Clostridium perfringens and the beta2 (CPB2) toxin: Development of a diagnostic ELISA for neonatal piglet enteritis, and distribution of the gene in isolates from selected animal species

by

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ABSTRACT

CLOSTRIDIUM PERFRINGENS AND THE BETA2 (CPB2) TOXIN: DEVELOPMENT OF A DIAGNOSTIC ELISA FOR NEONATAL PIGLET ENTERITIS, AND DISTRIBUTION OF THE GENE IN ISOLATES FROM SELECTED ANIMAL SPECIES

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Advisor:
Dr. John F. Prescott

The main objective of this work was to develop an antigen-capture enzyme-linked immunosorbent assay for detection of beta2-toxin in the intestine of neonatal piglets. The format of the assay comprised of capture antibodies (polyclonal), antigen (beta2-toxin), detecting antibody (labeled monoclonal) and a substrate. The ELISA was optimized using recombinant protein. After intestinal content samples were applied, the test protocol needed to be adjusted because of the presence of high background signal in some samples consistent with intestinal proteases. This was overcome by processing the samples at 4°C and using citrate buffer pH 6.1 containing 5% bovine serum albumin.

The second objective was to identify cpb2 in Clostridium perfringens type A isolates from selected animal species and to examine genotype-phenotype corelation. The study concluded that consensus cpb2, if present, was almost always expressed. In contrast, only about three-quarters of atypical cpb2, mostly was present in isolates of non-porcine origin, were expressed.
Acknowledgements

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I would like to express my gratitude to my family, Ljuba, Dusan and Nenad for being a supporting force along the way. Many thanks to my closest friends, Helena, Irena, Jennifer, Donna, Marie, Michelle, Giulia, Sonia…for keeping my spirits up. Finally, my deepest appreciation to Kathy.

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Declaration of Work

All the work described in this thesis was performed by me with the following exceptions:

1. Post mortem examination of piglets was performed by the Animal Health Laboratory, University of Guelph, which provided intestinal samples.
2. Native CPB2 was purified by Yanlong Pei.
3. Glenn Soltes did the work and provided data for Figure 2.3.
4. Samantha Whiteside provided preliminary data on CPB2 expression from various isolates.
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<th>Definition</th>
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<tr>
<td>AAD</td>
<td>Antibiotic-associated diarrhea</td>
</tr>
<tr>
<td>A-ATYPF</td>
<td>Forward primer for atypical <em>cpb2</em> – single reaction PCR</td>
</tr>
<tr>
<td>A-ATYPR</td>
<td>Reverse primer for atypical <em>cpb2</em> – single reaction PCR</td>
</tr>
<tr>
<td>APMSF</td>
<td>Amidinophenyl-methanesulfonyl-fluoride</td>
</tr>
<tr>
<td>BSA</td>
<td>Bovine serum albumin</td>
</tr>
<tr>
<td>CaCo-2</td>
<td>Carcinoma colon – 2 cell line</td>
</tr>
<tr>
<td>CFU</td>
<td>Colony forming units</td>
</tr>
<tr>
<td>CHO</td>
<td>Chinese hamster ovary cells</td>
</tr>
<tr>
<td>CPA</td>
<td><em>Clostridium perfringens</em> alpha toxin</td>
</tr>
<tr>
<td>CPB</td>
<td><em>Clostridium perfringens</em> beta toxin</td>
</tr>
<tr>
<td>CPB2</td>
<td><em>Clostridium perfringens</em> beta2 toxin</td>
</tr>
<tr>
<td>CPE</td>
<td><em>Clostridium perfringens</em> enterotoxin</td>
</tr>
<tr>
<td>DNA</td>
<td>Deoxyribonucleic acid</td>
</tr>
<tr>
<td>EIA</td>
<td>Enzyme immunoassay</td>
</tr>
<tr>
<td>ELISA</td>
<td>Enzyme-linked immunosorbent assay</td>
</tr>
<tr>
<td>ETX</td>
<td><em>Clostridium perfringens</em> epsilon toxin</td>
</tr>
<tr>
<td>FCS</td>
<td>Fetal calf serum</td>
</tr>
<tr>
<td>GI</td>
<td>Gastrointestinal tract</td>
</tr>
<tr>
<td>HRP</td>
<td>Horseradish peroxidase</td>
</tr>
<tr>
<td>IPTG</td>
<td>Isopropyl-β-D-thiogalactopyranoside</td>
</tr>
<tr>
<td>IgG</td>
<td>Immunoglobulin G</td>
</tr>
<tr>
<td>IS</td>
<td>Insertion sequence</td>
</tr>
</tbody>
</table>
ITX  
*Clostridium perfringens* iota toxin

LB  
Luria-Bertani medium

mAb  
Monoclonal antibody

M-ATYPF  
Forward primer for atypical *cpb2* – multiplex PCR

M-CONF  
Forward primer for consensus *cpb2* – multiplex PCR

M-REV  
Reverse primer for atypical and consensus *cpb2* – multiplex PCR

MLST  
Multi-locus sequence typing

mRNA  
Messenger ribonucleic acid

OD  
Optical density

ORF  
Open reading frame

P18  
Porcine *C. perfringens* isolate number 18

pAb  
Polyclonal antibodies

PBS  
Phosphate-buffered saline

PBST  
Phosphate-buffered saline Tween 20

PCR  
Polymerase chain reaction

PFGE  
Pulsed field gel electrophoresis

RFLP  
Restriction fragment length polymorphism

rCPB2  
Recombinant CPB2

RPM  
Revolutions per minute

rRNA  
Ribosomal ribonucleic acid

SD  
Sporadic diarrhea

SDS-PAGE  
Sodium dodecyl sulfate –polyacrylamide gel electrophoresis

TCA  
Tricarboxylic acid
TGY  Tryptic soy broth, D-glucose, yeast extract medium
Chapter 1

Literature Review

General introduction

*Clostridium perfringens* infection associated with gas gangrene has been identified in humans for over a century, but its role in human enteric disease took longer to recognize. This organism has been linked to human gastrointestinal (GI) disease since at least the mid-1940s. McClung *et al.* (1945) were the first to show that this bacterium has the ability to cause food poisoning. This was confirmed during the 1950s through the studies of Hobbs and others (Hobbs *et al.*, 1953) and pathogenesis of these infections was mediated by enterotoxin (CPE). After World War II, outbreaks of *enteritis necroticans* in Germany were associated with *C. perfringens* producing another toxin, with the same unusual human disease (‘Darmbrand’) recognized later as endemic in the highlands of Papua New Guinea, where it is locally called “pigbel”. The local diet is based mainly on sweet potato which contains a trypsin inhibitor, so that the beta toxin (CPB) ingested with contaminated pig meat during feasts is not destroyed in the intestine. In the 1980s, *C. perfringens* was linked to human non-food-borne GI diseases, in some cases associated with antibiotic use. The connection of *C. perfringens* and enteric disease in animals however occurred earlier than in humans, in the 1920s and 1930s, particularly because of the importance of pulpy kidney disease as a common and fatal infection of lambs. The understanding of enteric diseases caused by *C. perfringens* in animals has increased over the years, but there is still much to be discovered about its role in intestinal disease.
Phylogeny of the organism

The genus *Clostridium* consists of a distinct group of Gram-positive anaerobic bacteria with the ability to produce heat-resistant endospores. The genus represents almost 150 heterogeneous species (Dworkin et al., 2006). They are large rods with the distinct feature of production of several potent, mouse-lethal, extracellular toxins (Quinn et al., 2002; Hirsh et al., 2004). These toxins are considered to be major virulence factors.

Early taxonomic studies of anaerobic sporeforming Gram-positive rods, based on homology of rRNA molecules, led to recognition of four groups which are likely individual genera (Johnson and Francis, 1975). Group I contained well-defined species with a low G+C content (21 – 28%). *Clostridium perfringens* is a member of Group I. Later, 16S rRNA sequence analysis confirmed these findings and species clusters have been defined within these groups. It has been suggested that organisms clustered in phylogenic Group I are the true members of the genus *Clostridium*, referred to as the core cluster (Rood et al., 1997; Fischetti et al., 2006). The inexplicable failure to rename the other clostridial Groups as individual genera appears to relate to a conflict between modern taxonomy and the historic names of some classic clostridial pathogens.

*Clostridium perfringens* has an ubiquitous environmental distribution. It is also present as normal intestinal flora of humans and domestic animals, but has a wide toxin arsenal; 17 different currently recognized toxins of various types and potencies are encoded by genes in the chromosome or on plasmids (Songer et al., 1996). This feature explains its ability to cause a variety of infections with different symptoms. In contrast to other clostridia, *C. perfringens*
tolerates air and requires only moderate lowering of the oxidation-reduction potential for growth (Quinn et al., 2002; Dworkin et al., 2006; Gyles et al., 2010). Because of its fast growth rate in optimal conditions, its importance as a disease agent and to a lesser extent its ability to be genetically manipulated, \textit{C. perfringens} became one of the most important members of the genus for initial genetic studies of clostridia (Shimizu et al., 2002).

\textbf{Clostridium perfringens – types and genetic properties}

The mouse-lethal toxins or their combination expressed by individual isolates of \textit{C. perfringens} forms the basis of the toxin-typing scheme traditionally used in differentiating isolates, although this system is increasingly recognized as archaic. Using this typing system, \textit{C. perfringens} is divided into five toxinotypes (A-E), based on production of combinations of alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX) toxins, the four major \textit{C. perfringens} mouse-lethal toxins. Truly non-toxigenic strains of \textit{C. perfringens} are rare (Songer and Post, 1996). Potent toxins are dominant virulence factors. As new toxins and disease associations emerge, however, this classic differentiation of \textit{C. perfringens} is increasingly recognized as inadequate to describe such a versatile pathogen, so that the typing scheme will likely be replaced by one based on PCR or DNA microarray that describes a greater array of virulence genes and of the subtypes or varieties of the pathogen.

The most widespread toxinotype in the intestines of animals and the environment is \textit{C. perfringens} type A. This organism is associated with different enteric diseases in different animal species, including humans (Table 1.1). Some diarrheal diseases in animals may be associated with enterotoxigenic strains of this organism associated with the CPE. It has been suggested that
the pathogenesis of type B infection is due to the synergistic effects of CPB and ETX. Strains of type C *Clostridium perfringens* infect many domestic farm animal species. The most susceptible are newborn animals.

### Table 1.1 Toxin-types and diseases caused by *Clostridium perfringens*

<table>
<thead>
<tr>
<th>C. <em>perfringens</em> types</th>
<th>Animal species</th>
<th>Disease</th>
<th>Toxins: confirmed and suspected</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>Poultry</td>
<td>Necrotic enteritis</td>
<td>CPA, NetB</td>
</tr>
<tr>
<td></td>
<td>Piglets</td>
<td>Enterocolitis</td>
<td>CPA, CPB2</td>
</tr>
<tr>
<td></td>
<td>Horses</td>
<td>Neonatal hemorrhagic diarrhea</td>
<td>CPA, CPB2</td>
</tr>
<tr>
<td></td>
<td>Calves</td>
<td>Abomasal ulceration and tympany</td>
<td>CPA, CPB2</td>
</tr>
<tr>
<td></td>
<td>Humans</td>
<td>Gangrene</td>
<td>CPA, Theta</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Food poisoning</td>
<td>CPA, CPE-positive for food poisoning</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Antibiotic-associated diarrhea</td>
<td>CPE, CPB2-associated</td>
</tr>
<tr>
<td>B</td>
<td>Lambs</td>
<td>Dysentery</td>
<td>CPA, CPB, ETX</td>
</tr>
<tr>
<td></td>
<td>Sheep</td>
<td>Chronic enteritis</td>
<td>CPA, CPB, ETX</td>
</tr>
<tr>
<td>C</td>
<td>Poultry</td>
<td>Necrotic enteritis</td>
<td>CPA, CPB</td>
</tr>
<tr>
<td></td>
<td>Neonatal pigs, Lambs, Calves, Goats, Foals</td>
<td>Hemorrhagic or necrotic enterotoxemia</td>
<td>CPA, CPB</td>
</tr>
<tr>
<td></td>
<td>Adult sheep</td>
<td>Acute enterotoxemia</td>
<td>CPA, CPB</td>
</tr>
<tr>
<td></td>
<td>Humans</td>
<td>Enteritis necroticans</td>
<td>CPA, CPB</td>
</tr>
<tr>
<td>D</td>
<td>Calves, Goats, Adult cattle, Young lambs</td>
<td>Enterotoxemia</td>
<td>CPA, ETX</td>
</tr>
<tr>
<td>E</td>
<td>Calves</td>
<td>Enterotoxemia</td>
<td>CPA, ITX</td>
</tr>
</tbody>
</table>

Disease occurs on rare occasions in older animals, and usually follows alteration of intestinal flora due to sudden dietary changes. Infection caused by type D is the result of sudden changes to a rich diet or continuous feeding of a highly concentrated ratio (Songer *et al.*, 1996). Both type B and type C cause enteritis in the small intestine, which may be a consequence of descending infection in the GI tract. However, type D usually causes an ascending infection into the small intestine from the large intestine in response to poorly digestible carbohydrate diets. The large
number of bacteria in the large intestine may prevent development of *C. perfringens* infection in this site through the proteolytic cleavage of secreted, mouse-lethal proteins.

*Clostridium perfringens* forms spores that survive in the environment, from where they can be picked up by a host. In addition, it can be spread by the fecal-oral route. The organism is well adapted to different niches in animals or humans, with an apparent host predilection or association, although the basis of this has not been determined. It can be concluded that they are often members of the normal intestinal flora with the potential to cause disease. Outbreaks of clostridial enteric infection are often associated with known risk factors. These include such factors as lack of competing intestinal flora in the neonatal period, treatment with antibiotics in adult humans and horses, local intestinal damage by other infections such as coccidiosis, declining maternally-derived immunity in young animals, dietary changes permitting proliferation of this extraordinarily fast-growing bacterium in the small intestine, the lack of trypsin in the neonatal intestine or the presence of trypsin inhibitors in the small intestine so that toxins are not degraded, and other factors.

*Clostridum perfringens* was the first Gram-positive bacterium whose genome was fully sequenced (Shimizu *et al.*, 2002). The genome consists of a single chromosome of 3.6 Mbp (Rood *et al.*, 1998). The content of the G+C is low (28.6%), as is characteristic of clostridia (Rood *et al.*, 1997). Complete genome sequences are now available for three *C. perfringens* strains: CP13 (a cpe-negative type A soil isolate) (Shimizu *et al.*, 2002), ATCC strain 13124 (a type A gas gangrene isolate), and SM101 (a CPE-producing food poisoning isolate) (Myers *et al.*, 2006). A comparison of these genomes revealed the existence of many different “genomic
islands”; further analysis suggested that these islands were carried by numerous *C. perfringens* strains and were associated with different phenotypic characteristics (both virulence and metabolic) of the strains (Myers *et al.*, 2006).

The ability to ferment carbohydrates is determined by a full set of genes encoding glycolytic enzymes (anaerobic glycolysis). Some of these genes are strain-unique, which indicates different capabilities to exploit different environmental conditions (Myers *et al.*, 2006). An important characteristic in respect to energy metabolism is that *C. perfringens* lacks some genes encoding enzymes of the tricarboxylic acid (TCA) cycle and electron transport chain proteins (Shimizu *et al.*, 2002; Fischetti *et al.*, 2006). In addition, genes that normally encode enzymes for amino acid biosynthesis are missing (Shimizu *et al.*, 2002). Because of these losses, the organism requires that some sugars and many amino acids be acquired from the environment, a feature apparently responsible for the more than 200 transport-related genes, including 52 ATP-binding cassette transporter systems (ABC transporter systems). These findings imply that *C. perfringens* cannot grow in an environment where an amino acid supply is limited (Shimizu *et al.*, 2002; Fischetti *et al.*, 2006; Myers *et al.*, 2006), but that it can rapidly transport sugars and amino acids acquired by breakdown of tissues into the cell. Metabolism that does not include amino acid synthesis may have been an evolutionary “pressure” for the organism to create new mechanisms for surviving, or alternatively and more likely, the loss of these genes may have followed development of ways to rapidly acquire nutrients from the host. As an unusual “flesh-eating pathogen”, *C. perfringens* specialises in secretion of an extraordinary array of different potent toxins and non-toxic enzymes that degrade host tissue to acquire vital nutrients. For example, the unusually high number of five hyaluronidases causes degradation of
polysaccharides (hyaluronic acid, chondroitin sulphate) that not only help the organism to spread into deeper tissues but also to break down these complex sugar molecules. Hyaluronidase variation in the C-terminal sequences may change their substrate specificities for various host polysaccharides. Sialidases also contribute to the degrading of sialic acid from host tissues and cells (Shimizu et al., 2002).

Production of many toxins and extracellular enzymes is regulated by the two-component VirR/VirS regulatory system (VirR – response regulator; VirS – sensor protein). This regulatory system, in response to a quorum sensing peptide signal, rapidly activates the VirR regulon (DNA) which upregulates production of a mix of degradative enzymes. There is a tight linkage between multiple genes for virulence and nutrient acquisition, since the VirR/VirS (virulence) regulon regulates not only toxin genes but also genes involved in energy metabolism. Complete genome analysis of strain CP13 showed that *C. perfringens* has 48 genes encoding signal transduction systems, including 28 sensor kinases and 20 response regulator genes conserved in all three strains (CP13, ATCC13124 and SM101) (Shimizu et al., 2002). After complete CP13 genome sequencing, Shimizu *et al.* (2002) found four previously unidentified putative VirR binding sites. One is a member of the VirR/VirS system, located upstream of the *hyp7* gene, a positive regulator for CPA and kappa-toxin genes. The product of the *hyp7* region is VirR/VirS-regulated RNA (VR-RNA), which mediates the signal from the VirR/VirS system to control the expression of certain toxins, whereas theta-toxin (perfringolysin) is regulated directly by the VirR/VirS (Okimura *et al.*, 2008). The presence of an unusually large number of rRNA genes ensures rapid production of enzymes after VirR up-regulation. Toxins are produced during early rather than late logarithmic growth, an unusual characteristic among many pathogens,
emphasizing the extraordinarily rapid ability of this organism to cause tissue destruction in its acquisition of essential nutrients.

Identification of disease-causing strains

Detection of known or potential toxins produced by *C. perfringens* is crucial for a better understanding of *C. perfringens* infections. Because of the cost, animal welfare considerations and in some cases the complexity of toxin detection, use of animals for toxin typing has largely been replaced by genotyping of *C. perfringens* isolates; identifying genes does not mean, however, that they are expressed. Numerous PCR protocols have been developed to genotype different isolates to detect the presence of different toxin genes (*cpa, cpb, etx, iap, cpe, cph2*) (Songer *et al*., 1996; Meer *et al*., 1997), including protocols for multiplex PCR assays. Other than *cpb2*, discussed below, silent mouse-lethal toxin genes in animal isolates are apparently rare, and the genotype (presence of toxin genes) and phenotype (production of the toxin) correlate almost 100% (Songer *et al*., 1998). Exceptions to this generalization are type E isolates which can carry a silent copy of the *cpe* and the “atypical” *cpb2*, which are commonly out-of-frame (Songer *et al*., 1998).

Apart from conventional and multiplex PCR for detection of toxin genes, various strain genotyping systems have been developed for epidemiological purposes, such as multilocus sequence typing (MLST) (Turner *et al*., 2007), restriction fragment length polymorphism (RFLP) and pulsed-field gel electrophoresis (PFGE) (Engstrom *et al*., 2003; Nakamura *et al*., 2003; Lukinmaa *et al*., 2004), as well as DNA microarray (Al-Khalidi *et al*., 2004).
Toxins as virulence factors

One of the most important features of *C. perfringens* in pathogenesis is the ability to produce extracellular toxins and enzymes. A summary of the important toxins and their biological effects is presented in Table 1.2.
Table 1.2 Properties of important *Clostridium perfringens* toxins

<table>
<thead>
<tr>
<th>Toxin/Enzyme</th>
<th>Gene location</th>
<th>Activity</th>
<th>Biological effect</th>
</tr>
</thead>
<tbody>
<tr>
<td>Alpha</td>
<td>Chromosome (cpa)</td>
<td>Phospholipase C</td>
<td>Hemolytic, Cytotoxic, Necrotizing, Lethal</td>
</tr>
<tr>
<td>Beta</td>
<td>Plasmid (cph)</td>
<td>Pore-forming</td>
<td>Inflammation, Necrosis, Lethal</td>
</tr>
<tr>
<td>Epsilon</td>
<td>Plasmid (etx)</td>
<td>Increases cell-membrane permeability</td>
<td>CNS toxicity, Enterotoxemia, Lethal</td>
</tr>
<tr>
<td>Iota</td>
<td>Plasmid (itx)</td>
<td>Actin ADP-ribosylation</td>
<td>Cytoskeleton disruption, Increased vascular permeability, Dermonecrotic, Lethal</td>
</tr>
<tr>
<td>Enterotoxin</td>
<td>Chromosome/Plasmid (cpe)</td>
<td>Pore-forming (relaxes tight junctions, released with endospores)</td>
<td>Cytotoxic, Diarrhea</td>
</tr>
<tr>
<td>Beta2</td>
<td>Plasmid (cpb2)</td>
<td>Pore-forming</td>
<td>Necrosis (intestinal mucosa), Lethal</td>
</tr>
<tr>
<td>NetB</td>
<td>Plasmid (netB)</td>
<td>Pore-forming</td>
<td>Cytotoxic (chicken cells)</td>
</tr>
</tbody>
</table>

**Toxin-coding genes and plasmids (Location of toxin-coding genes)**

Genes encoding extracellular toxins and enzymes are located on the chromosome and on plasmids (Sawires *et al.*, 2006). Chromosomal enzyme- and toxin-encoding genes include *cpa* (encodes CPA), *pfoA* (perfringolysin O or theta-toxin), *colA* (encodes collagenase or kappa-toxin) and *nagH* (hyaluronidase or mu-toxin gene). The genes *nanH* and *nanI*, which encode different sialidases, are also located on the chromosome (Rood *et al.*, 1998; Sawires *et al.*, 2006). Comparison of the organization of the chromosomal regions where virulence genes are located in diverse *C. perfringens* isolates (of all toxin types) revealed the existence of three hypervariable regions (Rood *et al.*, 1998). Based on the 3 genomes characterized to date, all types of *C. perfringens* have a similar genomic organization, but their ability to produce different mouse-
lethal toxins is due to acquisition or loss of specific toxin genes through their ability to acquire (and lose) plasmids. Considerable further genome sequencing is required to fully characterize the genomic organization and the “pan-genome” of *C. perfringens*, but the work of Myers *et al.* suggests high diversity. PCR analysis has shown that these genetic islands are very variable and encode different virulence and other characteristics of *C. perfringens* (toxins, enzymes, sporulation factors, strain-specific capsule, metabolic characteristics) (Myers *et al.*, 2006).

Many of the major toxin-encoding and virulence-associated genes are located on extrachromosomal elements. Use of the I-CeuI endonuclease (which cuts only within rRNA operons in the chromosome) showed that *plc*, *pfoA*, *colA*, and *nagH* are located on the chromosome whereas *cpb* (CPB), *cpb2* (CPB2), *etx* (ETX), *iap/ibp* (ITX), *lam* (lambda-toxin), and *ure* (urease) are located on large plasmids (that do not contain I-CeuI sites) (Jost *et al.*, 2006). The *netB* gene has also recently been found to be plasmid encoded (Lepp *et al.*, 2010).

Different “classical” types of *C. perfringens* produce different toxin combinations in various amounts. This difference in toxin type production is the result of acquisition or loss of extra-chromosomal elements that contain genes for these toxins. *Clostridium perfringens* type B and type D have the ability to produce ETX (Rood *et al.*, 1997) as an inactive prototoxin, which is later activated in the GI tract by proteolytic cleavage. CPB is produced by *C. perfringens* type B and type C (Rood *et al.*, 1997). The structural gene (*cpb*) has been cloned and determined to encode a polypeptide, which has to be cleaved in order to acquire toxicity (Rood *et al.*, 1998). CPB has significant sequence similarity to *Staphylococcus aureus* alpha-toxin and components of the gamma-toxin and leukocidins (Hunter *et al.*, 1993). The *cpb2* gene is found on a plasmid
(Rood et al., 1998). A recent study (Li et al., 2007) showed that cpb2 was present on plasmids in seven of eight type E isolates. In all seven isolates the gene was found on a plasmid distinct from the iap/ibp plasmid, where the structural genes for ITX are located (Rood et al., 1998). The cpb2 sequence, which may or may not encode an intact protein, is present in other types of C. perfringens. For example, type D isolates can carry a single plasmid that may encode three different toxins (CPB2, ETX, and CPE) or three different plasmids, each encoding a single toxin (Sayeed et al., 2007).

Recently, another C. perfringens toxin, NetB, has been identified as a virulence factor crucial in the pathogenesis of necrotic enteritis in chickens (Keyburn et al., 2008), and its gene is found on a plasmid (Lepp et al., 2010). Phylogenetic analysis confirmed that NetB and CPB are two distinct toxins with low sequence identity (38%) but similar molecular sizes. The recently described novel toxin gene, tpeL, has also been shown to be plasmid-mediated in type A strains (Chalmers et al., 2008) and may contribute to the virulence of chicken necrotic enteritis isolates (Coursedon et al., 2011).

Clostridium perfringens CPE is only produced during sporulation, and is not secreted in the vegetative state. The structural gene (cpe) can be found either in the chromosome or on a plasmid (Li et al., 2007). Human isolates of C. perfringens from food poisoning outbreaks carry cpe on the chromosome, whereas cpe is located on a plasmid in the isolates of animal origin and isolates associated with non-foodborne human infections (Miyamoto et al., 2006).
Genomic analysis of *C. perfringens* indicated the presence of a gene equivalent to *ureC* of *Helicobacter pylori* (Dupuy *et al.*, 1997). Most animal isolates contained the *ureC* gene (*ureABC* operon similar to that in many other prokaryotes). This gene was located on a plasmid, which can also carry toxin genes including *cpb, cpe, etx* and *iap/ibp* (Li *et al.*, 2007). These findings suggest that urease activity may be a virulence-associated factor important in *C. perfringens* pathogenesis, although further work is needed to confirm this. Taken together, these findings show that plasmids have an important role in extending the pan-genome of *C. perfringens*.

**Clostridium perfringens-associated diseases in pigs**

*C. perfringens* type C is the most common cause of necrotizing enteritis in piglets. In contrast to *C. perfringens* type A, tissue damage is more severe when the cause of the disease is type C. The organism attaches to epithelial cells in small intestine (via adhesion proteins) and produces CPB, which causes necrotic changes of the enteric mucosa (Sayeed *et al.*, 2010).

*C. perfringens* type A is ubiquitous in the environment and a member of the normal flora in pig intestine. It is apparently a cause of enteric infections of piglets (Klaasen *et al.*, 1999; Bueschel *et al.*, 2003; Songer and Uzal, 2005), although this is less clear than the role of type C isolates. Songer and Uzal (2005) noted in a review that piglets inoculated orally with pure cultures developed prominent clinical signs as a direct consequence of intestinal necrosis, which is, however, uncommon in naturally-occurring cases. When only CPA was administered to neonatal piglets the results were mild enteritis and edema of the villi (Songer *et al.*, 1998). For
suckling piglets, sows are probably a source of infection with type A *C. perfringens*. The infection is characterised by its mild clinical signs, which vary from pasty to more watery diarrhea, and mild growth retardation. The gross lesions attributed to the infection vary from no change to mild inflammation of the small intestine (Klaasen *et al.*, 1999; Songer and Uzal, 2005; Jaggi *et al.*, 2009; Schwartz, 2009). The lesions are therefore quite unlike the classic lesions of haemorrhage and necrosis caused by *C. perfringens* type C. In addition to CPA, CPB2 was identified as a toxin with a still-unclear role in the pathogenesis of pig enteric disease.

The finding that most disease-associated pig strains are *cpb2* positive suggested a possible role in the pathogenesis of *C. perfringens* type A enteritis in pigs (Bueschel *et al.*, 2003; Waters *et al.*, 2003; Jost *et al.*, 2005). It has been reported that over 97% of pig diarrhea isolates produce CPB2 (Waters *et al.*, 2003). There is an apparent correlation between *cpb2*-positive *C. perfringens* type A and diarrhea in piglets (>90% of isolates from diarrheic piglets are *cpb2*-positive) (Schwartz *et al.*, 2009). The role of the toxin in enteric disease is however still uncertain, and it may only be a marker of virulence (Gibert *et al.*, 1997; Bueschel *et al.*, 2003). As discussed below, MLST (Urwin *et al*. 2003) has identified a related cluster to which swine enteritis isolates belong, reinforcing the concept that there is an association between these isolates and swine enteric disease. An alternative explanation is, however, that these isolates may be host-adapted normal flora.

In piglets, diagnosis of neonatal diarrhea associated with *cpb2*-positive *C. perfringens* is usually made by detecting large numbers of the organism in piglets with diarrheal illness and the exclusion of other pathogens (Songer and Uzal, 2005). Development of a CPB2 diagnostic
ELISA should be useful in diagnosing enteric disease associated with these bacteria since the current diagnosis of *C. perfringens* type A enteric disease is unreliable.

**CPB2 and disease**

**Discovery and identification of the *cpb2***

While trying to purify CPB from *C. perfringens* type C (strain CWC245) during the 1980s, Popoff *et al.* obtained a protein of approximately 28 kDa, which is significantly smaller than CPB (~35 kDa). Cloning and sequencing of this putative *cpb* indicated that the “new” protein was not in fact the product of *cpb*. Gibert *et al.* (1997) confirmed that the new protein was, in fact, a new *C. perfringens* toxin. The strain was isolated from a dead piglet with necrotic enteritis. It was thought at first that the newly discovered protein (27.6 kDa) was a product of CPB (34.8 kDa) proteolysis (Gibert *et al.*, 1997), but the CPB2 amino acid sequence showed minimal homology with CPB (15% identity). The protein and the encoding sequence were named CPB2 and *cpb2*, respectively, due to comparable biological effects to CPB (Gibert *et al.*, 1997). A role for CPB2 in enteric disease in animals and humans has still to be convincingly shown.

*cpb2*-positive isolates and, in some cases, CPB2 were found in diarrheic piglets (Klaasen *et al.*, 1999; Bueschel *et al.*, 2003), in horses with typhlocolitis (Herholz *et al.*, 1999; Bacciarini *et al.*, 2003), in diarrheic dogs (Thiede *et al.*, 2001) and in calves with enterotoxemia (Manteca *et al.*, 2002; Bueschel *et al.*, 2003; Lebrun *et al.*, 2007). There are interesting complexities relating to the gene and its expression. For example, *cpb2* can be differently expressed in isolates from different host species (Jost *et al.*, 2005), and in some cases the genes are not intact. However,
porcine *C. perfringens* type A and type C *cpb2*-positive isolates express CPB2 (Jost *et al.*, 2005). In other words, in pig isolates there is a strong correlation between CPB2 phenotype and genotype, but the correlation of phenotype and genotype among non-porcine isolates is less consistent (Jost *et al.*, 2005).

CPB2 is encoded by either a consensus or an atypical gene, and the amino acid identity is 62.3% (Jost *et al.*, 2005; Lebrun *et al.*, 2007). The gene carried mostly by non-porcine isolates is not only usually atypical in sequence but may be non-functional due to frame shift mutations (Bueschel *et al.*, 2003; Jost *et al.*, 2005).

Strains that produce CPA and CPB2 are found in horses suffering from typhlocolitis and other intestinal disorders (Herholz *et al.*, 1999), particularly those treated with the aminoglycoside antibiotic gentamicin. Herholz *et al.* (1999) described the incidence of *cpb2*-positive *C. perfringens* in horses with enteric diseases. They confirmed the presence of these strains in horses with typhlocolitis and other intestinal disorders and their absence from healthy horses or horses with disorders other than intestinal disease (Herholz *et al.*, 1999; van Asten *et al.*, 2008). The presence of CPB2 was demonstrated by immunohistochemistry (Bacciarini *et al.*, 2003). Very interestingly, *cpb2* was out-of-frame and not expressed; however, when the bacteria were grown in the presence of gentamicin, the mRNA was transcribed as an intact and toxic molecule. This transcription was the result of the aminoglycoside antibiotic causing a distortion in the ribosome and “misreading” of the transcript to produce intact protein. Although never proven, this remarkable finding may explain the close association between gentamicin use and typhlocolitis in gentamicin-treated and hospitalized horses (Vilei *et al.*, 2005). To date this is
perhaps the closest proof that CPB2 is involved in enteric disease in animals and humans (Table 1.3).

There is conflicting evidence as to whether or not CPB2 is associated with human enteric diseases. Recently cph2- and cpe-positive C. perfringens isolates have been linked to human antibiotic-associated/sporadic diarrhea (AAD/SD) (Fisher et al., 2005), but the production of CPB2 as an accessory toxin by human cpe-positive C. perfringens type A is still to be confirmed (Fisher et al., 2005; van Asten et al., 2008).

Table 1.3 Is CPB2 a virulence factor in enteric disease?

<table>
<thead>
<tr>
<th>CPB2 significant</th>
<th>Or just a coincidence?</th>
</tr>
</thead>
<tbody>
<tr>
<td>Toxic for CaCo-2 and CHO cells (Gibert et al., 1997)</td>
<td>Consensus toxin more toxic than atypical</td>
</tr>
<tr>
<td>Necrosis in intestinal loops (guinea-pig) (Gibert et al., 1997)</td>
<td></td>
</tr>
<tr>
<td>Lethal (mice) (Fisher et al., 2006)</td>
<td>CPB2 toxigenic strains present among healthy piglets</td>
</tr>
<tr>
<td>Most porcine isolates type A neonatal enteritis are cph2-positive (Bueschel et al., 2003)</td>
<td>Just host adapted normal flora, or linked to another virulence gene?</td>
</tr>
<tr>
<td>cph2 gene is controlled by VirR regulon (VR-RNA) (Ohtani et al., 2003)</td>
<td></td>
</tr>
<tr>
<td>Often found in human antibiotic-associated diarrhea isolates with cpe gene (Fisher et al., 2005; Gibert et al., 1997)</td>
<td>Low toxicity, varies 0.1 - 20.0 µg/ml</td>
</tr>
</tbody>
</table>

There is considerable work to be done to characterize the location of cph2 in isolates of C. perfringens from different animal and human sources. It seems apparent that many are on plasmids, but the plasmids of C. perfringens are complex, since many isolates possess several different large plasmids, carrying different virulence genes. There is potential for homologous recombination events between the plasmids, producing new plasmids. In addition, the plasmids
may be selected for their ability to encode features that promote the survival of the organism in the environment and within the intestine, not simply for virulence characteristics.

The expression of \emph{cpb2} is regulated by the VirR/VirS system, as well as by VR-RNA (Ohtani \textit{et al.}, 2003). This means that the VirR/VirS system regulates not just chromosomal but plasmid-borne virulence genes. Moreover, VirR boxes are present in \emph{cpb2} (e.g. ATCC13124 and SM101), which indicates that the VirR dependent system is both present and important in different \emph{C. perfringens} strains (Okimura \textit{et al.}, 2008). This is further evidence for a contributory role of CPB2 to disease (Table 1.3).

The CPB2 as a protein is unique, even though its function is similar to CPB. The toxin consists of 265 amino acids, the first 30 of which form a signal peptide. Once secreted \textit{in vitro} it can be cleaved into 15 and 13 kDa peptides (the approximate molecular weight is 28 kDa) (Lebrun \textit{et al.}, 2007a). It should be considered a toxin because of its cytotoxic activity for CHO, I407 (Gibert \textit{et al.}, 1997) and CaCo-2 cell lines (Vidal \textit{et al.}, 2009), as well as its lethality in mice (Fisher \textit{et al.}, 2006). It can induce hemorrhagic necrosis in ligated intestinal loops of guinea pigs. However, existing data about the lowest concentration of the toxin that can cause cell rounding vary greatly, with a range from 0.1 $\mu$g/ml to 20.0 $\mu$g/ml (Gibert \textit{et al.}, 1997). The variation in doses required for cell damage may be due to instability of CPB2, or might represent contamination by trace toxic material of different origin (e.g., CPB). In work reported by Gibert \textit{et al.} (1997), cells treated with CPB2 did not show significant disruption of the cytoskeleton, in contrast to other \emph{C. perfringens} toxins (i.e. ITX).
In summary, a possible role of CPB2-producing *C. perfringens* type A in pathogenesis of neonatal diarrhea in piglets, and in other animal species, remains unclear. These organisms have been isolated from piglets with enteric disease (Klaasen *et al.*, 1999; Waters *et al.*, 2003), but also from piglets without diarrhea (Songer and Uzal, 2005; Jaggi *et al.*, 2009). Considering this, no definite conclusions can be drawn whether neonatal piglet diarrhea caused by *C. perfringens* type A can be attributed to CPB2. An immunoassay for CPB2 and its application to diarrheic and healthy piglets might contribute to resolving this question.

**Immunooassays for antigen detection**

Immunooassays have greatly improved the study and diagnosis of infectious disease. Enzyme immunooassays (EIAs) are usually used for detection of soluble antigens and antibodies during immune responses. One of their advantages is the possibility to test easily large numbers of specimens.

Various forms of immunooassays are available, but enzyme-linked immunosorbent assay (ELISA) and sodium dodecyl sulfate polyacrylamide gel electrophoresis (SDS-PAGE) with Western blotting are widely used for antigen detection, mainly because they are fast, simple and highly sensitive and specific. SDS-PAGE with Western blotting technique includes determination of relative molecular weight of polypeptides in characterization of protein antigens. Even though Western blotting is a proven qualitative method, there is a possibility that the denaturation process, a necessary step in the method, might destroy an epitope for which antibodies are specific. This immunoassay is very useful for screening for antibodies that can be used in other systems (e.g. ELISA).
The basis of all EIAs is the same; one of the reagents used in the reaction is conjugated with an enzyme. Appropriate substrate addition results in a colour change that can be detected visually or by spectrophotometer.

**Enzyme-linked immunosorbent assay (ELISA)**

ELISA generally employs antibodies directed against specific proteins. Antibody populations can be very specific and bind only one specific epitope (monoclonal antibodies), or can bind multiple epitopes (polyclonal antibodies) on the protein of interest. In an antigen-detection ELISA, antigen is detected using antibodies labeled with an enzyme; addition of an appropriate substrate, produces a colour change proportional to the concentration of bound enzyme. This method is very useful in detection and quantitation of antigens.

Essentially, ELISA has four standard steps: coating, blocking, antigen-antibody reaction, and colour development. Generally, the two possible formats of ELISA are *competitive* and *non-competitive* (Crowter, 2001). Non-competitive ELISAs include two-stage indirect ELISAs and capture ELISAs (direct or indirect).

**Direct antigen-capture ELISAs**

This type of ELISA is frequently used as a method for detection and quantitation of specific proteins. The principle of this format is based on use of a combination of antibodies (capture and detecting) directed against different antigen epitopes, facilitating specific detection of the antigen of interest. The method makes use of a microtiter plate coated with capture antibodies specific for a particular antigen. After addition of the antigen, captured antigen is
detected by enzyme-labeled (detecting) antibodies. Washing steps are performed after each step in order to remove unbound proteins (antigen or antibodies). After addition of the enzyme substrate, the signal (colour) generated during the reaction is proportional to the amount of bound enzyme-labeled antibody, and is measured using an ELISA reader (spectrophotometer). Antigen-capture ELISAs have been developed for detection and quantitation of different toxins produced by *C. perfringens* (McClane *et al.*, 1984; Wimsat *et al.*, 1986) and *C. difficile* (Lyerly *et al.*, 1983; Deshpande *et al.*, 2010) in the GI tract.

ELISA assays can be relatively rapid, simple, accurate, specific and sensitive. The performance of the test depends, however, upon many variables, such as selection of a suitable antigen for specific antibody production, suitable reagents and buffers and assay conditions, and control of non-specific binding. Optimization of the ELISA is crucial for assay performance, including optimization of sample preparation. It is necessary during evaluation to assess the accuracy, sensitivity and specificity, reproducibility and detection limits of the assay.

There is much to be learned about the role of the CPB2 in enteric disease associated with *C. perfringens*, and about this organism as an enteric pathogen of humans and animals. Previous studies have shown that isolation of *cpb2*-positive *C. perfringens* type A from the intestinal tract and/or intestinal contents of diarrheic animals is not diagnostic. Other tests are necessary for the final diagnosis, particularly those that can determine whether the expressed toxin is present. SDS-PAGE and Western blotting are very useful after the organisms are isolated and grown under *in vitro* conditions. In contrast, antigen-capture ELISAs have the potential to detect and quantitate CPB2 produced *in vivo*. Such an assay is not currently available, however, the current
work addresses the role of CPB2 in enteric disease of piglets through the development of an ELISA that can be used in diagnosis of neonatal porcine enteritis associated with cpb2-positive type A C. perfringens.

Thesis proposal overview

Rationale

Clostridium perfringens is considered one of the major causes of piglet diarrhea. The role in infection of the novel toxin identified by Gibert et al. (1997), named CPB2, is still unclear. In most clinical cases of piglet diarrhea associated with C. perfringens type A, the isolates are cpb2-positive, and these isolates form a genetically homogenous group, which suggest its importance. Regardless, the mechanism of the disease and the role of CPB2 have yet to be elucidated.

Currently, there are opposing views whether CPB2 is involved in enteric disease in neonatal piglets. However, there has been considerable research with regard to CPB2 toxicity and its importance in infection (Gibert et al., 1997; Waters et al., 2003; Fisher et al., 2005; Songer and Uzal, 2005). Considering both views, my hypothesis is that CPB2 has a significant role in the pathogenesis of some enteric disease in neonatal piglets. Therefore, it is necessary to provide more information about this, as a background to the development and assessment of a practical test for detecting the toxin in neonatal piglets with C. perfringens-associated diarrhea.

Development of a diagnostic ELISA will be of use in understanding the role of CPB2 in enteric disease in piglets and potentially also in other animals, including humans.
Objectives

The specific objectives are:

1. Development of an antigen capture ELISA for *C. perfringens* CPB2.
2. Evaluation of a limit of detection and specificity for the consensus protein by ELISA using CPB2 positive *C. perfringens* from diarrheic and healthy piglets (clinical specimens).
3. To confirm the presence and expression of the consensus and atypical types of *cpb2* in *C. perfringens* isolated from selected animal species, and therefore of the potential applicability of the ELISA to species other than pigs.

Approach

A strain collection of *C. perfringens* will be made and isolates will be examined for consensus and atypical forms *cpb2*. The focus of the collection will be on the consensus CPB2 from pig isolates. Proteins will be obtained using His-tagged consensus CPB2 protein (plasmid pJGS197) and His-tagged atypical CPB2 protein (plasmid pJGS659) (obtained from Dr Songer). Consensus recombinant CPB2 (rCPB2) will be sent for preparation of polyclonal antiserum. The monoclonal antibody will be developed against recombinant rather than native protein. Both polyclonal and monoclonal antibody (Songer 9E4B) will be evaluated by Western blot for reaction against consensus and atypical CPB2.

Capture ELISA will be the format for antigen detection. Polyclonal antibodies will be used to coat ELISA plates and to capture the CPB2, and the monoclonal antibody Songer 9E4B will be used for detection of the captured protein. When monoclonal antibody is used for detection, the system is more specific for the protein of interest, which increases the possibility
of detecting only the targeted antigen. Most capture ELISAs use either two monoclonal antibodies that detect different epitopes, or polyclonal antibodies for capture and a monoclonal antibody for detection.

The limitation of the system is that antigens must have at least two epitopes, since both the capture and the detection antibodies need to bind. Topography of epitopes is also very important, which is the reason why recombinant proteins may not always be suitable for antibody production. Purified native protein will be used for assessment of the prepared ELISA.

**Expected outcome**

This research will lead to better understanding of the importance of CPB2 in the pathogenesis of the infection caused by CPB2-producing *C. perfringens* type A. The research will also improve understanding of the distribution and expression of the two forms of *cpb2* in *C. perfringens* type A from different animal species, and the potential application of the ELISA in animals other than neonatal piglets.
Chapter 2

Development of an antigen-capture ELISA for

Clostridium perfringens beta2 (CPB2) toxin in the

neonatal piglet intestine

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Investigation.
Abstract

Diagnosis of *Clostridium perfringens* type A as a cause of porcine neonatal diarrhea has been made mainly by exclusion of other pathogens. Many studies have shown that the CPB2 toxin gene (*cpb2*) is highly prevalent in isolates from diarrheic piglets. An ELISA was developed for detection and quantitation of CPB2 in neonatal piglet intestinal contents. Polystyrene plates were coated with polyclonal capture antibodies prepared against consensus recombinant CPB2 (rCPB2). The ELISA was developed using consensus rCPB2, atypical rCPB2, purified consensus native CPB2 (nCPB2), and field samples of neonatal porcine intestinal contents. Captured antigen was detected using a horseradish peroxidase-labelled (HRP) monoclonal antibody. The limit of detection of the ELISA for consensus CPB2 was between 2.0 and 3.5 ng/ml. The ELISA detected atypical rCPB2 only weakly. Optical density (OD) was protein concentration dependent. The test confirmed differences between consensus and atypical rCPB2, with similar results obtained when testing consensus rCPB2 and native CPB2 (nCPB2). Results obtained from intestinal content samples, particularly from the small intestine, were highly inconsistent, and suggested variable protease activity. Addition of protease inhibitors partially prevented degradation of the toxin; however, sample processing at 4°C, at a lower pH (citrate buffer with 5% of BSA, pH 6.1) and “cold incubation” of applied antigens, abolished protease activity. The recombinant toxin was preserved in spiked intestinal samples by freezing at -70°C, suggesting that necropsy samples could be stored for periodic testing. With appropriate sample preparation, antigen-capture ELISA can detect CPB2 in the intestinal contents of neonatal piglets.
Introduction

The worldwide distribution of *Clostridium perfringens* and its diverse arsenal of toxins and enzymes give it the ability to cause disease in several different animal species including humans (Songer, 1996). *Clostridium perfringens* is currently classified into five toxinotypes (A-E) (Petit *et al*., 1999), depending on its production of four mouse-lethal toxins; different types are linked with specific clinical diseases of domestic animal species. As discussed below, type A *C. perfringens* is considered by some to be one of the most important enteric pathogens in neonatal piglets (Klaasen *et al*., 1999; Bueschel *et al*., 2003).

While purifying CPB from type C *C. perfringens*, Gibert *et al.* (1997) isolated a protein of approximately 28 kDa. It was originally thought to be a cleavage product of the beta toxin and, because it was toxic for both CHO cells and for mice, it was named CPB2. Later the amino acid sequence was found to be distinct from that of CPB (Gibert *et al*., 1997). The toxin is lethal for mice (Gibert *et al*., 1997; Vilei *et al*., 2005) and for various cell lines (Gibert *et al*., 1997; Fisher *et al*., 2006), and causes haemorrhagic necrosis when injected into intestinal loops of guinea-pigs (Jolyvet-Reynaud *et al*., 1986). Subsequently, it was discovered that there are two forms of the CPB2 toxin, the consensus form first described by Gibert *et al.* (1997) and a less toxic “atypical” variant described by Jost *et al.* (2005). Despite extensive studies of the distribution of *cpb2* in isolates of *C. perfringens* from numerous animal sources, no general conclusions have been drawn as to the role of CPB2 in enteric diseases of animals or humans (van Asten *et al*., 2008).
In swine, in contrast to many other species, \textit{cpb2} is almost invariably the consensus type and is almost invariably expressed (Bueschel \textit{et al.}, 2003; Waters \textit{et al.}, 2003). In addition, and in contrast to isolates from the majority of other species, MLST has shown that porcine \textit{C. perfringens} type A strains belong to a common clonal complex (Jost \textit{et al.}, 2006a). Diarrheal disease isolates almost always carry \textit{cpb2}, in contrast to a smaller proportion of \textit{cpb2}-positive isolates from healthy pigs (Bueschel \textit{et al.}, 2003; Garmory \textit{et al.}, 2000; Waters \textit{et al.}, 2003). However, there is no convincing evidence to date that CPB2 itself is responsible for \textit{C. perfringens} type A diarrhea in neonatal piglets (Songer and Uzal, 2005). It is possible that \textit{cpb2} is a marker of, rather than directly involved in, virulence (Jost \textit{et al.}, 2006b). If type A \textit{C. perfringens} causes diarrhea in neonatal piglets, as is widely believed (van Asten \textit{et al.}, 2008), most aspects of the disease are poorly understood and the diagnosis is equivocal, made only by exclusion of other enteric pathogens in combination with the detection of large numbers of \textit{cpb2}-positive \textit{C. perfringens} in the small intestine.

As an approach to improving the diagnosis of \textit{C. perfringens} type A diarrhea of neonatal piglets, an antigen-capture ELISA was developed for detection of CPB2 in the neonatal pig intestine; its characteristics are described here.

\textbf{Material and Methods}

\textbf{Purification of recombinant and native CPB2}

Purification of His-tagged consensus and atypical rCPB2 protein from \textit{Escherichia coli} DH5\textalpha{} (containing plasmids pJGS197 and pJGS659, respectively) was performed as suggested by the manufacturer of the columns. Bacterial cells were grown in Luria-Bertani (LB) liquid
medium (BD Difco, Mississauga, ON, Canada). A 100 ml volume of the medium, supplemented with ampicillin (100 µg/ml), was inoculated with an overnight culture and incubated at 37°C until an optical density (OD) of 0.6 at 600 nm was reached. Expression of rCPB2 was induced by addition of 2.5 mM isopropyl-β-D-thiogalactopyranoside (IPTG), and the incubation was continued for an additional 3 hr at 37°C. The cells were harvested by centrifugation at 15,000 x g for 20 min at 4°C. The pellet was resuspended in 5 ml lysis buffer (20 mM Tris-HCl, 100 mM NaCl, pH 8.0), with the addition of protease inhibitor mix (GE Healthcare, Bio-Sciences Corp, Piscataway, NJ, USA) and lysozyme (100 mg/ml) (each 1% of the total volume) and incubated on ice for 2 hr. After incubation and sonication (60 cycles, 30 sec, 7 repetitions; after each repetition the liquid was cooled down for 2 min on ice), the cell extract was centrifuged at 12,100 x g for 20 min at 4°C. The supernatant (10 ml) was mixed with a nickel-nitrilotriacetic acid (Ni-NTA) agarose matrix (2 ml) and incubated at 4°C with shaking for 30 min. The mixture was poured into a column (Qiagen Inc., Mississauga, ON, Canada) and washed with lysis buffer containing 10 mM imidazole. Elution was performed by use of buffer containing increasing concentrations of imidazole (10, 20, 50, 100, and 250 mM).

Purification of consensus nCPB2 from a porcine isolate (P18) was performed using anion exchange, followed by size exclusion chromatography. The method was modified from Gibert et al. (1997) and Fisher et al. (2005). Porcine isolate P18 was grown anaerobically in TGY liquid medium (3% tryptic soy broth, 2% D-glucose, 1% yeast extract [BD Difco] and 0.1% L-cysteine [Sigma Aldrich, Oakville, ON, Canada]) overnight at 37°C. After centrifugation at 10,000 x g for 20 min at 4°C, the supernatant was incubated at 60% ammonium sulphate saturation under constant stirring overnight at 4°C. The solution was centrifuged (10,000 x g for 20 min at 4°C),
and the pellet was suspended in 10 mM Tris, pH 7.5 before dialysis overnight at 4°C against 10 mM Tris, pH 7.5. After dialysis, the solution was filtered through a 0.22 µm filter and loaded on a DEAE-650M column (Tosoh USA Inc., Grove City, OH, USA), pre-equilibrated in 10 mM Tris, pH 7.5. The column was washed with 180 ml 10 mM Tris, pH 7.5 and bound protein was eluted with a 0-100% 1 M NaCl gradient in 10 mM Tris, pH 7.5. All the phases of chromatography were performed at 4°C. Fractions containing CPB2 were pooled and concentrated using centrifugal filter units. Ion exchange purified and concentrated CPB2 was subjected to size exclusion chromatography at 4°C, using a Superose 12 10/300 GL column (GE Healthcare) equilibrated with PBS pH 7.4. Five molecular weight standards (thyroglobulin, globulin, ovalbumin, myoglobin, and vitamin B12) were used for calibration. Fractions showing a single band upon Coomassie blue staining were pooled and confirmed as being CPB2 by Western blotting (using monoclonal antibodies 9E4B).

Purification of consensus nCPB2 was confirmed by SDS-PAGE staining with Coomassie Brilliant Blue R250 (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada) staining and by Western blot (using monoclonal antibody Songer 9E4B). Purified protein was concentrated by use of centrifugal filter units, 10,000 Da molecular weight cut-off (Amicon Ultracentrifugal filter units, Millipore Inc., Billerica, MA, USA). Protein quantitation was performed using bovine serum albumin as the standard (BCA Protein Assay Kit Product, Pierce, Fisher Scientific Ltd., Ottawa, ON, Canada).
Preparation of polyclonal and monoclonal antibodies

Polyclonal rabbit antiserum was raised against consensus rCPB2 protein. Mouse monoclonal antibody Songer 9E4B (Jost et al., 2005) had been prepared using consensus rCPB2 as antigen. Immunoglobulins (polyclonal antibodies) were purified using protein A columns (GenScript, Piscataway, NJ, USA). Antibodies were aliquoted in small volumes and stored at –70°C until used.

Development and optimization of the antigen-capture ELISA

Optimization steps in the process of development of the antigen-capture ELISA included assessment of the optimal concentrations of polyclonal capture antibody (rabbit anti-rCPB2 pAb), of rCPB2, of HRP-labelled monoclonal detection antibody (mAb-HRP), optimization of incubation conditions, selection of blocking buffers, and selection of coating buffers (details not shown).

For the final ELISA, 96-well plates (MaxiSorp, Nunc, Roskilde, Denmark) were coated with 100 µl/well of polyclonal capture antibody (diluted 1:2500 in carbonate-bicarbonate buffer pH 9.6) for 2 hr at 37°C followed by overnight incubation at 4°C. After washing the plate twice with wash buffer (PBS, 0.05% Tween 20, 0.5% fish skin gelatin [Norland HiPure Liquid Gelatin, Norland Products Inc, Cranbury, NJ, USA]) and once using phosphate-buffered saline, pH 7.4 (PBS), the coated plate was blocked using blocking buffer (PBS, 0.05% Tween 20, 2% BSA) for 2 hr at 37°C. After washing 3 times with wash buffer, 100 µl/well of purified consensus rCPB2 (two-fold dilution series starting from 109.9 to 3.43 ng/ml in wash buffer) or processed intestinal contents samples from neonatal piglets (described below), were added to the plate (in triplicate,
or in duplicate when the sample volume was insufficient). The addition of antigen was performed on ice. Washing buffer with no antigen was used as a negative control. After incubation at 4°C overnight, followed by 5 washings on ice with cold washing buffer, 100 μl/well of enzyme-labeled detecting antibody (diluted 1:5000 in wash buffer) was applied, and the plate was incubated for 1 hr at room temperature. The plate was then washed 3 times with wash buffer and 100 μl/well of the chromogenic substrate 2,2'-azino-di-[3-ethylbenzthiazoline sulfonate (6)] diammonium salt (ABTS) (Roche Diagnostics, Laval, QC, Canada) was added. After 1 hr further incubation at room temperature, the reaction was stopped using 0.5% SDS (50 μl/well) and the OD measured at 405 and 490 nm in an ELISA reader (spectrophotometer) (BioTek Instruments Inc., Power Wave XS, Winooski, VT, USA). The mean dual wave OD values (OD at 490 nm subtracted from OD 405) of the replicates of each sample were used to calculate the ratios of the positive and negative samples. Two signal-to-noise ratios were calculated. One compared the OD values between wells with the antigen (intestinal sample or rCPB2) and wells with no antigen added (both wells coated with pAb); the other compared the OD values between the pAb-coated and uncoated wells (antigen was applied in both wells). To determine the optimal concentrations for capture and detection antibodies, the assay was performed with different concentrations of each. Concentrations selected were those that gave the best ratios comparing OD values.

Serial two-fold dilutions of rCPB2 standards, starting from 110 ng/ml, were included in each plate to generate a standard curve.
Source of intestinal samples for diagnostic development of the ELISA

Piglets from 10 swine farms with a history of suspected *C. perfringens* type A neonatal diarrhea were submitted for detailed diagnostic examination. Piglets from each of the farms were less than 3 days of age; at least three piglets (2 diarrheic and 1 healthy) from the same farm were submitted live to the diagnostic laboratory. Piglets were euthanized and immediately subjected to a detailed necropsy examination. Contents of the small and large intestine were collected on ice within 30 min of death and submitted to the laboratory within 1 hr. All subsequent intestinal content processing was done on ice, and centrifugation at 4°C. Briefly, each sample was centrifuged at 4°C at 13,000 RPM for 60 min. If the intestinal contents were of a thick consistency, they were diluted in sterile cold PBS and the dilution was recorded. These samples were vortexed vigorously before centrifugation. After supernatant was separated, it was filtered (0.45 µm pore diameter, low protein binding, Fisherbrand syringe filters, Fisher Scientific Ltd., Ottawa, ON, Canada), and was then ready for application onto the ELISA plate.

Effect of intestinal content and feces on CPB2 detection

*Clostridium perfringens* rCPB2 and in some cases concentrated supernatant of isolate P18 were used as a source of consensus CPB2 for spiking piglet fecal and intestinal content samples with undetectable *C. perfringens* counts (< 100 CFU/ml). In addition, small intestinal and colonic content samples, obtained at necropsy of the piglets, were spiked using the same procedure. Briefly, 3 ml of sterile cold PBS was added to 3 g of feces or intestinal content, mixed thoroughly and stored on ice. A volume of 3 ml of rCPB2 (1:250 dilution; starting concentration 1099 µg/ml) was added to the sample, vortexed vigorously for 5 min, and then stored on ice. The spiked sample was divided in four aliquots (F-0, F-4, F-8 and F-overnight). F-0 and negative
control (non-spiked feces) were processed and tested immediately, whereas F-4, F-8 and F-overnight were processed and tested after incubation at room temperature for 4 hr, 8 hr, and overnight, respectively.

To examine the presence of protease activity in the small intestinal contents, and how this could be overcome, three approaches were used. The first examined the effect of protease inhibitors. Two inhibitors, amidinophenyl-methanesulfonyl-fluoride (APMSF, serine protease inhibitor, Sigma Aldrich) and a protease inhibitor mix (GE Healthcare) were added to the samples, in the concentrations recommended by the manufacturer, before processing for ELISA testing. After protease inhibitors were added, samples were vortexed and the processing was continued according to the above described protocol. The second approach involved processing at low temperature. For this, all the steps of processing of the intestinal content, plate washing and application of the samples onto the plate were performed on ice. This was followed by incubation at 4°C overnight. The third approach examined pH effects. Two sets of four different pH (PBS pH 7.4; citrate buffers pH 4.0; pH 5.0; pH 6.0) buffers were used for sample preparation and sample spiking using rCPB2 (13.75 ng/ml). Bovine serum albumin (5%) and fetal calf serum (FCS, 50%) were added to each of the buffers in set one and set two, respectively.

**Effect of freezing feces and intestinal content on CPB2 recovery**

Spiked feces and necropsy intestinal samples previously tested immediately upon receiving and after spiking, were frozen at -70°C. After thawing, samples were assayed by ELISA following the protocol described above, and results compared to those of the freshly
Results

Initial evaluation of the ELISA using recombinant consensus CPB2

Initial experiments to optimize the ELISA used rCPB2, aimed at a desired ELISA sensitivity of 1-5 ng/ml. The lowest concentration of captured CPB2 detected was between 2.0 and 3.5 ng/ml. The data demonstrated that incubation of pAb, diluted 1:2500, for 2 hr at 37°C followed by overnight at 4°C was optimal for coating the wells, and that a 1:5000 dilution of mAb-HRP incubated for 1 hr at room temperature was optimal for detecting captured antigen. Figure 2.1 shows the means of triplicate OD values obtained when 0.43-220 ng/ml dilutions of the consensus rCPB2 antigen were applied to the coated plate and incubated for 2 hr at 37°C.

Comparison of recombinant versus native CPB2

The concentration of native purified consensus CPB2 was determined as approximately 1475 µg/ml. When consensus nCPB2 was used as antigen instead of consensus rCPB2, the ELISA results were similar. Figure 2.2 shows the minimal difference in assay performance between the two antigens.

In contrast, when atypical rCPB2 was used as antigen, the limit of detection was higher than for consensus protein. OD readings of the signal that resulted from the capture of atypical rCPB2 (when applied at the same concentration) were approximately 90% lower than for consensus rCPB2 (Appendix Figure 2).
Effect of intestinal content and feces on CPB2 recovery

When tested by ELISA, the supernatant from cultures of strain P18 containing consensus CPB2 showed comparable reaction kinetics to those of rCPB2 (Appendix Figure 3). Spiking piglet feces or piglet colonic or small intestinal contents with the native CPB2, using the concentrated supernatant of strain P18, showed that the amount of toxin that could be recovered decreased with storage at room temperature over a short time (Fig. 2.3). The loss of the toxin in colonic or fecal contents was about 30-50% after 4 hr at room temperature. If the added CPB2 was detected, as shown for fecal and colonic samples in Figure 2.3, the dose-response curve was similar to the standard curve; however the intensity of the signal was lower, indicating partial destruction of the toxin. Strikingly, the small intestinal contents from some piglets eliminated the toxin almost immediately, and also caused very high noise in control wells. The ODs in some cases were higher in uncoated than in coated wells (Fig. 2.3). This would be consistent with degradation of capture antibody and/or blocking agent, which probably led to non-specific binding to the ELISA plate by the labelled detection mAb.

Effect of freezing feces and intestinal content on CPB2 recovery

Freezing (-70°C) had no effect on CPB2 recovery from spiked samples (Appendix Figure 1.).

Detection of CPB2 in clinical samples

Initial ELISA testing of intestinal content samples, from both diarrheic and healthy piglets, showed highly inconsistent results. Some samples apparently contained highly active proteases, which resulted in high OD readings in the control wells lacking capture antibodies.
(noise). These results suggested that the presence of some other compounds (likely proteases) in the samples caused non-specific reactions in negative control wells by degrading the blocking agent. Mixing of small intestinal contents from some animals with CPB2 resulted in almost immediate loss of the toxin (Fig. 2.3). Of 68 intestinal content samples from neonatal piglets that originated from farms with a history of possible type A C. perfringens diarrhea, 41 samples showed acceptable results (13 positive and 28 negative samples), suggesting that the assay as developed could be applied in diagnosis in about 60% of cases. As described below, the acceptable OD value for the “noise” was determined to be 0.120. However, 16 samples gave false-positive results (identified by high ODs in the capture-antibody-free control wells, with concurrent high ODs in the capture-antibody-coated wells) due to the activity of interfering factors present in the intestinal contents (Fig. 2.4).

Figure 2.5 presents a summary of the “noise” in the capture antibody-free well of the ELISA for colonic and small intestinal samples from diarrheic and healthy piglets, processed before studies involving the addition of protease inhibitors. This was used to define a value above which the noise in the control (capture antibody-free) wells was judged unacceptable. Figure 2.5 shows that most of the sample values in the control wells ranged between 0.051 and 0.150 OD. An OD 0.120 was judged to be the “cut-off” value for noise, above which the test results would be considered unreliable. This value was determined by calculation and addition of 3 standard deviation of the OD obtained by the signal in wells where PBS was added instead of the test antigen (intestinal contents).
The addition of protease inhibitors reduced the noise observed in some wells without capture pAb, confirming that there was protease activity in the samples with noise in their control wells. Inclusion of protease inhibitors reduced interference by proteases, but the effect appeared to depend on the amount of proteolytic activity present. Only 4 of 15 samples showed improvement of the test performance, but these protease-inhibitor induced improvements in control wells often resulted in lower signal OD by wells with capture antibody, which did not result in overall signal-to-noise ratio improvements. The protease activity in the small intestinal content during ELISA testing could also be partially controlled by lowering the temperature to 4°C during the initial sample incubation period. Twenty-one samples previously incubated at 37°C for 2 hr were retested using initial incubation at 4°C overnight. Of these 21 samples, 20 had improved signal-to-noise ratio when coated and “uncoated” (control) wells were compared (data not shown). The results showed that low temperature had a beneficial effect on the overall test performance, presumably by lowering activity of the proteases present. An example of the effect of incubation in the cold on a small intestinal sample with proteolytic activity is shown in Figure 2.6.

As another approach to controlling and decreasing the activity of proteases, the effect of pH on test performance was evaluated and, in addition, protein saturation (high protein concentration in a diluent can “serve” as a substrate for active protease) of diluents used in intestinal sample processing was assessed. A sample that contained reactive proteases (Fig. 2.3) was used to determine if low pH and protein saturation of the diluents improved the reaction. Table 2.1 shows the results of rCPB2 recovery after the small intestinal content spiking using eight different diluents. Use of citrate buffer with 5% BSA (pH 6.1) markedly increased recovery
of rCPB2 measured by ELISA compared to the other diluents used. Overall, rCPB2 recovery was considerably higher when diluents with 5% BSA were used, compared to diluents with 50% FCS (Table 2.1). These results indicated that, by adjusting the conditions and saturating the diluents, protease activity could be overcome and their activity controlled (Fig. 2.7).

Discussion

Design of the ELISA initially involved optimization using consensus recombinant CPB2 and showed that, with plates precoated overnight at 4°C, the assay could be finished during one usual working day and detect as little as 2-3 ng/ml of purified rCPB2 (Fig. 2.1). The limit of antigen detection of the ELISA was almost identical for the purified consensus native CPB2 protein (Fig. 2.2), but was far lower for the atypical rCPB2 protein, probably because the mAb does not detect the relevant epitope of the atypical protein. Consensus and the atypical CPB2 proteins have only 62.3% amino acid identity (Jost et al., 2005). The most obvious application of this ELISA is in the diagnosis of type A-associated enteric disease in neonatal swine, since C. perfringens isolated from these piglets are commonly consensus cpb2-positive and this gene is commonly expressed, in contrast to isolates from the intestine of other species where the cpb2 gene is often both atypical and apparently not expressed (Jost et al., 2005).

As others have found, optimizing an antigen-capture ELISA by using intestinal content and fecal samples can involve some difficulties because intestinal or fecal extracts may affect toxin recovery, either by degradation through proteases (Viscidi et al., 1984; Hanavich et al., 1985) or because of the necessity for dilution (Bartholomew et al., 1985; Wimsatt et al., 1986). However, dilution was not an issue since most of the intestinal contents used for the development
of the current assay were liquid, and so were applied undiluted or minimally diluted. Optimization results showed that the toxin could be recovered from feces or intestinal contents (Fig. 2.3), though with some loss after storage at room temperature for a few hours, and that (with exceptions related to protease activity in the small intestine) the ELISA was able to quantitate CPB2 toxin in the intestinal content extracts.

The major problem identified in development of the ELISA was the presence of protease activity in the small intestinal content of some neonatal piglets (Fig. 2.3). The samples that displayed excessive desorption of the wells, presumably as a result of protease activity, caused a high proportion of nonspecific reactivity. The use of protease inhibitors showed that some intestinal contents had protease activity (likely pancreas-derived trypsin) responsible for “uncoating” antibody coated wells and/or degrading the blocking agent (BSA) from the polystyrene well. In the absence of coating or blocking, it is possible that mAb-HRP bound to uncoated wells and gave false-positive signals (“noise”). A “cut-off” of acceptable and unacceptable noise was developed based on the OD of control wells incubated with buffer rather than intestinal samples. Applied to intestinal samples from piglets at necropsy it showed that, in the absence of protease inhibition, about 40% of samples gave unacceptable noise (Figs. 2.4, 2.5).

Use of protease inhibitors, diluents of different pH saturated with 5% BSA or 50% FCS, and low temperature processing, were evaluated to address the proteolysis/desorption problem. Lowering the antigen capture temperature largely, but not completely, eliminated protease activity (Fig. 2.6), with the variability apparently dependent on the amount of proteolytic activity.
present. By lowering the test antigen capture temperature from 37°C (2 hr) to 4°C (overnight) the incubation time was prolonged so that the ELISA testing time became 2 days (with precoated plates). An alternative approach assessed was inclusion of protease inhibitors in the assay, although this was found to be expensive and only partially effective. An approach considered was the use of heat inactivation of proteases, as shown by Hanvanich et al. (1985) for detection of *Clostridium difficile* toxin A in feces. However, since the CPB2 toxin is very unstable (Gibert et al., 1997) this would likely have had a destructive effect on the toxin itself, so this approach was not examined.

The most effective way of abolishing protease activity was the use of a low pH buffer (6.1) combined with high protein concentration in the buffer and with processing at 4°C. Saturation of four different pH buffers showed that 5% BSA gave higher and more consistent recovery of the rCPB2 from spiked small intestinal content than 50% FCS. This approach was developed by Viscidi et al. (1984) for prevention of desorption by intestinal proteases during the detection of *C. difficile* toxin A in feces (Viscidi et al., 1984; Hanvanich et al., 1985). These studies compared several different sample diluents to determine one with the best effect on the ELISA performance. Their results showed that 50% FCS and citrate buffer containing 5% BSA pH 4.7 significantly reduced desorption of the plates (protease activity) and improved recovery of the toxin A from the samples, compared to the other tested diluents (including citrate buffer alone [pH 4.7], 5% BSA alone, phenylmethylsulfonyl fluoride (PMSF), and soybean trypsin inhibitor). Fetal calf serum contains various protease inhibitors and high concentration of proteins; in addition to inhibition, it may provide substrate in excess for protease activity. Since the optimal pH for protease activity in the intestinal or fecal content is alkaline or neutral, an acid
pH inhibits the activity of intestinal proteases.

One of the difficulties in developing a CPB2 capture ELISA was the absence of a “gold standard”. *Clostridium perfringens* is considered to be a component of a normal microflora (Songer and Uzal, 2005) and the isolation of the organism does not necessarily mean that it is a cause of the disease. Without a gold standard, a receiver operating characteristic (ROC) curve could not be used in order to compare the diagnostic performance of the “new” test (CPB2 ELISA) with a well-established laboratory or diagnostic test (gold standard). An acceptable OD for noise was therefore determined from the control, PBS-treated, “uncoated” wells.

In conclusion, an antigen-capture ELISA was developed to detect the *C. perfringens* consensus CPB2 toxin in feces and in the intestinal contents of piglets. The ELISA was initially confounded by the presence of proteases in the intestinal contents but this effect was overcome. Control wells lacking capture antibodies allow detection of proteolytic activity that in some cases cannot be overcome by the developed method, and identify the samples to which the ELISA cannot be applied. Detection of CPB2 in the intestinal content samples using this ELISA may be valuable in diagnosis of the role of consensus *cpb2*-associated diarrheal illness in neonatal swine. The advantage of an ELISA over an alternative approach, such as real-time PCR quantitation of *cpb2* in the intestine, is that the ELISA detects expression of the protein, not simply the presence of the DNA or mRNA. Further work is required to apply the ELISA to assess the role of type A *C. perfringens* in enteric disease in neonatal swine.
Table 2.1 Recovery of rCPB2 in the small intestinal content with high protease activity by using eight different diluents.

<table>
<thead>
<tr>
<th>Buffers/pH</th>
<th>Recovery (%)</th>
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<tbody>
<tr>
<td></td>
<td>5% BSA</td>
<td>50% FCS</td>
<td></td>
</tr>
<tr>
<td>PBS (7.1)</td>
<td>48.8</td>
<td>33.8</td>
<td></td>
</tr>
<tr>
<td>Citrate buffer (6.1)</td>
<td>85.1</td>
<td>18.3</td>
<td></td>
</tr>
<tr>
<td>Citrate buffer (5.1)</td>
<td>75.5</td>
<td>36.0</td>
<td></td>
</tr>
<tr>
<td>Citrate buffer (4.5)</td>
<td>62.2</td>
<td>25.0</td>
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Figure 2.1 Standard curve for *Clostridium perfringens* recombinant consensus CPB2 determination by capture ELISA test.

The ELISA detected as little as 2-3.5 ng/ml consensus CPB2 using a preliminary protocol (antigen incubation phase at 37°C for 2 h).
Figure 2.2 ELISA detection of recombinant consensus versus native (consensus) CPB2.

The ELISA detected native consensus CPB2 as well as the recombinant protein using a preliminary protocol (antigen incubation phase at 37°C for 2 h).
Figure 2.3 CPB2 detection in spiked samples.

Feces, and colon and small intestinal contents spiked with the P18 isolate concentrated supernatant were tested immediately (T=0) and after 4 hr (T=4h) at room temperature using a preliminary protocol (antigen incubation phase at 37°C for 2 h). Storage for 4 hr in feces, colonic or small intestinal content reduced CPB2 detection. For some samples of small intestinal contents, mixing of toxin with the contents resulted in almost immediate destruction of CPB2, likely attributable to the presence of proteases in the samples.
Using a preliminary protocol (antigen incubation at 37°C for 2 h), the tested samples fell into four groups depending on OD values of the signal and the noise (three standard deviations were added to the OD values of the noise in the wells where no antigen was added (the acceptable noise was therefore ≤ OD 0.120)): 1. Samples with high signal but unacceptable noise in the control wells; 2. Positive samples (no noise in control wells); 3. Samples with low signal but unacceptable high noise in the control wells; 4. Negative samples (no noise in control wells).
Figure 2.5 OD range of "noise" in the capture antibody-free control wells.

Colonic and small intestinal samples from 68 diarrheic and healthy piglets. Most of the samples clustered between 0.051 and 0.150 OD values.
Figure 2.6 Effect of the temperature on protease activity.

Examples of samples with active proteases tested using a preliminary protocol with the exception of the temperature and the duration of the antigen incubation phase (samples tested in parallel 4°C and at room temperature over night). “Cold” incubated samples showed higher OD readings.
Figure 2.7 The small intestinal content sample spiked and processed under different conditions (temperature) using different buffers (pH).
Chapter 3

Distribution and expression of the *cpb2* gene in *Clostridium perfringens* isolates of different domestic animal origin

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Abstract

The high prevalence of *cpb2* and expression of CPB2 in isolates from diarrheic piglets suggests that it may be an important marker of virulence, but the prevalence and expression of the “consensus” and the “atypical” *cpb2* genes in isolates from other domestic animal species are less well characterized. This study examined the prevalence and expression of these genes in *C. perfringens* isolates from cattle, chickens, dogs, goats, horses and sheep using PCR, SDS-PAGE and Western blotting. Almost all porcine isolates (12/14) carried and expressed the consensus form of *cpb2* but, when the gene was present in non-porcine isolates was usually the atypical form (39/108 versus 9/108 consensus). The consensus form of *cpb2* was also detected in a small number of non-porcine isolates, being most common in horse isolates (5/12). Western blotting assays confirmed CPB2 expression by almost all the consensus *cpb2*-positive isolates, regardless of source. Expression in atypical *cpb2*-positive isolates was more variable, in 30 of 39 isolates (76%), and was most consistent in isolates from chickens (10/10). Expression of the atypical gene was considerably more frequent than reported previously by others.
**Introduction**

*Clostridium perfringens* is a Gram-positive, spore-forming, anaerobic pathogen, widely distributed in and isolated from soil, water, and the intestinal tract of animals and humans (Songer and Uzal, 2005). *Clostridium perfringens* isolates are currently divided into five toxinotypes, depending on the ability to produce different combinations of four major toxins (McClane *et al.*, 2000). This typing scheme is inadequate to describe the diseases associated with this bacterium, particularly for type A strains. For example, CPE is associated with enteric disease in humans (Lindstrom *et al.*, 2011) and other species, NetB is associated with necrotic enteritis of chickens (Keyburn *et al.*, 2008), and TpeL has also been associated with some chicken necrotic enteritis isolates (Chalmers *et al.*, 2008).

*Clostridium perfringens* enteric infection has been associated with necrotizing enteritis in animals and humans, including enterotoxemia in cattle, goats and sheep, and food poisoning in humans (Rood, 1998; Manteca *et al.*, 2002; Bueschel *et al.*, 2003; Schotte *et al.*, 2004; Lebrun *et al.*, 2007). The role of CPB, ETX and ITX are well described in the pathogenesis of enteric diseases (Soger, 1996), but the role of CPA and of CPB2 is still unclear.

CPB2 was first identified by Gibert *et al.* in 1997 in a type C isolate from a piglet that had died of necrotic enteritis (Gibert *et al.*, 1997). The gene (*cpb2*) has been shown to be plasmid-borne and has been detected in isolates of all the *C. perfringens* toxinotypes recovered from various animal species, including humans (Gibert *et al.*, 1997; Jost *et al.*, 2005; Gurjar *et al.*, 2010). In isolates from humans with AAD or SD *cpb2* may be found in association with *cpe* on plasmid. Antibiotic-associated diarrhea or SD isolates carried the two genes on the same
plasmid when IS1151 sequences were present downstream of cpe, but on different plasmids when IS1471-like sequences were found downstream of cpe (Fisher et al., 2005). One explanation for the wide distribution of the cphb2 gene is that it is carried on the conjugative plasmids of C. perfringens (Fisher et al., 2005). The CPB2 protein consists of a 265 amino acid polypeptide, which after secretion loses 30 amino acids giving it a molecular mass of 27.6 kDa. It is unstable, being susceptible to proteolytic cleavage (Gibert et al., 1997). Early studies of CPB2 toxin showed that it was lethal for mice and toxic for CHO and I407 cells (Lebrun et al., 2007a). There is only 15% identity between CPB and CPB2 amino acid sequences and the two toxins are not immunologically related (Gibert et al., 1997). Since Caco-2 (human colon carcinoma) cells are sensitive to CPB2, it was suggested that the toxin might play a role in human intestinal disease (Gibert et al., 1997). To date no structure-function analysis has been conducted; however Smedley et al. (2004) suggested that it is most likely a pore-forming toxin.

Subsequent to the description of cphb2, a second form of the gene was identified (Jost et al., 2005; Fisher et al., 2005). To distinguish these two sequences, the original gene was identified as the “consensus” gene (Gibert et al., 1997), whereas the newer gene is called the “atypical” gene (Jost et al., 2005). The atypical gene has 70% DNA identity to the consensus gene sequence but, with minor exceptions, 99% identity between all atypical genes. About 44% of atypical genes examined (4 of 9) isolates had a frameshift mutation that resulted in a loss of expression (Jost et al., 2005). The role of the CPB2 in enteric disease in animals and humans has not been confirmed, but the most convincing evidence for a possible role is in enteric diseases of neonatal piglets (Klaasen et al., 1999; Waters et al., 2003), in gentamicin-associated diarrhea in horses (Herholz et al., 1999; Bacciarini et al., 2003; Vilei et al., 2005) and possibly enteric diseases of humans (Fisher et al., 2005; Lindstrom et al., 2011). Porcine cphb2-positive C. perfringens
isolates almost invariably carry the consensus gene and, when it is present, the gene is usually expressed (Jost et al., 2005). In contrast, most non-porcine isolates carry the atypical gene (Jost et al., 2005); when present, the consensus gene was reported as usually not expressed (Jost et al., 2005).

The present study was carried out in order to examine further the distribution of consensus and atypical cpb2 gene amongst porcine and non-porcine isolates originating from healthy and diseased animals, and the correlation between genotype and phenotype (CPB2 production) of cpb2-positive isolates.

Material and Methods

Clostridium perfringens isolates tested in this study were all type A isolates of avian (n=10), bovine (n=11), canine (n=49), caprine (n=11), equine (n=12), ovine (n=15), and porcine (n=14) origin. Most of the isolates were from animals with enteric disease diagnosed clinically or at necropsy, although the final diagnosis was rarely C. perfringens-associated enteritis. Porcine isolates were from neonatal pigs, but isolates from other species were from animals of unknown ages, and were convenience sample isolates from the laboratory collection maintained in freezing medium at -70°C. Positive identifications of C. perfringens were based on the presence of the characteristic double zone of hemolysis and colonial morphology as well as the demonstration of cpa by conventional (single reactions) or multiplex, gel based, PCR (Baums et al., 2004).
Polymerase chain reaction for *cpb2*

DNA extraction was performed by using InstaGene Matrix according to the manufacturer’s protocol (Bio-Rad Laboratories Ltd., Mississauga, ON, Canada). Supernatant fluid was removed and stored at -20°C until use. All the samples were screened by PCR for both consensus and atypical *cpb2* and typed as *cpb2*-positive or *cpb2*-negative. PCR targeting *cpa* was conducted as a control to confirm a successful DNA extraction. All strains in this study carried *cpa*.

The multiplex PCR was conducted following the protocol developed by Jost *et al.* (2005), which used two forward primers one specific for the consensus gene (M-CONF, 5’- CAATTGGGGGAGTTTATCCAA-3’, expected product size 304 bp), and another forward primer specific for the atypical gene (M-ATYPF, 5’- ATTATGTAGGAATACAGTTA-3’, expected product size 741bp) and one reverse primer that was common to both (M-REV, 5’- CAATACCTCCACCAATACTC-3’). The alternative PCR consisted of two separate PCR reactions, one for the consensus gene using the same primers as the multiplex without M-ATYPF, and another for the atypical gene using A-ATYPF (5’-AATGGTGCTATATACAATATGG-3’) and A-ATYPR (5’-TTAATCGATGCTGGAGTATAAT-3’) (expected product size 442bp).

All PCR assays were performed under the same conditions using Taq DNA polymerase with ThermoPol Buffer (New England Biolabs Inc., Pickering, ON, Canada). A touch-down PCR program was used: 94°C for 3 min, 35 cycles of 94°C for 30 s, 60°C to 50°C for 30 s/cycle
(the annealing temperature is decreased by 1°C every cycle until 50°C), 72°C for 1 min, and finally, 72°C for 5 min.

PCR products were separated electrophoretically in a 1% agarose gel and a PCR amplifications were visualized on GelDoc (BioRad) by using Ez-Vision Two DNA dye marker (AMRESCO Inc., CedarLane Laboratories Ltd).

**CPB2 protein preparation and Western blotting**

Since CPB2 is a secreted protein (Gibert et al., 1997), concentrated culture supernatant fluids of the isolates carrying the consensus or the atypical cpb2 gene were tested for gene expression. For this purpose *C. perfringens* was grown anaerobically overnight at 37°C in TGY media that contains 3% tryptic soy broth (Difco, Voigt Global Distribution Inc., Lawrence, KS, USA), 2% D-glucose (Difco), 1% yeast extract (Difco) and 0.1% L-cysteine (Sigma-Aldrich Canada Ltd., Oakville, ON, Canada). The culture supernatant fluid was collected by centrifugation at 12,100 x g for 20 min at 4°C. The supernatant was then filtered using a 0.2 µm Nalgene Bottletop Filter (Nalgene Labwear, Fisher Scientific Ltd., Mississauga, ON, Canada) and concentrated by using a 10kDa cut-off Amicon Ultra-15 10K Centrifugal Filter Device (Millipore Inc., Billerica, MA, USA) at 4,000 x g for 60 min at 4°C. Protease inhibitor mix was added to the approximately 30-fold concentrated supernatant in the amount of 1% of the total volume. Samples were used for SDS-PAGE and Western blot analysis of secreted proteins and stored at -20°C for further use.
Proteins present in concentrated supernatants were prepared using a 1:1 ratio of Laemmli Sample Buffer (Bio-Rad) and separated by sodium dodecyl sulphate–polyacrylamide gel electrophoresis (SDS-PAGE) under reducing conditions in 12.5% acrylamide gel at room temperature. Samples were compared to Precision Plus Protein Western C Standards (Bio-Rad). The gels were stained with Coomassie blue (R-250) (Bio-Rad).

Proteins were transferred to a nitrocellulose 0.45 µm transfer membrane (BioTrace NT, Gelman Laboratory, Laurent, QC, Canada) for 50 min at constant power supply of 90 V. After transfer was completed, membrane was blocked overnight using blocking buffer (PBS, 0.05% Tween 20, 0.5% fish skin gelatin [Norland HiPure Liquid Gelatin, Norland Products Inc, Cranbury, NJ, USA]). The blocking period was followed by incubation with primary antibody. For all the samples two Western blots were performed since two types of primary antibodies were used, polyclonal rabbit antibody (GenScript, Piscataway, NJ, USA) raised against purified consensus rCPB2 and monoclonal (Songer 9E4B, mouse anti-CPB2; GenScript) raised against consensus rCPB2, at 1:10,000 and 1:500 dilutions (v/v) respectively. Membranes were incubated for 90 min at room temperature. After washing, the membranes were incubated for 60 min at room temperature by using alkaline phosphatase-conjugated goat anti-rabbit IgG and alkaline phosphatase-conjugated goat anti-mouse IgG (Jackson ImmunoResearch Laboratories Inc., West Grove, PA, USA) at 1:10,000 and 1:1,000 dilutions, respectively. Specific protein bands were visualized using the Alkaline Phosphatase Conjugate Substrate Kit (Bio-Rad).
**Statistical analysis**

A Kappa test was used as a method for calculation of agreement between PCR and Western blot tests.

**Results**

**PCR**

A total of 122 *C. perfringens* isolates from different hosts were screened by PCR for the presence of consensus and atypical *cpb2* (Table 3.1). In the multiplex PCR, amplified consensus and atypical *cpb2* generated 304 bp and 741 bp sized amplicons, respectively (Fig. 3.1). However, the multiplex PCR gave variable results for atypical *cpb2*, and thus an alternative PCR was also used. For the alternative PCR, isolates were considered positive for the atypical gene if amplicons were present at approximately 442 bp (Fig. 3.1).

Results for the presence of consensus and atypical *cpb2* in the 122 isolates tested are shown in Table 3.1. In total, *cpb2* was detected in 60 isolates (21 consensus, 39 atypical) and 62 isolates were negative (Table 3.1). Porcine isolates largely possessed the consensus gene, and avian isolates invariably the atypical *cpb2*; isolates from other sources were more inconsistent in the presence or absence of the two genes (Table 3.1).

**CPB2 expression**

A 25 kDa immunoreactive band was detected in isolates expressing the consensus protein and a 24.6 kDa band in isolates expressing the atypical protein. Results confirmed that almost all porcine isolates carried the consensus gene, and that it was invariably expressed. All consensus
*cpb2*-positive *C. perfringens* isolates, with the exception of one of canine origin, showed 25 kDa immunoreactive bands with either the monoclonal or polyclonal antibodies. Both variants of the gene, consensus and atypical, were detected in non-porcine isolates, but the atypical *cpb2* was the dominant variant. Out of 47 *cpb2*-positive non-porcine *C. perfringens*, 9 carried the consensus *cpb2* and 38 the atypical *cpb2*. The monoclonal antibody detected the consensus protein (20/21) better than it did the atypical protein (17/39).

Atypical *cpb2*-positive isolates produced a protein that was smaller (24.6 kDa) (SDS-PAGE and Western blot) compared to the consensus protein. All the consensus proteins appeared as a 25 kDa band in the Western blot. Figure 2.2 shows the detection of both consensus and atypical CPB2 (equine isolates) by monoclonal and polyclonal antibodies.

Statistical calculation (Kappa test) showed that there was a strong agreement between PCR and Western blot for consensus *cpb2* with kappa = 0.97 (P<0.001). Agreement between PCR and Western blot for atypical *cpb2* was moderate with kappa = 0.78, (P<0.001).

**Discussion**

This study identified a number of important differences from earlier studies of the presence and expression of consensus and atypical *cpb2* in isolates from different animal species. Most notably, it was found that the majority of atypical *cpb2* were expressed. By contrast, in an earlier study by Jost *et al.* (2005), the atypical *cpb2* was not expressed by any of the isolates from several animal species tested (whether they carried the gene with a frameshift mutation or with an intact atypical gene). The reason for this remarkable discrepancy between earlier and the
current findings is unclear, since in the current study over three-quarters of atypical \textit{cpb2} positive isolates were found to produce CPB2 when polyclonal antibodies were used for detection. Our data are consistent with the reports of Lebrun et al. (2007b) in which 9 (69\%) of 13 bovine isolates expressed the atypical \textit{cpb2} gene. Possible reasons why the earlier study (Jost et al. 2005) did not detect the expression of the atypical gene include \textit{C. perfringens} growth time differences from the current study and the type of antibodies used in Western blotting for detection of gene expression. The incubation time of 48 hr in anaerobic conditions used by Jost et al. (2005) may have been too long, possibly creating the opportunity for proteases to degrade the atypical protein, which is unstable and can easily be degraded (Gibert et al., 1997). In the current study and those of others (Waters et al., 2003; Fisher et al., 2005; Vilei et al., 2005), incubation was overnight in anaerobic conditions. Differences in media used might also be responsible for the differences observed. Another potential reason for different results of Jost et al. (2005) was the different monoclonal antibody used for Western blotting (9E10B). Our study showed that the monoclonal antibody 9E4B detected the atypical protein less well than the consensus protein, and the same may have been the case for 9E10B. Western blots using polyclonal antibodies raised against consensus rCPB2 detected atypical CPB2 well in the current study (30/39). Jost et al. (2005) attributed the lack of expression of atypical \textit{cpb2} to the presence of frameshift mutations in about 44\% of a small number (9) of genes sequenced. We did not sequence genes in those isolates that failed to express atypical \textit{cpb2}, but this is a likely explanation for inability of expression in the current study (9 out of 39 isolates). However, Fisher et al. (2005) sequenced \textit{cpb2} from two human \textit{C. perfringens} isolates that carried intact atypical \textit{cpb2} open reading frames (ORFs), and showed that frameshift with formation of premature stop codons was not the explanation for failure of \textit{cpb2} expression. Waters et al. (2005) who showed
also that mRNA levels were in some cases too low to produce a protein detectable by Western blotting. In the current study, the expression of 77% of the atypical cpb2, using pAb, in isolates from different animal species challenges the earlier conclusions that these genes are mostly unexpressed.

Another difference between this study and those of others (Bacciarini et al., 2003; Jost et al., 2005; Vilei et al., 2005) is that consensus cpb2 was usually expressed (Table 3.1). These findings are however on only a small number of consensus cpb2 isolates, and are skewed by the presence of porcine consensus positive cpb2, which are well recognized to be expressed (Waters et al., 2003; Jost et al., 2005). The consensus gene was found to be present most commonly in equine isolates, and their expression also contrasts with the studies of Vilei et al. (2005) which found frameshift mutations in 18 of 22 genes sequenced. These differences may relate to source including geographic differences. Our study confirmed the relative low frequency of the consensus cpb2 in non-porcine isolates (Bueschel et al., 2003; Jost et al., 2005; van Asten et al., 2008), but suggests that there are differences between isolates from different species, with horse isolates being the most effective in expressing consensus CPB2. The contribution of the consensus cpb2 product to the virulence of C. perfringens is still unclear.

An additional difference between the present study and those of others was the difference in the prevalence of the atypical cpb2 in isolates from different sources, although the numbers of isolates examined in this study were low. The most notable finding was the presence of this gene or its product in all avian isolates. Further work is required to determine whether the presence of the atypical cpb2 is a characteristic of avian isolates, as the consensus cpb2 appears to be of
porcine isolates. The atypical *cpb2* was also present in isolates from sheep (Table 3.1), although it was only expressed in 40% of these isolates. This study was the first to examine the distribution and expression of the CPB2 toxin in *C. perfringens* in isolates from dogs, sheep and goats.

Porcine *C. perfringens* strains predominantly carry and express the consensus *cpb2* gene (Jost et al., 2005, Klaasen et al., 1999; Bueschel et al., 2003; Waters et al., 2003), and the results of the current study supported this well-accepted observation. Further work is, however, required to understand the role of this toxin in enteric disease of swine.

Interestingly, the monoclonal antibody failed to detect expression of the atypical gene in 57% of the isolates that were positive when examined with the polyclonal antibodies (Table 3.1). This difference may relate to a difference in the epitope recognized by the monoclonal, possibly combined with differences in strength of expression of the protein. The CPB2 protein immunoreactive consensus band was smaller (25 kDa) than described by Gibert et al. (1997) (27.6 kDa mature protein), but the reason for this difference in size is unclear.

In conclusion, this study has identified a number of important differences and discrepancies about the prevalence and expression of consensus and atypical *cpb2* from earlier studies. Further work is required to illuminate these differences and to determine their relevance to the epidemiology of *C. perfringens* in isolates from different animal sources and the contribution of *cpb2* to enteric disease in these species.
Table 3.1 Presence of consensus and atypical \( cpb2 \) gene in \textit{C. perfringens} from different animal sources.

<table>
<thead>
<tr>
<th>Origin</th>
<th>Consensus ( cpb2 )</th>
<th>Atypical ( cpb2 )</th>
<th>Negative</th>
<th>Consensus CPB2 (pAb)</th>
<th>Atypical CPB2 (mAb)</th>
<th>(pAb)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Porcine</td>
<td>12/14</td>
<td>1/14</td>
<td>1/14</td>
<td>12/12</td>
<td>1/1</td>
<td>1/1</td>
</tr>
<tr>
<td>Equine</td>
<td>5/12</td>
<td>3/12</td>
<td>4/12</td>
<td>5/5</td>
<td>3/3</td>
<td>3/3</td>
</tr>
<tr>
<td>Bovine</td>
<td>1/11</td>
<td>5/11</td>
<td>5/11</td>
<td>1/1</td>
<td>2/5</td>
<td>2/5</td>
</tr>
<tr>
<td>Avian</td>
<td>0/10</td>
<td>10/10</td>
<td>0/10</td>
<td>0/0</td>
<td>0/10</td>
<td>10/10</td>
</tr>
<tr>
<td>Ovine</td>
<td>0/15</td>
<td>10/15</td>
<td>5/15</td>
<td>0/0</td>
<td>3/10</td>
<td>4/10</td>
</tr>
<tr>
<td>Caprine</td>
<td>0/11</td>
<td>3/11</td>
<td>8/11</td>
<td>0/0</td>
<td>2/3</td>
<td>2/3</td>
</tr>
<tr>
<td>Canine</td>
<td>3/49</td>
<td>8/49</td>
<td>38/49</td>
<td>2/3</td>
<td>6/8</td>
<td>8/8</td>
</tr>
<tr>
<td>Total</td>
<td>21/122</td>
<td>40/122</td>
<td>61/122</td>
<td>20/21</td>
<td>17/40</td>
<td>30/40</td>
</tr>
</tbody>
</table>
Figure 3.1 Agarose gel electrophoresis of PCR products of consensus and atypical *cpb2*

(A) New England Biolabs 100 bp DNA Ladder, (B) Positive *cpa* control, (C) Positive multiplex PCR atypical *cpb2* control, (D) Positive multiplex PCR consensus *cpb2* control, (E) Positive alternative PCR atypical *cpb2* control, (F) Positive alternative PCR consensus *cpb2* control.
Figure 3.2 Western blot of consensus and atypical CPB2 detected using monoclonal antibody

Chapter 4

General Discussion and Conclusions

The work described in this thesis has made novel contributions to investigating the role of *C. perfringens* CPB2 toxin in enteric disease of animals and *cpb2* expression in isolates originating in different animal species.

The enteric disease associated with *C. perfringens* type A in neonatal piglets is not well defined although it appears to be one of the most frequently diagnosed causes of diarrhea in neonatal piglets (Klaasen *et al*., 1999; Bueschel *et al*., 2003, Jost *et al*., 2005). Diagnosis is made by exclusion of other causes of diarrhea in piglets combined with the isolation of large numbers of *cpb2*-positive *C. perfringens* (Songer and Uzal, 2005). A more specific diagnostic approach is needed, and the work described in this thesis provides one potential tool. To date, there have been no attempts to design, evaluate and apply an antigen capture ELISA test as a diagnostic test in order to detect CPB2 in intestinal contents or feces. Although there are many described ELISA tests for detecting *C. perfringens* enterotoxin (McClane and Strouse 1984; Bartholomew *et al*., 1985; Wimsatt *et al*., 1986) or *C. difficile* toxin A (Lyerly *et al*., 1983; Viscidi *et al*., 1984) in fecal samples, this is the first study to develop an assay specific for *C. perfringens* consensus CPB2 toxin, and to detect CPB2 in intestinal contents. In addition, my results showed that the assay could be used for detection and determination of the toxin concentration in *C. perfringens* culture supernatants fluids.
To date, the methods used in studies linking CPB2 with diarrhea and enteric disease in different animal species are based on detection and identification of cpb2-positive *C. perfringens* by PCR (Garmory *et al.*, 2000; Thiede *et al.*, 2001; Bacciarini *et al.*, 2003; Baums *et al.*, 2004; Jost *et al.*, 2005) and the expression of the gene by Western blotting using polyclonal and monoclonal antibodies (Gibert *et al.*, 1997; Bueschel *et al.*, 2003; Jost *et al.*, 2005) or immunohistochemistry (Bacciarini *et al.*, 2003). However, these methods lack the ability to detect the toxin in the live animal and thus are insufficient to make a precise diagnosis. As recognized for some cpb2-positive isolates, there are discrepancies between the genotype and the phenotype. The presence of the gene does not necessarily mean expression; therefore, diagnosis of the disease based solely on PCR results should be made with caution. In addition, if the gene is expressed (which in porcine isolates is almost always the case), there is still the question of whether expression is the same *in vivo* and *in vitro*. This emphasises the necessity for a test that can detect the toxin in the intestinal contents and/or fecal samples from diarrheic piglets, and potentially of other animal species. However, studies reported here, and those of others, suggest that the consensus cpb2 gene is relatively uncommon in *C. perfringens* isolated from other species, and that the atypical protein was less well detected than the consensus protein by the monoclonal antibody used in the ELISA (Chapter 2) and Western blot (Chapter 3). Also, because of the low frequency of the consensus CPB2 in isolates from animals other than swine, the ELISA would not be suitable for routine detection of CPB2 in non-porcine species with possible type A *C. perfringens* enteritis.

Based on this knowledge and the work described, I showed that the antigen capture ELISA test detected CPB2 in the intestinal contents of neonatal piglets. Since antigens in the GI
tract can be present in very small quantities, it was important to create an assay with a low limit of detection, and the ELISA detected as little as 2-3 ng/ml of protein. This is about the same level of detection as ELISAs for *C. perfringens* enterotoxin (Bartholomew et al., 1985; Wimsatt et al., 1986). Samples derived from the GI tract are rich in enzymes (including proteases) and extracts that potentially can display desorbing activity when applied onto coated ELISA plates, thus giving false positive results, and/or interfering with toxin recovery from a sample, giving false negative results. During the development of the ELISA, I encountered such difficulties and effectively addressed them by adjusting sample processing and the test protocol. Studies were focused on protease activity, since the conclusion was that false positive results were the outcome of enzyme desorbing activity. The approach to overcome this was partially adopted from Viscidi et al. (1984) and Hanvanich et al. (1986), including the change of pH and protein saturation of the sample diluent. Additionally, protease activity was abolished by lowering the temperature and prolonging the time of the antigen (sample) incubation phase. However, despite all the difficulties overcome during the development of the antigen capture ELISA test, there was no “gold standard” against which to compare the new test since *cpb2*-carrying *C. perfringens* type A are commonly found in the intestinal tract of piglets and since detailed study is required to identify piglets with enteric disease attributable only to *C. perfringens* type A. For this reason, a working definition of “positive” in this assay was based on three standard deviations above the “background” reading in wells with no antigen.

Earlier studies have suggested that CPB2 has a significant role in neonatal enteric disease in piglets. For example, a study in Europe, The Netherlands and Switzerland (Klaasen et al., 1999) showed a high prevalence of *cpb2*-positive *C. perfringens* type A isolated from piglets
with diarrhea. Hence, they speculated that CPB2 had a role in the neonatal diarrhea. This conclusion was supported by the work of Bueschel et al. (2003), who found cpb2-positive C. perfringens more commonly in piglets with diarrhea than in a small number of a healthy piglets. However, a more recent study conducted in Switzerland (Jaggi et al., 2009) detected a high prevalence of C. perfringens type A harbouring cpb2 in non-diarrheic piglets. The data are inadequate to draw a solid conclusion on the association of CPB2 and the disease. It was the intention of this research to use the ELISA to try to resolve the issue of whether type A C. perfringens cause morbidity in neonatal piglets; in the event, development of the ELISA itself to the point where it could be used for this purpose took the time available for this thesis research.

The results reported in Chapter 3 confirm the results of other studies that show that porcine isolates almost always carry and express consensus cpb2 (Gibert et al, 1997; Klassen et al., 1999; Garmory et al., 2000; Bueschel et al., 2003), supporting the potential application of the ELISA to assess the role of CPB2 in enteric disease. As others have found, non-porcine C. perfringens isolates were more likely to test positive by PCR for the atypical gene and these did not always express the gene. One example is the study performed by Lebrun et al. (2007b) where they showed that bovine C. perfringens carrying the consensus or atypical cpb2 expressed the gene (64% and 69% respectively of cpb2-positive isolates). However, in contrast to the data of Jost et al. (2005), I found (Chapter 3) that the atypical gene carried by non-porcine isolates is commonly expressed. This finding is consistent with that of Lebrun et al. 2007b for bovine isolates. The work reported in Chapter 3 also identified apparent differences between isolates from different sources in the presence of cpb2 genes; for example, a notable finding was that all avian isolates carried and expressed the atypical variant of the gene. Another difference from
other studies, albeit with small numbers of isolates, was that consensus *cpb2*, when present in non-porcine isolates, was usually expressed. There were also intriguing differences in prevalence of consensus and atypical *cpb2* in isolates from different species, which may reflect differences in disease associations of these genes, which are invariably found on plasmids.

Overall, the research described in this thesis provides an important tool to address the question of whether CPB2-producing *C. perfringens* type A causes neonatal piglet diarrhea. In addition, it suggests that this ELISA is really only applicable to enteric disease in neonatal piglets, rather that to *C. perfringens* type A enteric disease in other species, where the consensus gene is generally uncommon. The antigen capture ELISA development showed that CPB2 could be detected in the intestinal contents of piglets, once the problem of protease activity was resolved. In addition, the study of distribution and expression of consensus and atypical *cpb2* identified substantial disagreement with a previous report. As such, this study was successful in creating a baseline of knowledge that will be useful in focusing future research on the potential role of atypical CPB2 toxin in the pathogenesis of *C. perfringens* enteric disease in species other than pigs.

Among the many questions raised by this work that need to be addressed are: How different is the expression of *cpb2* in *vitro* and in *vivo*? If the atypical *cpb2* is expressed in *vivo*, how much is expressed? Is atypical CPB2 significant in enteric disease in animals other than swine? Is the CPB2 really a significant enteric toxin or just a marker of virulence in swine isolates?
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Appendix

Appendix Figure 1. Comparison of the ELISA curves for the piglets (piglet A and C) fresh and frozen intestinal samples.

Both samples with GE protease inhibitor (PI). The rCPB2 ELISA curve is included for comparison (moved from a higher dilution but un-scaled). The Y axis units are dual wave absorbance (405-490nm) and the X axis units are sample dilutions.
Appendix Figure 2. ELISA detection consensus versus atypical recombinant CPB2.
Appendix Figure 3. ELISA of the supernatant from a culture of P18 porcine isolate containing consensus CPB2.

Concentrated supernatant containing native consensus CPB2 showed comparable reaction kinetics to those of rCPB2.
Appendix Figure 4. Western blot native purified concentrated consensus CPB2 from a porcine isolate detected by monoclonal antibody (Songer 9E4B).

1. Native concentrated purified consensus CPB2 from a porcine isolate (P18); 2. Native purified consensus CPB2 from a porcine isolate (P18); 3. Normal rabbit serum; 4. Recombinant concentrated purified CPB2; 5. Molecular weight standards (kDa).