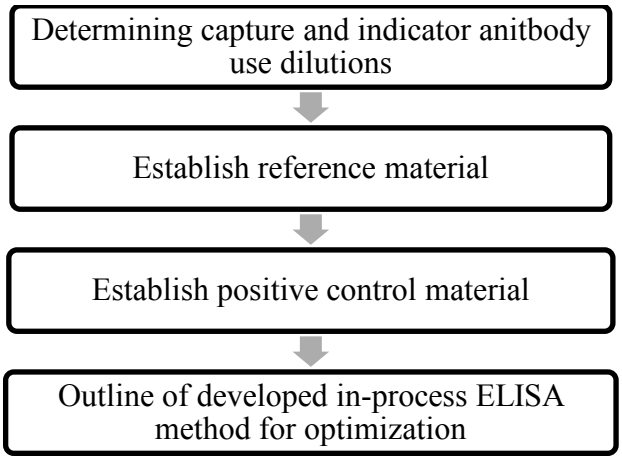


Fig. 7: Monoclonal antibodies 23 and 37 produced the greatest signal relative to positive control, 10A2, when evaluated by indirect ELISA to *C. perfringens* type C. All investigational antibodies were negative when evaluated by indirect ELISA to *C. perfringens* type A. Data is representative of mean OD (OD_{405} - OD_{490}) for each dilution completed in duplicate. The individual monoclonal antibodies were diluted 2-fold across the plate with a 1:500 starting dilution.

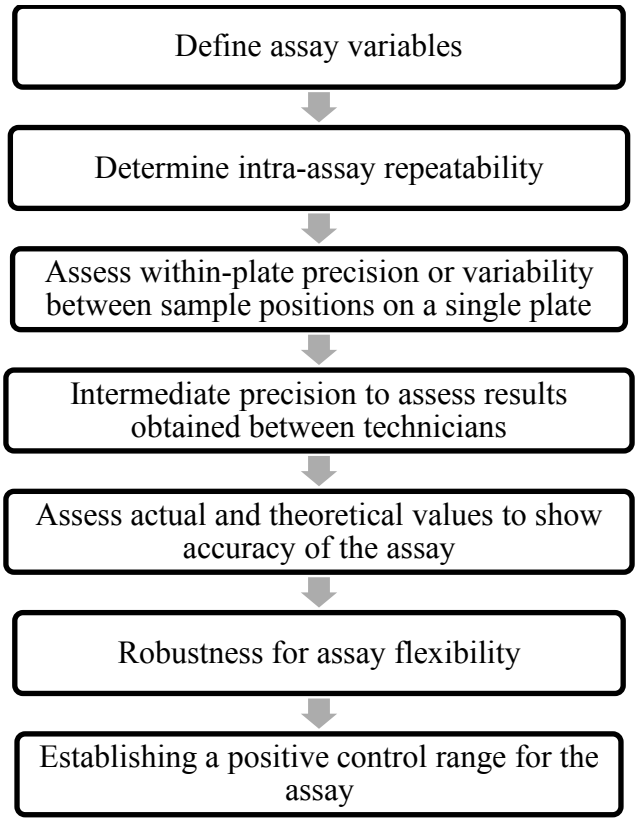
2.3.3 IN-PROCESS ELISA DEVELOPMENT AND OPTIMIZATION STEPS

The following are two flow charts outlining the steps used during the development and optimization of an in-process ELISA.

Development Steps:



Optimization Steps:



2.3.4 IN-PROCESS ELISA DEVELOPMENT STEPS

2.3.4.1 CAPTURE AND INDICATOR ANTIBODY USE DILUTION

The appropriate antibody dilutions for use in the in-process ELISA need to be determined initially to optimize the signal to background ratio. This is important so the background does not interfere with the signal from the antigen being measured. The dilutions of the capture antibody (*i.e.,* goat anti-*C. perfringens* type C 31) and indicator antibody (*i.e.,* mouse anti-*C. perfringens* type C clone 23) were determined by performing a checkerboard assay for evaluating optimal dilutions. Capture antibody dilutions down the y-axis of a 96-well plate (row A through row H) and the indicator antibody dilutions were along the x-axis (column 1 through column 12). The plates were read and the average OD's were plotted for both capture and indicator antibodies to determine optimal use dilutions. The optimal use dilution for the capture antibody was determined to be 1:500, while the indicator antibody optimal use dilution was determined to be 1:2,000 (Fig. 8 and 9).

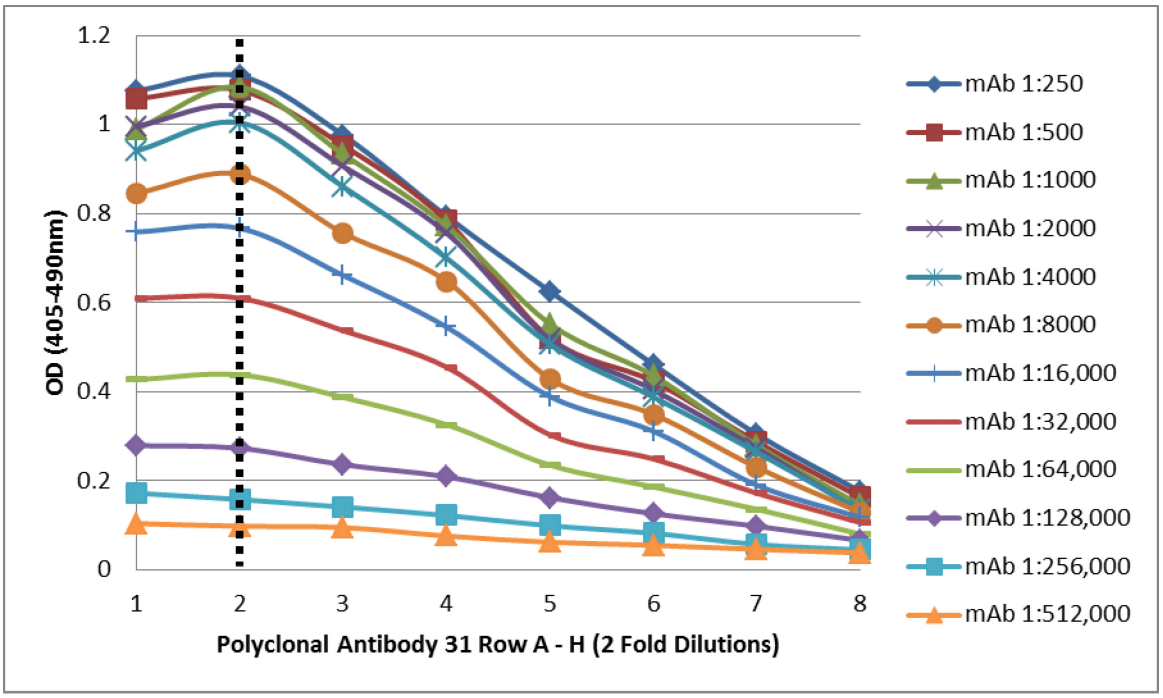


Fig. 8: Optimal dilution for polyclonal antibody 31 was determined to be 1:500 by checkerboard assay. Black dashed line highlights the 1:500 dilution. The capture starting dilution was 1:250 in row A and diluted two fold to 1:32,000 in row H.

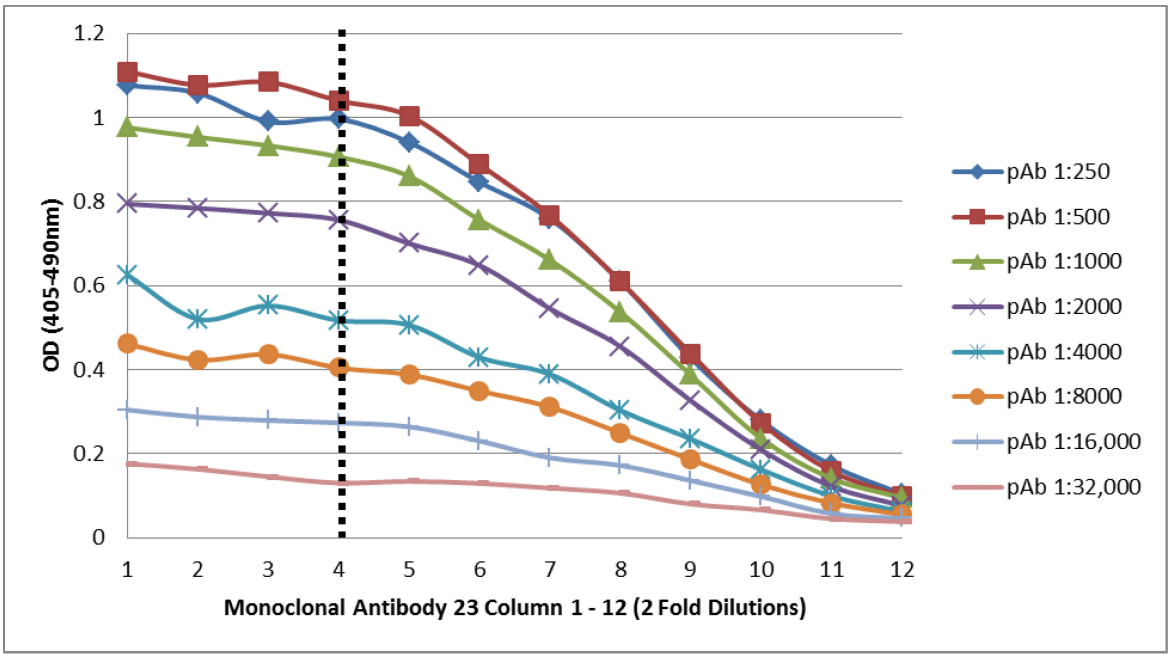


Fig. 9: Optimal dilution for monoclonal antibody 23 was determined to be 1: 2,000 by checkerboard assay. Black dashed line highlights the 1:2,000 dilution. The indicator starting dilution was 1:250 in column 1 and diluted two fold to 1:512,000 in column 12.

2.3.4.2 FULL CURVE ANALYSIS OF REFERENCE

Reference material must be representative of the samples that will be analyzed in the ELISA. Since in-process samples are cultures of *C. perfringens* type C, a representative lot was chosen as the reference. The reference material is used to generate a standard curve for determining fermentation yields. To establish the linear range of the reference, a full curve analysis containing both the upper asymptote and lower asymptote was performed. For this purpose, *C. perfringens* type C toxoid bulk antigen lot 166263 was selected as the reference material. Full curve was achieved by starting the bulk material at a 1:2 dilution (diluted in 1% casein sodium in PBST) on the plate and serially diluted three-fold from columns 1 through 12 and ran in triplicate (Fig. 10).

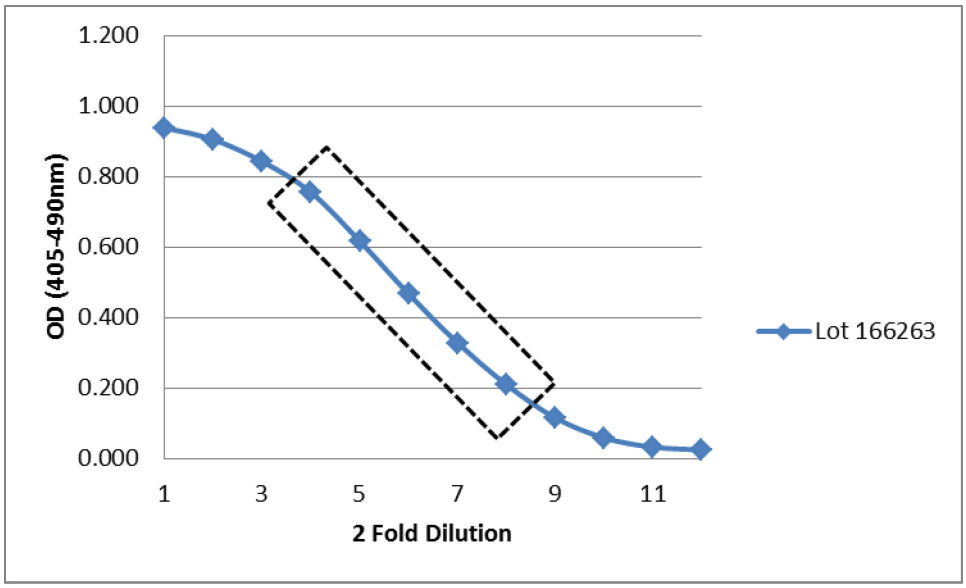


Fig. 10: The linear portion of the reference material was established by full curve analysis using dilution 1:54 through 1:4,374 (boxed area). Data is representative of mean OD (OD_{405} - OD_{490}) for each dilution completed in triplicate. The starting dilution of reference was 1:2 with three fold dilutions across the plate.

The reference was given an arbitrary stock concentration of 8,040 relative units per mL (RU/mL), which was based off the L+ value of the bulk antigen. The first point of the linear region was a 1:54 dilution; this established the starting dilution of the reference for the in-process ELISA. With a reference stock concentration of 8,040 RU/mL, the starting dilution of the reference at 1:54 would be 148.89 RU/mL. After a reference has been determined and assigned a stock concentration value, the next step was to establish a positive control to be used in the ELISA. The positive control ensures that the reagents have not changed or degraded from assay to assay.

2.3.4.3 SELECTING A POSITIVE CONTROL

A positive control for an ELISA assay establishes the repeatability of the assay and stability of the reagents. The positive control should be representative of the samples that will be analyzed in the assay and similar to the reference material, but from a different lot. *C. perfringens* type C toxoid bulk antigen lot 183502 was selected as the positive control. The positive control and the reference must be linear to each other as determined by calculating the slope ratio. The slope ratio is calculated by dividing the slope of the positive control by the slope of the reference (Fig. 11). Linearity is usually established with slope ratios within 0.8 – 1.25.

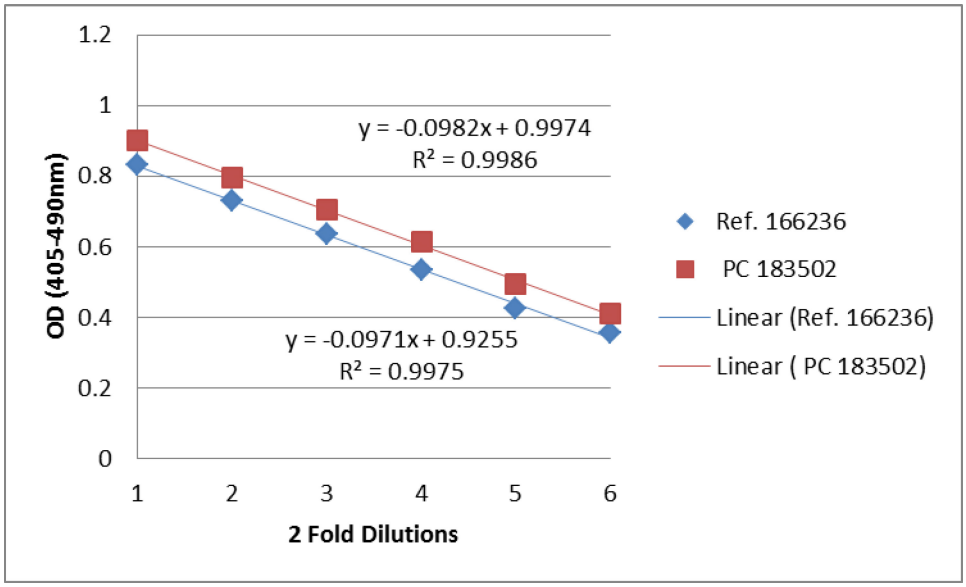


Fig. 11: The linear equations for the reference and positive control contained similar slopes, yielding a slope ratio of 1.01. Starting dilutions of reference (Ref.) and positive control (PC) was 1:54.

The slope for the positive control was -0.0982 and -0.0971 for the reference resulting in a slope ratio of 1.01, which was within the acceptable range. After the use dilutions of the capture and indicator antibodies, and the reference and positive control

have been established, the next step is putting them into an appropriate assay format for assay optimization.

2.3.4.4 IN-PROCESS ELISA FOR OPTIMIZATION

The following briefly describes the method used to optimize the in-process ELISA (as described in section 2.2.10). The polyclonal capture antibody was dispensed onto a polystyrene 96-well plate and incubated overnight at 4°C. The next day, the plate was blocked and incubated. During incubation the reference, positive control and test articles were prepared for diluting onto a dilution plate. The samples from the dilution plate were transferred to respective wells of the blocked plate and incubated. The indicator antibody was added to the plate and then incubated. Next, the peroxidase conjugate antibody was added to the plate and incubated. ABTS peroxidase substrate was added to all wells on the plate. The plate was read at 405 nm minus 490 nm targeting a ≥ 0.8 OD for the first dilution of the reference. This targeted OD is used during the in-process ELISA optimization; during optimization steps an OD read range is established.

2.3.5 IN-PROCESS ASSAY OPTIMIZATION STEPS

2.3.5.1 MEAN, STANDARD DEVIATION AND COEFFICIENT OF VARIATION

To evaluate the in-process ELISA, three parameters are determined: the mean and standard deviation (STD) of replicate samples that are used to calculate the percent coefficient of variation (%CV). The %CV is calculated by taking the standard deviation divided by the mean and multiplying by 100. Low %CV indicates less variation between

replicates and higher precision of the assay. A ≤15% CV would indicate the assay reagents are performing with acceptable consistency between replicates and in accordance with Veterinary Services Memoranda 800.112 (United States Department of Agriculture, 2017b). Variability can arise from any step of the assay and these can be defined by five different methods for optimizing an assay as described below.

2.3.5.2 INTRA-ASSAY PRECISION OR REPEATABILITY

One optimization parameter is to show the in-process ELISA intra-assay precision, which is a measure of how repeatable the assay is from plate to plate. To assess precision and repeatability, one bulk lot was used in one sample position on the assay plate (*i.e.,* T01, Fig. 1) on six different plates to achieve six replicates. The RU/mL is calculated by individually applying a linear regression model to the reference, positive control and test samples. If the slope ratio for the reference and positive control criteria are met, then the relative potency of the sample is calculated based on the difference in intercepts and common slopes of the reference and sample. The linear regression model then calculates a RU/mL. In order to estimate the repeatability, the %CV of the RU/mL was calculated to determine if the six replicates demonstrated a %CV ≤ 15%.

Table 5: Intra-assay precision of the in-process ELISA.

Antigen Lot	**Plate #**	**RU/mL**
169196	1	6230
	2	6307
	3	7023
	4	6163
	5	6447
	6	6365
Mean RU/mL		6423
STD		311
%CV		5

Intra-assay precision of the assay method was evaluated with bulk lot 169196 and the RU/mL results were calculated by measuring the bulk lot against the known reference (Table 5). The %CV of the six replicates was 5%, meeting the criteria for intra-assay precision.

2.3.5.3 WITHIN-PLATE PRECISION

The next step in assay optimization is to assess within-plate precision, which evaluates the variability between sample positions on a single plate using the same sample bulk. A similar RU/mL result should be achieved regardless of which sample position (T01, T02 or T03) the test article is placed on the plate. The RU/mL data from all three samples positions on two plates were analyzed in order to estimate and calculate the %CV for the within plate precision (Table 6).

Table 6: Within-plate precision of the in-process ELISA.

Antigen Lot	**Position**	**Plate 1**	**Plate 2**
		RU/mL	
183502	T01	12252	12289
	T02	12210	11891
	T03	12699	12277
Mean RU/mL		12387	12152
STD		271	226
%CV		2	2

Within-plate precision of the assay method was evaluated with bulk lot 183502 by testing all three sample positions on two separate plates. Testing was completed on one day and the RU/mL was calculated by measuring the bulk lot 183592 against the known reference. The %CV's were less than 5% for the two plates, meeting the validation criteria for within-plate precision ≤ 15%.

2.3.5.4 INTERMEDIATE PRECISION

Another optimization step for the in-process assay is to show different personnel can achieve the same results with the same sample. Intermediate precision is established by comparing assay results obtained from different technicians within the same laboratory. The intermediate precision was assessed by evaluating one bulk lot at 100% and diluted to two different concentrations. Two different analysts each performed two replicate plates. Precision was evaluated by calculating the %CV for each preparation (Table 7).

Table 7: Intermediate precision of the in-process ELISA.

			Sample		
			T01 (100%)	**T02 (70%)**	**T03 (30%)**
Antigen Lot	**Replicate**	**Technician**	**RU/mL**		
169196	1	1	6697	4289	1888
		2	5863	3731	1692
	2	1	6420	4448	2035
		2	7074	4495	1811
Mean RU/mL			6514	4241	1857
STD			510	352	144
%CV			8	8	8

Intermediate assay precision evaluated one bulk lot at three different concentrations which were 100%, 70% and 30%. Intermediate CV's for RU/mL was calculated by measuring the bulk lot 169196 against the known reference. The %CV's were 8%, meeting the validation criteria for intermediate precision of ≤ 15%.

2.3.5.5 ACCURACY

After establishing intermediate precision, the next step for the in-process assay is to assess the accuracy of the assay. Accuracy is the closeness of the assay value to the

predicted value based on analysis of the sample to the reference. For example, a bulk lot can be measured relative to a standard curve to obtain a value for that bulk lot. The same sample can then be diluted and the value should be known. For instance, the accuracy of the assay was determined by comparing the measured RU/mL to the theoretical RU/mL of three bulk lot preparations. The measured RU/mL of the non-diluted, 100%, bulk lot (T01) and its two diluted preparations, 70% and 30% (T02 and T03) were compared to their theoretical RU/mL. The calculated difference (percent of theoretical) in RU/mL between measured and theoretical values were compared (Table 8). The difference (percent of theoretical) in RU/mL between the measured and theoretical values for T02 and T03 were calculated as follows;

Percent of theoretical RU/mL = 100 * (measured RU/mL ÷ theoretical RU/mL)

Table 8: Comparison of measured RU/mL to theoretical RU/mL values demonstrating accuracy of the in-process ELISA.

Antigen Lot		**169196**			**173883**			**182291**		
		T01	**T02**	**T03**	**T01**	**T02**	**T03**	**T01**	**T02**	**T03**
Rep	**Tech**	**RU/mL**								
1	1	6697	4289	1888	8426	5687	2490	7976	4862	2234
	2	5863	3731	1692	8707	6035	2514	9181	6122	2843
2	1	6420	4448	2035	7792	5358	2256	7758	5576	2195
	2	7074	4495	1811	6649	5120	1905	9024	6151	2128
Mean RU/mL		6514	4241	1857	7894	5550	2291	8485	5678	2350
Theoretical RU/mL			4560	1954		5525	2368		5939	2545
% Theoretical			93	95		100	97		96	92

The acceptance criterion for the accuracy of an assay has been internally established to fall within the range of 85 – 115 % theoretical. The theoretical RU/mL is calculated by multiplying the non-diluted sample (T01) mean RU/mL result by 70% or 30% for samples T02 and T03 respectively (Table 8). Samples T02 and T03 for each of

the three bulk lots show the measured RU/mL relative to the calculated theoretical RU/mL and resulted in % theoretical values within the acceptable range.

2.3.5.6 ROBUSTNESS

Determining robustness of an assay gives the operator a defined range of OD to yield confidence in the assay output. Robustness of the assay with regard to plate reading range was evaluated by one analyst assaying one plate per day and repeating this over three days. Each plate was read at a range of 0.6 – 1.1 OD for the reference lot 166263 and the RU/mL data is analyzed by comparing %CV (Table 9).

The minimum and maximum read range was evaluated by one analyst. The positive control (lot 183502) was used as the sample bulk measured for T01, T02 and T03. On each of the three assay days, a single plate was read at an early (0.6) through late (1.1) OD. The data shows that a reading range of the first reference dilution points, 1:54 dilution (wells B2 and B7 in Figure 1), from 0.6 – 1.1 OD is an acceptable range for potency assay robustness, generating an overall average %CV of 3% across the three assay days.

Table 9: Variation between the minimum and maximum read range values.

Day	OD[a]	PC	T01	T02	T03	Mean RU/mL	Average %CV
		RU/mL					
1	0.6	11608	11903	11311	11502	11572	2
	0.7	11622	11795	11478	11498	11590	
	0.8	11751	11877	11879	11577	11778	
	0.9	11825	11890	11733	11902	11842	
	1.0	12135	12223	12054	11648	11975	
	1.1	12190	12594	12447	11931	12324	
2	0.6	12720	12165	11448	13069	12227	3
	0.7	12637	12477	11444	12714	12212	
	0.8	12896	12252	12210	12699	12387	
	0.9	13133	12580	11788	13278	12549	
	1.0	13179	12830	12011	13429	12757	
	1.1	13959	12872	13100	13121	13031	
3	0.6	10689	9966	10107	10859	10311	5
	0.7	10508	10111	10183	10461	10252	
	0.8	10575	11135	10350	10838	10774	
	0.9	10736	11324	10993	11050	11122	
	1.0	11626	11487	11235	11497	11406	
	1.1	11713	11599	11164	11221	11328	

[a]Average OD reading of reference first dilution points in wells B2 and B7.

2.3.5.7 ESTABLISHING POSITIVE CONTROL RANGE

After the other assay parameters had been optimized, the last step was to establish a positive control range. The positive control range is another measurement used to ensure day to day and operator to operator consistency. The assay is satisfactory when the positive control is within the range for each assay plate assessed. Establishing the positive control range was achieved by three different technicians running six plates on three separate days. The standard deviation of the mean was determined and the range was established by plus and minus two times the standard deviation of the mean. The mean RU/mL from each of the three technicians and the positive control range was established to be 9,556 – 13,838 RU/mL (Table 10).

Table 10: Positive control range for the in-process ELISA was established using mean variation between three individual runs.

Day/Tech	Plate #	PC lot 183502 (RU/mL)
Day 1 Tech 1	1	11277
	2	11161
	3	11051
	4	11168
	5	10532
	6	11956
Day 2 Tech 2	7	11599
	8	12297
	9	10087
	10	11749
	11	11908
	12	11631
Day 3 Tech 3	13	11699
	14	11550
	15	12311
	16	11096
	17	15284
	18	12199
Mean RU/mL		11698
Standard Deviation (STD)		1070
%CV		9
Positive Control Range Ru/mL[a]		9,556 – 13,838

[a]Values determined by ± two times the standard deviation of the mean.

2.3.6 EVALUATION OF ELISA COMPARED TO L+ ASSAY

Eleven bulk antigens of *C. perfringens* type C toxoid with known L+ values were measured using the optimized in-process ELISA. These results were plotted to determine the correlation between the two different assays (Fig. 12). This analysis was done to determine if the in *vitro* ELISA could directly replace the mouse L+ assay as the in-

process assay. The cumulative R^2 value was 0.4048 indicating low correlation of L+ values to measured RU/mL values.

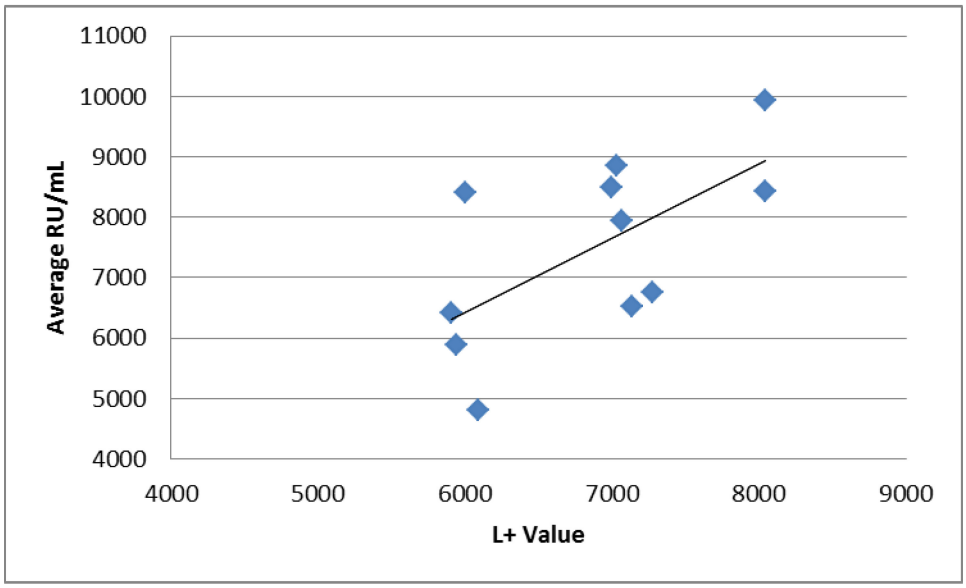


Fig. 12: L+ values compared to measured RU/mL values of eleven bulk antigen lots showed low correlation between L+ assay and in-process ELISA; R^2 value of 0.4048 (trend line).

2.4 DISCUSSION

Clostridial species importance is due in part to the fact that they can be found in many environments including soil, water and mammals. While they frequently exist as commensals, *C. perfringens* can be the cause of severe disease in both humans and animals. Outbreaks of *C. perfringens* causing necrotic enteritis in neonates can have a great economic impact on livestock producers. Effective vaccines have been developed to help prevent outbreaks, but they can be expensive due to the test methods involved in the production and release of the vaccines. Currently, the beta toxoid form is a common immunogen used in *C. perfringens* type C vaccines. To determine the potency of

production runs and facilitate blending of vaccines products, the potency test methods involve the use of lethal mouse assays. An in *vitro* method for testing the beta toxoid of *C. perfringens* would reduce the expense of vaccine production by eliminating or reducing the use of laboratory animals. The data presented in this thesis details the development of a robust and precise in-process ELISA method (below the industry standard of 15% CV) for detecting and quantifying beta toxoid in cultures of *C. perfringens* type C.

In this study, a monoclonal antibody specific to the beta toxoid from *C. perfringens* type C was generated and selected for developing an in *vitro* in-process ELISA assessing beta toxoid yield. This monoclonal antibody had high specificity to the beta toxin via western blot, produced a high ELISA signal to the beta toxoid and strong neutralizing activity with no cross reactivity to *C. perfringens* type A alpha toxoid. This monoclonal antibody, and a selected polyclonal antibody, allowed for the development of an in-process ELISA that is specific to the beta toxoid.

The development of in *vitro Clostridium* toxoid assays has produced a diverse collection of protocols with varying utility. Redhead, K., *et al*. described a *C. septicum* in-process assay using Vero cells, which are sensitive to the toxin from *C. septicum*, and showed a correlation between their cell-based assay and mouse L+ test assay results. They concluded the mouse L+ test could be replaced by their cell-based assay (Redhead et al., 2011). While the cell-based assay was more accurate than the mouse L+ test and eliminated the use of mice, this method measures toxin but cannot quantify toxoid in production fermentation batches. In addition to cell line-based assays, ELISA methods (*i.e.,* sandwich, blocking, and competitive assays) have been used to develop serological

assays for measuring the presence of anti-*C. perfringens* beta toxin in rabbits, pigs, cattle, sheep and horse serum. ELISA methods have the potential to reduce the use of mice for assessing the presence of serum antibodies in the final potency test, but does not eliminate animal usage altogether (Krt, 1999). Another ELISA method tried to replace the mouse neutralization test for estimating levels of beta toxin antitoxin in the sera of immunized rabbits for final potency testing. This in *vitro* method uses an ELISA where a monoclonal antibody to beta toxin is coated on the plate to capture *C. perfringens* beta toxin, which then binds beta antitoxin in rabbit sera (Ebert et al., 1999). While this method had a good correlation between in *vivo* and in *vitro* for testing sera potencies, rabbits are still vaccinated first in order to obtain final potency results. To date, the available assays were not designed for quantifying antigen for vaccine input. The sandwich ELISA described in this thesis is an alternative to the current L+ test that will potentially reduce the use of experimental animals. An ELISA would be a high through put advantage able to test greater number of samples in a shorter period of time, more economical and have better consistency of batch to batch vaccine assembly in comparison to assembling vaccines based on in *vivo* testing. This ELISA would also have the potential to replace the current potency testing of *C. perfringens* type C vaccines as described in 113.111 of the USDA 9CFR and in monograph 363 of the EP.

The research described here has shown the development of an optimized ELISA method for detecting and quantifying *C. perfringens* type C beta toxoid. However, the in *vivo* L+ values were shown not to correlate with the ELISA RU/mL values. Figure 12 illustrated there was low correlation between the L+ values and the average measured RU/mL values. Values generated by the L+ assay are not directly indicative of the

quantity of beta toxin present in the *C. perfringens* type C material, which could be why variability is seen in animal testing. The L+ is measuring the antigenicity of toxin verses the toxoid being quantified with a specific antibody in the in-process ELISA.

In order to replace the L+ test with the developed ELISA, additional experiments would need to be completed. Using the developed ELISA, one approach would be to perform the in-process ELISA method on formulated vaccines. If the in-process ELISA could also detect beta toxoid in final vaccine, then this would aide in quantitatively determining the protective dose in current vaccines. Another approach would be to obtain toxoid antigen with known L+ value and measure the toxoid material in the in-process assay to determine the respective RU/mL value of the bulk. Vaccines could be prepared with toxoid antigen at different RU/mL levels for vaccinating mice and the mice would be challenged with a known amount of beta toxin that is lethal in mice. By repeating this with multiple antigen bulks of type C toxoid, one could define the potential protective dose in mice. After mouse challenge studies had been completed, large animal studies, species specific, would need to be conducted showing the protective minimum immunizing dose (MID). This is now possible due to the generation of reagents which are the monoclonal and polyclonal antibodies to beta toxoid material as well as the method for use of those reagents.

In my research, I assessed and selected a polyclonal and a monoclonal antibody and optimized the concentrations used to detect the *C. perfringens* type C toxoid. These antibodies had specificity to beta toxin and toxoid and exhibited neutralizing activity; hence, can to be used as the capture and indicator for developing an in-process ELISA. The USDA requires the reagents in these types of in *vitro* methods correlate to protection

either by specificity to protective epitopes or by the ability to neutralize antigens that cause disease. Through a combination of western blots, neutralization assay and ELISA's, I identified which antibodies would be appropriate for the assay. This optimized in-process ELISA could potentially replace the mouse L+ test which would reduce the use of animals to measure the type C toxoid material and potentially improve *C. perfringens* type C vaccine production through batch to batch consistency, decreased testing time and could improve cost of the product.

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