TOXIGENIC CLOSTRIDIUM DIFFICILE IN THE BOVINE CALF GUT: ASSOCIATION WITH ENTERIC DISEASE IN CALVES AND SIGNIFICANCE TO PUBLIC HEALTH

A Dissertation in Pathobiology by Beth A. Houser

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**ABSTRACT**

*Clostridium difficile* is an emerging pathogen in humans and animals. A real-time multiplex PCR assay was developed and standardized for the detection of genetic elements of toxigenic *C. difficile*. The assay targeted genes encoding TcdA, TcdB, and CDT toxins of *C. difficile* and was carried out in two duplex reactions. Reaction 1 targeted *tcdA* and *tcdB* while reaction 2 targeted *cdtA* and *cdtB*. The detection limit of the standardized real-time multiplex PCR assay for the toxin genes of *C. difficile* was 10³ cells/g and 10¹ cells/g for non-enriched and enriched fecal and ground meat samples, respectively. When DNA is extracted directly from fecal and ground meat samples, toxin genes of *C. difficile* may be detected in as little as 4 hours, although, sample enrichment is recommended to increase sensitivity. The multiplex real-time PCR assay was used throughout the study.

The role of *C. difficile* in enteric disease in calves was investigated. The findings of the study revealed that young calves were nearly 11 times more likely to have enteric lesions when toxins TcdA and/or TcdB were detected in the feces. Toxigenic *C. difficile* was detected in 43% of calves with no enteric lesions or clinical symptoms of enteric disease. This observation confirms previous reports that calves could be asymptomatic carriers of *C. difficile*. Isolates collected from calves were characterized using genotypic and phenotypic methods. Genes encoding TcdA, TcdB, and CDT were detected in 86.6%, 85.1%, and 83.6% of isolates, respectively. Deletions (100 bp) in the tcdC gene, a negative regulator of toxin expression, were detected in 73.1% of toxigenic isolates. All isolates were resistant to cefoxitin, a third generation cephalosporin, and 52.2% of the isolates were resistant to tetracycline.

Nine representative toxigenic *C. difficile* isolates were examined for *in vitro* toxin production in the presence of sub-inhibitory concentrations of oxytetracycline, an antibiotic commonly administered to calves during the first two weeks of life. It was observed that
exposure to oxytetracycline increased TcdA and/or TcdB production of one isolate. Exposure to oxytetracycline also resulted in TcdA and/or TcdB production by an isolate that did not produce toxin under control conditions.

A longitudinal study was conducted to determine the prevalence of toxigenic *C. difficile* in veal calves. Fifty veal calves from 4 herds (n=200) were followed for 18-22 weeks from the time of arrival on the veal farm to the time of slaughter. It was observed that 58 (29%) calves tested PCR positive for genetic elements of toxigenic *C. difficile* at least once over 18-22 week period. Calf age (p=0.011) and season (p=0.28e-6) influenced the prevalence of *C. difficile* toxin genes in calf fecal samples. Carcasses of calves (n=100) from two herds were sampled to determine the incidence of toxigenic *C. difficile* contamination. Carcass swabs were screened for toxigenic *C. difficile* contamination using culture and multiplex real-time PCR methods. Toxin genes of *C. difficile* were detected in 1 carcass swab by multiplex real-time PCR. Toxigenic *C. difficile* was detected by PCR and culture in 4 (8%) and 2 (4%) ground veal samples, respectively.

In summary, the findings of the study reveal that *C. difficile* is as an enteric pathogen in calves and calves could be asymptomatic carriers of toxigenic *C. difficile*. It is recommended that veterinary diagnostic laboratories should consider testing for *C. difficile* and TcdA/B toxins in fecal and necropsy samples from calves with suspected enteric disease. The study provided strong evidence to suggest that sub inhibitory concentrations of oxytetracycline could increase or induce production of toxins TcdA and TcdB. We have provided insight on the emergence of *C. difficile* in community acquired infections by identifying ground meat as a source of human exposure. Although the incidence of carcass contamination was low, viable toxigenic *C. difficile* was detected in finished ground veal product. Although other factors contributing to epidemiology of *C. difficile*-associated disease remain to be identified, this work has provided a basis for
continued public health investigation. A full understanding of the changing epidemiology of *C. difficile* will provide valuable information needed for designing and implementing disease management and prevention strategies in veterinary and public health.
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Chapter 1

Introduction
1.1 Introduction

*Clostridium difficile* is a well established gastrointestinal pathogen in humans and animal species. Human diseases attributed to *C. difficile* include antibiotic-associated diarrhea and pseudomembranous colitis. Infections can be complicated by toxic megacolon and bowel perforation and can result in death. The primary risk factor for disease is broad spectrum antibiotic treatment, which diminishes colonization of the susceptible normal intestinal flora population. Depletion of normal flora allows the opportunity for overgrowth of *C. difficile*, which can cause severe damage to the intestinal epithelium through the production of toxins TcdA, TcdB, and CDT.

Calf mortality associated with enteric disease continues to be a source of significant losses to producers in the dairy industry prompting researchers to identify etiologic agents associated with the disease. Although researchers have attempted to gain information on the pathogenesis of *C. difficile* in calves using experimental infection, little data exists on natural infection and intoxication of the calf gut with *C. difficile* (and its toxins) or factors influencing toxin expression in the calf gut.

To date, *C. difficile* related disease in humans has been mainly associated with debilitated populations and infections have historically been considered nosocomial. Recent reports of community acquired cases in low risk populations have caused concern. Changes in the epidemiology of *C. difficile* infection in humans have led researchers to attempt to identify sources of exposure outside the healthcare setting. Based on the genotypic similarity between human and animal isolates, it is thought that toxigenic *C. difficile* may be disseminated from food animals to humans through consumption of contaminated meat. Recent reports suggest calves can be asymptomatic carriers of toxigenic *C. difficile*, therefore, fecal contamination of veal meat products may be a public health risk. Although toxigenic *C. difficile* has been isolated from many
ground meats meant for human consumption, no investigation has been carried out on the incidence of *C. difficile* contamination of meat throughout the production process.

The emergence of hypervirulent strains and increasing human mortality rates associated with *C. difficile* infections necessitate a better understanding of the changing epidemiology of *C. difficile*. Current methods for detection of toxigenic *C. difficile* include culture and immunoassay. Culture is time consuming and has been associated with false negatives due to the low survival rate of *C. difficile* in samples. Immunoassays are expensive and have been criticized for inferior sensitivity. A high-throughput, sensitive, and specific detection method is needed for screening and monitoring possible sources of human exposure in the community and for more accurate and timely diagnosis of infection.

### 1.2 Statement of the Problem

The changing epidemiology and emergence of hypervirulent strains of *C. difficile* has presented new challenges in controlling infections attributed to this well-established pathogen. Reports of community-acquired human infection as well as infection of animal species has drawn considerable attention from the public health community. It is crucial to generate data on the epidemiology of toxigenic *C. difficile* to determine its significance in animal disease and to understand the public health risks posed by possible dissemination to humans via the food supply.
1.3 Research Objectives

1. To develop, standardize, and validate a multiplex real-time PCR assay for the detection of toxigenic *C. difficile* to be used for monitoring contamination of ground meat and aid in the diagnosis of *C. difficile* infection.

2. To investigate the role of *C. difficile* in enteric disease in calves

3. To assess factors relevant to the calf gut environment that influence toxin expression.

4. To determine the public health significance of *C. difficile* in veal calves by monitoring the prevalence of toxigenic *C. difficile* throughout the veal production process.
Chapter 2

Review of Literature
2.1 Clostridium difficile

The Clostridium genus is comprised of nearly 120 species of Gram positive, anaerobic, spore producing bacteria. Clostridia can be found in soil and water and commonly inhabit the mammalian intestinal tract (Finegold and Martin, 1982). While most species are non-toxigenic, others are known for production of exotoxins which are highly associated with enteric disease in both humans and animals. Hall and O'Toole were the first to describe the species, Clostridium difficile (Hall and O'Toole, 1935). Clostridium difficile cells are rod shaped and the presence of terminal spores gives them a characteristic tennis racket appearance (Finegold and Martin, 1982). Colonies grown on solid media are flat, irregular, non-hemolytic, and fluoresce yellow under ultraviolet light (Hall and Toole, 1935; McClane et al. 2006). Metabolism of C. difficile is similar to that of other species of the colonic flora. It utilizes monosaccharides including glucose, fructose, mannitol, mannose, and xylitol (Bongaerts and Lyerly, 1997). The organism also secretes collagenase, protease, and mucopolysaccharide-hydrolysing enzymes which breakdown substrates for utilization of nutrients in the mucosa (Bongaerts and Lyerly, 1997).

Phylogenetic research based on 16S rDNA homology studies have resulted in the organization of clostridial species into 19 clusters (Collins et al., 1994). The pathogenic clostridial species are contained in seven of these clusters (Figure 2-1). The major pathogenic species including C. perfringens, C. botulinum, and C. tetani belong to cluster I. Clostridium difficile is relatively divergent from the major clostridial pathogens and can be found in cluster X along with C. sordellii (Lyas and Rood, 2006)(Figure 2-1). Phylogenetic studies have provided evidence that favors the reclassification of C. difficile and several other closely related species into a separate genus, although, such a reclassification has not yet been instituted (Collins et al., 1994; Lyas and Rood, 2006).
Figure 2-1: Phylogenetic tree of the pathogenic *Clostridium* species. Based on 16S rDNA homology (Lyras and Rood, 2006).
For many years, *C. difficile* was thought to be a non-pathogenic species because it was first observed in the feces of healthy infants (Hall and O'Toole; 1935). Bartlett et al. (1977a) identified *C. difficile* as an etiologic agent of antibiotic-associated colitis in 1977. They noted the association between enterocolitis in hamsters and administration of clindamycin and lincomycin (Bartlett et al., 1977a; Small, 1968). They also observed that following clindamycin treatment the predominant bacterial species in the cecum included several species of *Clostridium*. In an attempt to reproduce clindamycin-associated enterocolitis, Bartlett et al. (1977a) challenged hamsters with various *Clostridium species* through intracecal injection. One species was able to produce enterocolitis and phenotypic characterization of the species revealed similarities to *Clostridium difficile*. Upon observation that cell free supernatant from this strain was able to produce enterocolitis, it was concluded that *C. difficile* produced toxin that elicited pathogenesis.

In the same year Larson et al. (1977) described a case of pseudomembranous colitis (PMC) associated with an unknown toxin. When no pathogenic bacteria were found in the feces of a patient who had developed PMC, tissue cultures were inoculated with a fecal suspension in an attempt to isolate a virus. No viruses were detected, although the fecal suspension had a cytopathic effect on HeLa and Rhesus monkey kidney cells. Larson et al. (1977) described cell rounding and dissociation from the glass plate after 48 hours of incubation. The toxic effect was seen with fecal suspensions from four of five other patients with PMC as well, but was not seen in fecal suspensions from six patients with ulcerative colitis or salmonellosis.

Bartlett et al. (1978) detected cytopathic toxin in stools from 26 out of 27 antibiotic-associated PMC patients. The toxin was neutralized by antibody to *C. sordellii*, a species closely relate to *C. difficile*. Taylor and Bartlett (1979) purified and characterized a cytotoxin from the supernatant of a *C. difficile* culture and compared it to cytotoxin from the feces of a patient with antibiotic-associated PMC. Toxin from both the pure culture and the fecal sample had the same molecular weight and both were inactivated by trypsin, but not DNase or RNase. George et al.
(1978) found supernatant from 14 out of 16 *C. difficile* isolates from antibiotic-associated colitis cases was cytotoxic to human amniotic cell cultures.

In 1981, Taylor et al. (1981) isolated a second toxin, which was designated toxin A, from a culture of *C. difficile* that was able to be biochemically separated from the cytotoxin described by other researchers. The second toxin also differed antigenically and in biological activity. By producing antitoxin to both proteins, Libby and Wilkens (1981) showed toxin A (TcdA), and toxin B (TcdB), the cytotoxin originally described, are immunologically distinct. Later, TcdA and TcdB were determined to be 308 kDa and 270 kDal in size, respectively (Barroso et al., 1990; von Eichel-Streiber and Sauerborn 1990). It was noted that most *C. difficile* strains produce both toxins (Taylor et al., 1981). Toxin A elicited toxicity when injected into a ligated rabbit ileal loop where as the cytotoxin gave a negative result. Although TcdB had a cytotoxic effect in cell culture, further studies also showed no response in intestinal loop assays elicited by the cytotoxin in guineapigs, hamsters, rats, rabbits or mice (Lima et al., 1988; Sullivan et al., 1982; Taylor et al., 1981). Based on the results of these early studies it was thought that TcdA, now considered an enterotoxin, was mainly responsible for the pathogenesis associated with *C. difficile* infection (Bartlett, 1997).

The clinical significance of TcdB was revisited when researchers reported TcdA negative, TcdB positive strains capable of causing disease (Alfa et al., 2000; Borriello et al., 1992; Kuijper et al., 2001; Lyerly et al., 1992; Riegler et al., 1997). Lyerly et al. (1992) described a TcdA negative/TcdB positive strain of *Clostridium difficile*. Toxin B isolated from this strain showed more enterotoxic activity than TcdB from a strain that produced both TcdA and TcdB toxins. Cytotoxic activity was also higher for the TcdA negative strain after 24 h exposure of Chinese hamster ovary K-1 cells. Borriello et al. (1992) described a TcdA negative/TcdB positive strain that caused hemorrhage and fluid accumulation in ligated rabbit ileal loops.
Riegler et al. (1997) reported that TcdB was 10 times more potent than TcdA in human intestinal mucosa. They found mannitol permeability to increase 16 times upon exposure to 32 nM of TcdA with similar results seen for TcdB at a concentration of only 3 nM. Similar results were reported by Savidge et al. (2003). They reported that concentrations of TcdB, equivalent to that of TcdA, were able to cause damage to the intestinal epithelium, increase permeability of the mucosa, and stimulate IL-8 expression and neutrophil recruitment. Also, nosocomial human outbreaks of *C. difficile*-associated diarrhea have been attributed to TcdA negative, TcdB positive *C. difficile* strains (Alfa et al., 2000; Kuijper et al., 2001; Pituch et al., 2001). It is suggested that TcdA and TcdB may act in synergy, the enterotoxic effects of TcdA allowing TcdB access to the intestinal mucosa (Lima et al., 1988). Today both TcdA and TcdB are considered to be clinically relevant because they are both proinflammatory and both are cytotoxic and enterotoxic in the human gut (Drudy et al., 2007).

A third toxin produced by *C. difficile* was described by Popoff et al. (1988), who noticed toxins of *C. difficile* shared a similar mechanism to that of binary toxins of other Clostridial species. Although it was apparent that TcdA and TcdB caused disaggregation of actin filaments, the actual molecular mechanisms of the toxins were unknown at the time. Popoff et al. hypothesized that *C. difficile* toxins acted as ADP-ribosyltransferases as seen in iota toxin of *C. perfringens* and C2 toxin of *C. botulinum*. They screened 15 *C. difficile* strains and were able to detect ADP-ribosyltransferase activity in one of those strains. Upon analysis, *C. difficile* toxins A and B (TcdA/B) showed no ADP-ribosyltransferase activity, confirming they had discovered a third toxin. Further, Popoff et al. (1988) were able to purify the ADP-ribosyltransferase which they designated CDT (*C. difficile* transferase). They found CDT is not cytotoxic, is not lethal in mice, and is antigenically different from C2 toxin, TcdA, and TcdB. Antibodies raised against CDT cross reacted with iota toxin from a *C. perfringens* type E strain, suggesting CDT belongs to the iota toxin family.
Perelle et al. (1997) later cloned and sequenced the CDT locus of the ADP-ribosyltransferase producing strain described by Popoff et al (1988). They confirmed that CDT (43 kDal) is a binary toxin by showing it is encoded by two genes, \textit{cdtA} and \textit{cdtB}. They also noted the sequences and organization of the genes encoding CDT were similar to the genes encoding iota toxin of \textit{C. perfringens}, also a binary toxin. The deduced amino acid sequences of CDTa and CDTb were found to be 84.3% and 81.2% homologous with respective \textit{C. perfringens} iota toxin subunits. A wide range in the prevalence of CDT producing strains has been reported. Stubbs et al. (2000) reported 6.4% of \textit{C. difficile} isolates from UK hospitals carried genes that encoded for CDT. Goncalves et al. (2004) reported a similar prevalence of 6% for \textit{C. difficile} isolates from human infections. Geric et al. (2003) reported 15.5% of \textit{C. difficile} strains from a United States collection of human isolates encoded CDT.

Geric et al. (2006) found that a TcdA negative/Tcd B negative/CDT positive strain was able to cause fluid accumulation in ligated rabbit ileal loops, but was not able to cause disease in hamsters. Barbut et al. (2005) conducted a retrospective study on \textit{C. difficile}-associated disease (CDAD) cases in humans involving strains with and without genes encoding for CDT. They found community acquired cases, abdominal pain, and liquid stools were more likely to be associated with strains encoding for CDT than strains that were \textit{cdtA} and \textit{cdtB} negative. Based on this data, it is thought CDT may influence severity of CDAD, although the true clinical significance of CDT has not yet been defined.

\textit{Clostridium difficile} has emerged as a successful enteric pathogen due to several important characteristics. The first is resistance to broad spectrum antibiotics which provides an ecological advantage in the gut of antibiotic treated hosts. The second characteristic is the production of potent cytotoxin and enterotoxin. Lastly, is the ability to produce spores which allow survival outside the host and facilitate transmission to new hosts through exposure to contaminated environments (Deneve et al., 2009). Due to increasing virulence, high morbidity
and mortality rates, and changing epidemiology, *Clostridium difficile* continues to be an important and challenging enteric pathogen in both humans and animals (Deneve et al., 2009).

### 2.2 Pathogenesis and Comparative Pathology

In the presence of normal gut flora *C. difficile* is not able to replicate in due to pH, the presence of short-chain fatty acids, lack of space, and nutrient limitations (Bartlett, 1997, Bongaerts and Lyerly, 1997). Only when colonization resistance provided by the resident gut flora is eliminated is *C. difficile* able to colonize the gut allowing for the initiation of toxin mediated pathogenesis (Borriello and Barclay, 1986). Long term treatment with broad spectrum antibiotics including ampicillin, amoxicillin, clindamycin, flavroquinolones, and cephalosporins is highly associated with *C. difficile* infection (Bartlett, 1997; Kelly and LaMont, 1998; Weese et al., 2009b). When susceptible gut flora is inhibited, highly resistant *C. difficile* is allowed to proliferate, colonize the gut, and produce toxin (Bartlett, 1997).

Thelestam and Bronnegard (1980) observed *C. difficile* cytotoxin in tissue culture disrupted the organization of actin in human lung fibroblasts using immunofluorescence staining. Wedel et al. (1983) confirmed their findings using electron microscopy, which showed shortening of actin filament bundles in rabbit aorta smooth muscle cells treated with TcdB. Pothoulakis et al. (1986) used DNase I inhibition and Triton extraction to show a dose dependent increase in cytoplasmic actin of TcdB intoxicated fibroblasts compared to control cells. They noted TcdB treatment resulted in an increased G-actin to F-actin ratio. Mitchell et al. (1987) showed cytoplasm collapse in TcdB treated HeLa cells and also showed an increase in monomeric actin and a decrease in F-actin. They did not observe any effect following exposure of purified actin to TcdB, suggesting the toxin acts indirectly to cause disaggregation of actin in the cell.
The molecular mechanisms for toxicity of TcdA and TcdB were first described by Just et al. (1994; 1995a; 1995b; 1995c). Just noted the effects of TcdB on actin were similar to those caused by *C. botulinum* C3 toxin, which is known to act on Rho proteins. G-actin subunits polymerize to form the F-actin filaments in order to assemble the cytoskeleton (Lodish et al., 2000). The Rho proteins (RhoA, B, and C) are important in cytoskeleton regulation by inducing the formation of actin filaments (Paterson et al., 1990; Ridley, 1997).

Just et al. (1994) observed inhibition of ADP-ribosylation of purified Rho-GST fusion protein from TcdB treated cells, showing TcdB is able to modify RhoA. They further confirmed their findings when they observed the effects of TcdB were inhibited in cells over-expressing RhoA. Just et al. (1995c) later showed TcdA also acts on Rho proteins. A decrease in migration of RhoA from treated cells in a non-denaturing gel as compared to control cells, confirmed the toxins act through Rho modification. They were also of the opinion that a cellular cofactor must be involved in the mechanism, because no toxic effect was seen the absence of cell lysate. Their subsequent experiments ruled out ADP-ribosyltransferase activity as a means of TcdA modification of Rho as seen with C3 toxin (Just et al., 1995c).

Just et al. (1995a and 1995b) later confirmed both TcdA and TcdB act on the Rho proteins through monoglucosylation. In order to confirm the cofactor incorporated into Rho proteins by TcdA, Just et al. (1995b) used electrospray mass spectroscopy and found the RhoA modification to be 162 Da, suggesting the incorporation of a hexose. The researchers then screened several hexoses for their ability to serve as a co-substrate for the TcdA induced RhoA modification. They were able to confirm that TcdA incorporates UDP-glucose in Rho family proteins at Thr-37. They were able to prevent Rho modification by mutating Thr-37 to Ala. The same mechanism was later confirmed for TcdB (Just et al., 1995a).

Popoff et al. (1988) reported CDT, the binary toxin produced by *C. difficile*, is an actin-specific ribosyltransferase. The binary toxin is formed by two subunits CDTa, the enzymatic
component, and CDTb, the receptor binding portion (Perelle et al., 1997). Gulke et al. (2001) showed the enzymatic component of CDT catalyzes ADP-ribosylation of G-actin causing depolymerization of F-actin and disregulation of the cytoskeleton.

von Eichel-Streiber and Sauerborn (1990) analyzed the gene encoding TcdA. They found C-terminal repeats with homology to carbohydrate binding regions of Streptococci glucosyltransferases. Toxin A binds to carbohydrate receptors on the surface of target cells via C-terminal repeats and is then endocytosed (Florin and Thelestam, 1983). To date, receptors for TcdB and CDT have not been identified. Once inside the cell, glucosylation of RhoA by TcdA and TcdB and ADP-ribosylation by CDT disrupts actin regulation leading to loss of cytoskeletal integrity. The result is cell rounding and loss of tight junctions between intestinal epithelial cells (Keel and Songer, 2006).

Toxins cause enterocytes to release cytokines and other mediators initiating an inflammatory response causing further tissue damage and fluid accumulation (Pothoulakis and LaMont, 2001). Upregulation of NF-κB expression in target cells results in the release of IL-8 and neutrophil chemotaxis. It has been observed that neutrophils significantly contribute to intestinal necrosis associated with *C. difficile* infection (Triadafilopoulos et al., 1989). Cells also respond to toxin through up-regulation of RhoB, a pro-apoptotic protein (Genth et al., 2008). Pothoulakis et al. (1994) observed substance P, able to increase vascular permeability, is released by neurons in response to TcdA resulting in release of water into the intestine further contributing to *C. difficile*-associated diarrhea.

Lesions associated with *C. difficile* are well documented for humans and a few animal species. Ample data exists on hamsters, guinea pigs, and rabbits as they are commonly used as models for studying CDAD. Data on animals associated with agriculture is limited to horses and pigs, which are the most commonly affected animal species. Locations and severity of lesions in animals differ greatly from that reported in humans (Figure 2-2).
Human CDAD is almost always limited to the descending colon, although in most human cases, CDAD is limited to diarrhea and no signs of colitis are observed (McFarland et al., 1989). In more severe cases colitis can develop and may be associated with the formation of a pseudomembrane on the colon epithelium (Hurley and Nguyen, 2002)(Figure 2-3). Pseudomembranes (or raised yellow plaques) are described as mushroom-like mucosal eruptions containing fibrin, mucin, necrotic debris, and immune cells (Froberg et al., 2004). Histologic examination of pseudomembranous colon epithelium shows a characteristic “volcano eruption” appearance (Hurley and Nguyen, 2002)(Figure 2-3). Kelly and LaMont (1998) report a small fraction of human *C. difficile* infections progress to fulminant colitis or toxic megacolon. Toxic megacolon is characterized by inflammation and accumulation of immune cells into the smooth muscle and serosa. Immune cells release nitric oxide which leads to loss of smooth muscle tone.
and bowel distension (Sheikh et al., 2003). Severe CDAD cases can be fatal due to bowel perforation and peritonitis.

Figure 2-3: Pseudomembranous colitis. A: pseudomembranous plaques on colon epithelium. B: Section of colon epithelium showing volcano-like eruption associated with pseudomembranous colitis. C: toxic megacolon of a pseudomembranous colitis patient (Hurley and Nguyen, 2002).

Lesions in pigs have been observed in the colon and cecum and vary from inapparent to transmural necrosis (Keel and Songer, 2006). Yeager et al. (2002) observed mesocolonic edema and colitis in 100% and 72% of piglets with *C. difficile* toxin in the colon contents, respectively.
The presence of CDAD in many of the piglets included in the study was associated with prophylactic or therapeutic antibiotic treatment. In another study, Yaeger et al. (2007) found *C. difficile* toxin was associated with 75% and 76% of piglets where loss of goblet cells and neutrophil infiltration was observed, respectively. They also noted 76% piglets with multifocal erosive lesions were positive for TcdA and TcdB.

As seen in pigs, *C. difficile* associated lesions in adult horses are limited to the cecum and ascending colon (Keel and Songer, 2006). Songer et al. (2009b) gave a description of a case study of a horse euthanized after 48 h of colic and diarrhea. At necropsy they found congested colonic and cecal mucosae, segmental edema, and thickening of the intestinal wall. They also observed 2-4 mm ulcers in the distal colon. Histological examination of colonic sections showed edema in the colonic submucosa. They also observed Gram positive rods and infiltration of mononuclear cells in the submucosa and lamina propria. Lesions in juvenile horses are usually found in the small intestine and are usually more severe than those observed in adult horses. Foals affected by CDAD can be hemorrhagic and have mucosal erosions or ulcers with fibrin and adherent ingesta (Keel and Songer, 2008). Colitis in foals associated with *C. difficile* has been reported but is rare (Magdesian et al., 2002).

There are very few case reports of *C. difficile* infection in cats and dogs, although chronic diarrhea in dogs has been associated with *C. difficile* (Keel and Songer, 2006). Similarly, documentation of *C. difficile* pathogenesis in bovine species is limited. Hammitt et al. (2008) conducted a histologic examination of intestines from diarrheic calves with high fecal toxin levels. They noted erosions with fibrinous exudates as well as distended crypts in the colon containing degenerate epithelial cells and neutrophils. Neutrophil and eosinophil infiltration was observed in the lamina propria of the jejunum, ileum, and colon. Gram staining showed Gram positive rods in the colonic lumen.
Further, Hammitt et al. (2008) inoculated ligated loops of the duodenum, jejunum, ileum, and upper and mid spiral colon of Holstein calves (confirmed toxin negative feces) with TcdA, TcdB, or both toxins. The calves remained anesthetized for 4-6 hours after inoculation and were then euthanized. They found TcdA resulted in accumulation of neutrophils in the lumen, epithelial necrosis, and lamina propria hemorrhage and edema in the duodenum. They also noted mucosal erosion and infiltration of neutrophils associated with TcdA in the jejunum, ileum, and colon. More severe lesions were observed with TcdB including multifocal, ulcerative enteritis in the duodenum and jejunum and necrosis in the ileum. Toxin B resulted in hemorrhagic epithelium, necrosis, and infiltration of neutrophils and lymphocytes in the colon. Innoculation with both toxins showed the same lesions as inoculation with a single toxin except the duodenum and ileum had severe necrosis of the villi. They also noted distended lacteals in the jejunum containing lymphocytes. The ileum showed mucosal edema and the lamina propria was infiltrated with inflammatory cells. Mucosal erosion, hemorrhage, mucus and cellular debris accumulation was observed in the upper spiral colon. Necrosis, vacuolization, and loss of lamina propria was seen in the mid spiral colon and inflammatory cells infiltrated the submucosa. The results of this study clearly showed \textit{C. difficile} toxins are able to cause enteric disease in calves, yet more research is needed to gain an understanding of the pathogenesis of \textit{C. difficile} disease in naturally infected calves.

\textit{Clostridium difficile} causes a wide spectrum of disease ranging from mild diarrhea to psuedomembranous colitis and life threatening toxic megacolon. Research has shown \textit{C. difficile} is able to infect and cause disease in many different animal species and locations and severity of lesions are highly variable. Although infections in horses and pigs are well documented, more research is needed to determine the significance of \textit{C. difficile} as an enteric pathogen in bovine and other agricultural animal species.
2.3 Role of *Clostridium difficile* in Calf Enteritis

In 2007, the National Animal Health Monitoring System reported that 56% of preweaned dairy calf mortality is due to diarrhea (USDA, 2007). In United States, the mortality rate of preweaned heifer calves was 7.8% in 2006. Over 50% of calf death was due to scours, diarrhea, or other digestive diseases (USDA, 2007). Virtala et al. (1996) followed 410 dairy calves over the first three months of life and recorded all disease conditions. They found 118 (28.8%) developed diarrhea and of these, 82 developed diarrhea before they reached 2 weeks of age. Diarrhea was by far the most common non-respiratory disease among young calves when compared to other diseases recorded: umbilical infection (14.2%), umbilical hernia (15.1%), and ringworm (3.9%). They also reported a mortality rate of 5.6%, with the highest mortality observed during the second week after birth. In a similar study, Gulliksen et al. (2009), identified risk factors associated with calf mortality. They found diarrhea significantly increased the risk of death during the first month of life.

*Clostridium difficile* is a well established enteric pathogen in pigs and horses (Keel and Songer, 2006; Ruby et al., 2003; Songer et al., 2009b; Yeager et al., 2002 and 2007). Recently published data suggests *C. difficile* is also associated with enteric disease in calves (Rodriguez-Palacios et al., 2006; Hammitt et al., 2008). Although limited data exists on the significance of *C. difficile* as an enteric pathogen in calves, recent studies have shown an association between diarrhea in calves and the presence of TcdA/B (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). Rodriguez-Palacios et al. (2006) collected fecal samples on 102 dairy farms from 144 and 134 calves with diarrhea and control calves, respectively. They were able to isolate *C. difficile* from 31 calves representing 25 farms. *Clostridium difficile* was found more frequently in control calves, suggesting calves may be an asymptomatic reservoir. Toxins A and B were detected in the
The presence of toxin was associated with diarrheic calves (p=0.0002). Interestingly, toxin and the organism were detected in the same calf on only 6 occasions. The detection of toxin was influenced by factors such as the month of sampling (p=0.008) and the age of the calf (p=0.005). Younger calves were more likely to be positive for toxin than older calves. The administration of antibiotics in feed was not associated with the presence of *C. difficile* or its toxins.

Hammitt et al. (2008) conducted a similar study in which they screened fecal samples from 253 diarrheic and 53 nondiarrheic calves for *C. difficile* and its toxins. *Clostridium difficile* and toxins A and/or B were detected in 13.2% and 30.2% of nondiarrheic calves and in 25.3% and 22.9% of diarrheic calves, respectively. Although toxin was found more commonly in feces of nondiarrheic calves, calves with diarrhea were more likely to have culture positive stools.

Rodriguez-Palacios et al. (2007a) set out to find a causal relationship between *C. difficile* and enteric disease in calves. Eight calves were orally inoculated with toxigenic *C. difficile* (isolated from a diarrheic calf), euthanized on day 6, and compared to 6 control calves who received sterile culture broth. None of the calves developed CDAD. They were able to isolate *C. difficile* from 60.5% of the innocualted calves, but toxin was not detected in any of the inoculated calves at any time during the study. They noted *C. difficile* was isolated from the cecum more often than from the ileum and duodenum. It was confirmed through ribotyping that 84.6% of isolates from inoculated calves were the same strain used for inoculation. Necropsy showed mild to moderate intestinal mucosa congestion, increased intestinal fluid, mesenteric edema, and edematous lymph nodes in 4 control calves and only 2 experimentally inoculated calves. Mild to moderate inflammatory changes were seen more frequently in inoculated calves than control calves. Although oral inoculation with toxigenic *C. difficile* resulted in colonization, it did not result in toxin production or clear evidence of enteric disease. These results suggest certain conditions in the calf gut may influence toxin expression.
Previous research suggests *C. difficile* may be an important enteric pathogen in calves. Unfortunately, a causal relationship between *C. difficile* infection and enteric disease in calves has not yet been established. All studies to date have been based on experimental infection and intoxication or did not include sufficient pathologic and histologic data. More research is needed on the pathology associated with *C. difficile* infection in calves.

### 2.4 Toxin Expression

The genes encoding *C. difficile* toxins TcdA and TcdB were both sequenced in 1990 (Barroso et al., 1990; Dove et al., 1990). The *tcdA* and *tcdB* genes are approximately 8kb and 7 kb, respectively, in length. The two toxin genes are located on a 19.6 kb chromosomal toxigenic element, or pathogenicity locus (PaLoc), with *tcdA* located 1.4 kb downstream of *tcdB* (Figure 2-4) (Hammond et al., 1995). Dove et al. (1990) found an open reading frame, later designated *tcdE*, between the two genes which is translated in the same direction as the toxin genes. A second open reading frame, later designated *tcdC*, was found downstream of *tcdA* which is translated in the opposite direction (Dove et al., 1990). Hammond et al. (1995) found a third open reading frame, referred today as *tcdD* or *tcdR*, upstream of *tcdB*.

Moncrief et al. (1997) showed the 22 kDa protein encoded by *tcdC* had sequence identity with DNA binding regulatory proteins. They showed expression of *tcdD* (or *tcdR*) resulted in 800 fold and 500 fold increases in expression of the reporter gene fused with promoters of *tcdA* and *tcdB*, respectively. These results showed *tcdD* is a positive regulator of *C. difficile* toxin expression. The TcdD protein allows RNA polymerase to recognize *tcdA* and *tcdB* promoters. Mani et al. (2002) determined TcdD is autoregulated by showing it was able to initiate transcription of a reporter gene fused with its own promoter. They also observed expression of TcdD was growth phase and nutrient dependent. High levels of expression were seen in the
stationary growth phase and TcdD expression was repressed in the presence of excess glucose. These results suggested toxin regulation may be controlled, in part, by environmental cues.

Figure 2-4: Organization of the *Clostridium difficile* pathogenicity locus

Hundsberger et al. (1997) suggested the protein encoded by *tcdC* is a negative regulator of *tcdA* and *tcdB* expression. They observed high levels of TcdC protein during the early exponential growth phase which correlated with low levels of the other four proteins (TcdA, TcdB, TcdD, and TcdE) encoded on the PaLoc. During the stationary growth phase they showed TcdC levels dropped and TcdA, TcdB, TcdD, and TcdE levels rose. Matamouros et al. (2007) confirmed TcdC is able to inhibit toxin production by indirectly interfering with the ability of TcdD to recognize the promoters of *tcdA* and *tcdB*.

Identifying factors that may effect toxin production is important because disease severity is correlated with toxin levels in the gut (Akerlund et al., 2006; Vernet et al., 1989). It has been suggested that mutations or deletions in the *tcdC* gene may result in hypervirulence. Curry et al. (2007) found point mutations in the *tcdC* gene that result in a stop codon severely truncate TcdC protein. An epidemic strain associated with human outbreaks contains an 18 bp deletion in the *tcdC* gene (McDonald et al., 2005). Warny et al. (2005) showed this strain produced 16 and 23
fold higher levels of TcdA and TcdB, respectively, than other genetically unrelated isolates. Other researchers claim deletions in the tcdC gene does not effect expression of toxin genes (Murray et al., 2009; Verdoorn et al., 2010). Murray et al. (2009) studied clinical C. difficile isolates and found that tcdC deletions did not correlate with level of toxin production. Verdoorn et al. (2010) found deletions in the tcdC gene were very common among C. difficile isolated from human patients. They did not find a difference in severity of disease between patients carrying strains with tcdC deletions and those carrying strains without deletions.

Tan et al. (2001) carried out a computational amino acid sequence analysis and found that TcdE protein showed similar structural characteristics to those seen in holins, a class of bacteriophage proteins that aid in the release of phage from bacterial host cells. They observed that the site of action of TcdE protein was the bacterial cell membrane using electron microscopy. They concluded that TcdE most likely has a lytic function which enables the release of TcdA and TcdB from C. difficile cells.

Variations in the PaLoc have been reported including differences in sequences of the five genes and the complete absence of tcdA or tcdB genes (1998). Rupnik et al. (1998) developed a strain typing system based on these variations in 1998, which defined 10 different toxinotypes based on differences in the PaLoc as compared to reference strain VPI 10463. Five new toxinotypes were reported in 2001 and five more novel toxinotypes were described in 2003. To date, 20 different toxinotypes have been identified (Rupnik et al., 2001 and 2003). No differences in virulence have been established based on toxin gene variation among strains (Voth, 2005).

Perelle et al. (1997) cloned and sequenced the CDT genes, cdtA and cdtB. The genes encoding CDT are not found on the PaLoc, but elsewhere on the chromosome. Carter et al. (2007) found a third functional gene on the cdt locus (CdtLoc), cdtR. They determined CdtR is required for CDT expression by incorporating cdtA and cdtB in cdtR+ and cdtR- strains. Transformants containing intact cdtR produced more CDT than strains with cdtR mutations.
Many bacterial species alter gene expression patterns to support survival as environmental conditions change (Vorob’eva, 2003). Induction of virulence related genes by environmental signals has been reported for many species of bacteria (Cotter and Miller, 1998; Mekalanos, 1992). It has been shown that toxin expression by *C. difficile* can be regulated by environmental signals (Figure 2-5) (Dupuy et al., 2008; Drummond et al., 2003; Gerber et al., 2008; Karlsson et al., 2003; Onderdonk et al., 1979; Yamakawa et al., 1996).

**Figure 2-5:** Regulation of toxin expression by environmental signals (Dupuy et al., 2008)

Onderdonk et al. (1979) showed higher toxin production upon incubation at 45°C when compared with a 37°C incubation. The same effect was seen when the oxidation reduction potential of the media was changed from -360 mV to 100 mV. Yamakawa et al. (1996) and Karlsson et al. (2003) both observed that the reduction of biotin, a nutrient essential for growth, in growth media resulted in decreased growth of *C. difficile* and increased toxin production. Karlsson et al. (2003) also observed that providing increased amounts of essential amino acids
increased the growth rate of *C. difficile*, but did not result in a marked increase in toxin production. These results suggest toxin production may be a stress related response.

Sub-inhibitory concentrations of antibiotics used in human medicine have been shown to increase mRNA levels of TcdA/B as well as toxin levels in culture supernatant when added to growth media (Dupuy et al., 2008; Gerber et al., 2008; Onderdonk et al., 1979). Drummond et al. (2003) observed an extension of the lag phase in growth of *C. difficile* when exposed to sub-inhibitory concentrations of vancomycin, metronidazole, clindamycin, amoxicillin, cefoxitin or ceftriaxone. This extension in lag phase was most dramatic at one half of the minimum inhibitory concentration (MIC) of antibiotic. This effect was not seen in the presence of clindamycin for a strain with a very high MIC for the drug. Drummond et al. (2003) also noticed an increase in toxin production and/or earlier toxin production with respect to the growth curve upon antibiotic exposure, although different strains of *C. difficile* reacted differently to the same antibiotic.

Similar results have been published by Gerber et al. (2008). They observed a delay in growth of *C. difficile* and detected TcdA/B toxins much earlier in the growth curve upon exposure to sub-inhibitory concentrations of metronidazole, vancomycin, and linezolid. They did not see any increase in toxin production with sub-inhibitory concentrations of clindamycin.

Understanding how antibiotics may affect the pathogenesis of *C. difficile* is critical in calves. Young calves are commonly administered antibiotics for treatment and prevention of illness, improvement of digestion, and growth promotion. The most common antibiotics used in calves are tetracyclines and sulfonamides (Frost, 1991; Sawant et al., 2005). Other antibiotics commonly used on dairy farms include ampicillin, erythromycin, and florfenicol (Gow et al., 2009, Sawant et al., 2005). Oxytetracycline is incorporated in milk replacer at doses of 0.05-0.1 mg/lb body weight per day to promote growth and improve digestion. A dose 10 mg/lb body weight per day (for 7-14 days) is administered in milk replacer for treatment of enteritis (USDA, 2008).
Oxytetracycline is a bacteriostatic antibiotic which inhibits growth of both Gram positive and Gram negative bacteria by targeting the 30S subunit of the ribosome. Protein synthesis is inhibited by preventing the binding of aminoacyl-tRNA to the ribosome (Bryskier, 2005). Sub-inhibitory concentrations of clinamycin, an antibiotic used in human medicine which also interferes with protein synthesis, has been shown to increase toxin expression in certain C. difficile strains (Drummond et al., 2003).

Fractions of orally administered oxytetracycline are excreted in the feces (Bryskier, 2005; Kuijper et al., 2001; Kunin and Finland, 1960). Experiments in rats showed close to 80% of orally administered tetracycline was excreted in the feces over 7 days following treatment (Kelly and Buyske, 1960). In humans, excretion of orally administered tetracyclines in the feces has been reported as highly variable. Excretion at a range of 23% to 73% of the dosage has been observed, most likely due to variable absorption rates in the gastrointestinal tract (Kunin and Finland, 1960). When administered with foods containing calcium, such as milk, absorption of tetracyclines is reduced due to ion binding (Bryskier, 2005). The result is that gut microflora is exposed to a larger fraction of the administered dose, although antimicrobial activity may be reduced (Chiang, 1981; Nouws, 1992).

It is well documented that administration of antibiotics is a risk factor for the development of CDAD in both humans and animals (Bartlett, 1997; Clooten et al., 2008; Kelly and LaMont, 1998; Weese et al., 2009b; Yeager et al., 2002). Although antibiotics have been shown to increase C. difficile toxin production in vitro, the significance of excreting antibiotics in feces with respect to severity of CDAD has not been determined.
2.5 Detection, Diagnosis, and Typing Methods

Methods for diagnosing *C. difficile* infection routinely include culture, cytotoxicity assay, or immunoassay for the detection of toxigenic *C. difficile* in fecal samples (Bartlett, 1997). The cytotoxicity assay is considered the gold standard for CDAD diagnosis. In most cases diluted fecal samples are used to inoculate tissue culture. When a fecal sample is cytotoxic, the presence of TcdB is confirmed through neutralization of the toxin with *C. sordellii* antitoxin (Bartlett, 1997). Cytotoxicity assays are time consuming with a turnaround time of more than 48 hours (Johnson and Gerding, 1998; Oldfield, 2004; van den Berg et al., 2007). Culture methods are also time consuming and considered inferior in sensitivity and specificity. According to Weese et al. (1999), low survival rate of the organism in feces over time can lead to a false negative culture result if there is a delay in sample processing. Culture may also result in false positive results because there is no specificity for toxin producing strains. Immunoassays provide a more rapid sample throughput and are widely used in diagnostic settings, although specificity of such assays has been questioned (Barbut et al., 1993; Shanholtzer et al., 1992). Toxin is susceptible to proteolytic degradation in fecal samples which increases the likelihood of false negative results if samples are not stored properly or if delays in sample testing occur (Bartlett et al., 1997).

Due to the emergence of TcdA negative/TcdB positive strains as well as TcdA negative/TcdB negative/CDT positive strains an assay capable of detecting more than one toxin is needed (Drudy et al., 2007; Geric et al., 2003). Nucleic acid amplification methods for the detection of microbes have been found to have unrivaled sensitivity and specificity and yield results much faster than culture-based methods (Louie et al., 2000). The specificity of primers to a DNA target weighs heavily on the accuracy of a PCR reaction. The amplification of non-specific products can yield false positive results. The use of a fluorescent labeled probe in real-time PCR assays minimizes the risk of amplification of non-specific targets since the binding of
the probe as well as the primers to the target sequence is needed for a signal to be produced. Real-time PCR assays are gaining popularity over traditional PCR methods in diagnostic settings because of their increased sensitivity and specificity, increased sample throughput, and because the use of low resolution gel detection methods can be excluded.

Recent studies have shown that real-time PCR based assays are superior to traditional PCR, immunoassays, and culture based methods for detection of genetic elements of toxigenic *C. difficile* in fecal samples (van den Berg et al., 2007). van den Berg et al. (2006) reported sensitivity, specificity, positive predictive value, and negative predictive value of a real-time PCR assay targeting the *tcdB* gene to be 87%, 96%, 57%, and 99% when compared to the cytotoxicity assay, respectively. In a more recent study, the same researcher reported culture concordance values of 53.6%, 55.4%, and 71.4% for the VIDAS immunoassay, the PTAB immunoassay, and a real-time PCR assay, respectively (van den Berg et al., 2007). Stamper et al. (2009a) assessed a commercially available real-time PCR assay for *tcdB*. When compared to culture, the real-time PCR assay and a cytotoxin detection assay had sensitivity, specificity, positive predictive values, and negative predictive values of 83.6%, 98.2%, 89.5%, and 97.1% and 67.2%, 99.1%, 93.2%, and 94.4 %, respectively. Stamper et al. (2009b) later evaluated a different real-time PCR assay for the *tcdB* gene and found a sensitivity, specificity, positive predictive value, and negative predictive value of 83.3%, 95.6%, 69.4%, and 98%, respectively, when compared to an immunoassay targeting TcdB protein. Eastwood et al. (2009) reported similar findings when comparing toxin detection methods to a real-time PCR assay targeting the *tcdB* gene. The real-time PCR assay had the highest sensitivity and highest negative predictive value when compared to other assays. Kvach et al. (2010) also found real-time PCR to be more sensitive (*p*=0.0001) than *C. difficile* toxin immunoassay.

Many PCR based methods for the detection of *C. difficile* toxin genes have been reported (Antikainen et al., 2009; Belanger et al., 2003; Lemee et al., 2004; Peterson et al., 2007). Most of
these assays only include detection of genes encoding TcdA and TcdB or require gel electrophoresis analysis. Peterson et al. (2007) designed a real-time PCR assay for the detection of genetic elements of toxigenic *C. difficile* with sensitivity, specificity, positive predictive value, and negative predictive value of 93.3%, 97.4%, 75.7%, and 99.4%, respectively (Peterson et al., 2007). The assay only targeted the *tcdB* gene. Guilbault et al. (2002) reported specificity and sensitivity of 100% and 91.5% for a PCR assay they developed. This was a traditional PCR assay and also only targeted the *tcdB* gene. Another PCR assay targeting the *tcdB* gene only was developed by van den Berg et al. (2006). They reported sensitivity, specificity, positive predictive value, and negative predictive value of 100%, 97%, 55%, and 100%, respectively, for the real-time PCR assay when compared to the cytotoxicity assay. Lemee et al. (2004) developed a multiplex assay for the detection of toxin genes *tcdA* and *tcdB* as well as *tpi*, a housekeeping gene specific to *C. difficile*.

Until recently, symptoms related to *C. difficile* infection were mostly attributed to toxins TcdA and TcdB (Barbut et al., 2005). The role of CDT in disease is not well understood, although researchers have reported strains that produce CDT but do not produce TcdA and TcdB. It has been shown that CDT protein increases the adherence abilities of *C. difficile* suggesting CDT may be considered a virulence factor (Schwan et al., 2009). Geric et al. (2003) reported that 15.5% of *tcdA* and *tcdB* negative *C. difficile* isolates examined were positive for both *cdtA* and *cdtB*. Therefore, assays that detect toxigenic *C. difficile* based on the presence of only *tcdA* and *tcdB* may yield false negative results. There is a need for a reliable, high throughput, multiplex real-time PCR assay capable of detecting genes encoding for all three toxins.

Although both genotypic and phenotypic typing methods have been employed for characterizing *C. difficile* isolates, genotypic methods are preferred (Braizer, 2001). Plasmid profiling has been used to type *C. difficile* strains, but sparse distribution of plasmid DNA among isolates proved this method impractical (Braizer, 2001). Restriction fragment length
polymorphism analysis has also been used, but was quickly replaced by less labor-intensive PCR methods. Arbitrarily primed PCR assays have been developed but are criticized for their lack of reproducibility (Collier et al., 1995). One of the most popular PCR based typing methods is ribotyping. Gurtler (1993) showed C. difficile isolates can be distinguished by the number and fragment sizes of the spacer regions between 16S and 23S rRNA genes. Rupnik et al. (1998) developed toxinotyping, another popular PCR-based typing method, which compares polymorphisms in the PaLoc. It has been demonstrated that PCR-ribotyping and toxinotyping correlate well (Rupnik et al., 2001).

Pulsed field gel electrophoresis (PFGE) has been used in many epidemiologic studies for typing C. difficile strains (Clatyon et al., 2009; Kuijper et al., 2006; Limbago et al., 2009; Talon et al., 1995). Researchers prefer PFGE over ribotyping because it offers superior discriminatory power (Bidet et al., 2000). More recently, molecular methods such as multilocus variable-number tandem-repeat analysis, multilocus sequence typing, and amplified fragment length polymorphism techniques have been employed for typing C. difficile isolates (Griffiths et al., 2010; Marsh et al., 2010; van den Berg et al., 2004). Although some studies have shown these methods have better discriminatory power, PFGE, ribotyping, and toxinotyping are still most commonly used in epidemiological studies (Killgore et al., 2008).

Many C. difficile typing methods exist and researchers must weigh the advantages and disadvantages of all the methods when choosing which method they prefer. Struelens (1998) states that the suitability of any typing method is based on typeability, reproducibility, stability, discriminatory power, and epidemiological concordance. Although many studies have been carried out to compare which typing methods are best for C. difficile, no unanimously preferred method has been identified (Brazier, 2001).
2.6 Epidemiology

*Clostridium difficile* infections in humans are classically considered nosocomial and mainly affect elderly hospital patients. Spores produced by *C. difficile* contaminate the hands of healthcare workers and the hospital environment and are transferred via the fecal-oral route (Sunenshine and McDonald, 2006). Infection can only occur when spores are ingested and colonization resistance is inhibited through disruption of the normal gut flora (Barbut and Petit, 2001). This disruption is commonly caused by broad spectrum antibiotic treatment. In fact, over 90% of *C. difficile* infections are associated with antibiotics (Barbut and Petit, 2001). *Clostridium difficile* infections were first associated with patients in healthcare settings who had been administrated clindamycin (Bartlett et al., 1978; Climo et al., 1998; Pear et al., 1994). Today, CDAD has been associated with almost all antibiotics except aminoglycosides (Sunenshine and McDonald, 2006). Use of proton pump inhibitors is also considered a risk factor for CDAD in humans (Dial et al., 2005).

Nosocomial *C. difficile* infections are also an issue in veterinary hospitals. Clooten et al. (2008) found cats and dogs that acquired *C. difficile* in veterinary hospitals were 4.05 times more likely to develop diarrhea than *C. difficile* negative animals. They determined administration of antibiotics (p=0.018) prior to hospitalization and administration of immunosuppressive (p=0.006) drugs during hospitalization were risk factors for developing a nosocomial *C. difficile* infection.

Bartlett et al. reports 15-25% of nosocomial antibiotic-associated diarrhea and 95% of PMC in humans is attributed to *C. difficile* infection (Bartlett, 1994; Bartlett and Gerding, 2008). It is estimated that nosocomial *C. difficile* infections cost the United States $1.1 billion per year (Kyne et al., 2002). Clinicians have been finding human disease increasing in severity and more difficult to treat than in the past (Hookman and Barkin, 2007; Sunenshine and McDonald, 2006). Among patients diagnosed with CDAD, relapse or re-infection occurs in 12%-24% within 2
months (Sunenshine and McDonald, 2006). Mortality rates of disease associated with \textit{C. difficile} have risen in the United States from 5.7 per million to 23.7 per million from 1999 to 2004 (Redelings et al., 2007).

Although \textit{C. difficile} infections have been limited to healthcare settings in the past, reports of community acquired cases are now being reported. In 2005, the CDC reported cases of serious \textit{C. difficile} infections in individuals from populations not thought to be at risk including young children and pregnant women (CDC, 2005). Out of 33 cases, 8 (24\%) had not been exposed to antibiotics within 3 months of infection. Within these 8 cases, 3 had close contact with individuals with diarrhea, two of which were confirmed to be \textit{C. difficile}-associated. Three (9\%) of the 33 cases had only received 3 or less doses of antibiotics before becoming infected, two of which received only one dose of clindamycin. The CDC found \textit{C. difficile} was transmitted peripartum to children of \textit{C. difficile} infected patients in two cases. In one of these cases, \textit{C. difficile} was also transmitted to a family friend. One case had been caring for an elderly \textit{C. difficile} patient. Another case had visited a parent with \textit{C. difficile} infection in a nursing home.

Hirschhorn et al. (1994) reported the incidence of community acquired CDAD to be 7.7 cases per 100,000 person years. Wilcox et al. (2008) conducted a case-control study on community acquired \textit{C. difficile} infections and found only half of the cases included in the study had received antibiotic therapy within four weeks of infection and one third had not been exposed to antibiotics or recently hospitalized. They also reported community acquired infection rates were higher in urban and semi-rural areas of the United Kingdom (Wilcox et al., 2008). Forward et al. (2003) found \textit{C. difficile} cytotoxin in 0.6\% of fecal specimens from community cases of diarrhea. Factors associated with cytotoxin in feces of community diarrhea cases included antibiotic therapy, age of 60 years or older, and elderly people living in the household.

Many environmental sources of exposure to \textit{C. difficile} in the community have been reported. Weese et al. (2009b) detected \textit{C. difficile} in 5.3\% of sites tested in 31\% of households.
al Saif et al. (1996) isolated *C. difficile* from 2.2%, 2.3%, and 2.0% of nursing homes, family houses, and student residences, respectively. They also found *C. difficile* in 21.4%, 36.0%, and 2.3% of soil, water, and raw vegetable samples, respectively. Recent reports suggest toxigenic *C. difficile* may be a foodborne pathogen. Many researchers have reported the contamination of raw meats with *C. difficile* (Bouttier et al., 2010; Jobstl et al., 2010; Rodriguez-Palacios et al., 2007b; Songer et al., 2009a; von Abercron et al. 2009; Weese et al., 2009a; Weese et al., 2010a). It has been shown that *C. difficile* spores survive in meat even after heating to proper cooking temperature (Rodriguez-Palacios et al. 2007).

The increasing virulence and changing epidemiology of *C. difficile* has resulted in major public health concerns. Once considered a nosocomial pathogen, community acquired *C. difficile* infections are becoming more common. This change in disease pattern requires a reassessment of current strategies for CDAD prevention and control. Although CDAD has been well researched over the past 40 years, factors contributing the virulence and epidemiology of CDAD in humans and animals remain to be identified.

### 2.7 Zoonosis and Food Safety

A study by Lemee et al. (2004) reported 27 out of 622 of animal stool samples contained toxigenic *C. difficile*. Reports of outbreaks of enteric disease associated with fairs and petting zoos have increased in the US and common sources of infection include cattle (Steinmuller et al., 2006). Borriello et al. (1983) reported 23% of household pets carried *C. difficile*. Weese et al. (2009b) isolated *C. difficile* from 10% of dogs tested and found carriage in dogs was associated with immunocompromised owners. al Saif et al. (1996) found *C. difficile* in 10%, 2%, 1%, 1%, and 2% of fecal samples from dogs, cats, horses, sheep, and poultry, respectively. They also reported *C. difficile* in 16.7% of environmental samples collected from veterinary clinics. A study
in Zimbabwe found feces from 29% of chickens at live markets were positive for *C. difficile* and 89.7% of isolates were toxigenic (Simango and Mwakurudza, 2008). They also reported 22.0% of soil samples collected at these markets contained *C. difficile* and 95.5% of isolates were toxigenic.

Previous research has shown genotypic similarity between *C. difficile* isolates of human and animal origin, suggesting animals should be considered an important source of exposure to *C. difficile* relevant to public health (Indra et al., 2009; Jhung et al., 2008; Rodriguez-Palacios et al., 2006; Rupnik et al., 2007). Jhung et al. (2008) found five isolates from pigs to be indistinguishable from human isolates when comparing PFGE patterns of *SmaI* digests. Indra et al. (2009) characterized *C. difficile* isolates from cows, pigs, and broiler chickens in Austria. They reported a pig isolate with ribotype 126, which is very similar to ribotype 078, a human outbreak strain. Rodriguez-Palacios et al. (2006) observed 8 different ribotypes among 31 *C. difficile* isolates from calves. Four of these isolates were ribotype 027, a North American human outbreak strain considered to be highly virulent (Loo et al., 2005; Rodriguez-Palacios, 2006; Warny et al., 2005).

Food animals including cows, calves, pigs, and broiler chickens have been identified as carriers of toxigenic *C. difficile* (Avbersek et al., 2009; Indra et al., 2009; Jhung et al., 2008; Songer and Anderson, 2006). *Clostridium difficile* has been isolated from raw meats such as beef, veal, pork, chicken and turkey intended for human consumption (Bouttier et al., 2010; Jobstl et al., 2010; Rodriguez-Palacios et al., 2007b; Songer et al., 2009a; von Abercron et al. 2009; Weese et al., 2009a; Weese et al., 2010a). Weese et al. (2009a) reported 12% of Canadian retail ground beef and pork samples contained toxigenic *C. difficile*, although upon enumeration, contamination levels were found to be low. In another study *C. difficile* was detected in 20.8% and 14.3% of Canadian retail ground beef and veal, respectively, and most isolates encoded for TcdA, TcdB, and CDT (Rodriguez-Palacios et al., 2007b). Songer et al. (2009a) found a much higher incidence
of *C. difficile* in retail ground meat in Tucson, Arizona. In their study 42.4%, 41.3%, and 44.4% of beef, pork, and turkey were found to contain toxigenic *C. difficile*, respectively. Weese et al. (2010a) detected *C. difficile* in 9.0%, 18.0%, and 15.0% of chicken thighs, wings, and legs, respectively. Other studies report a low prevalence of *C. difficile* in ground meat. A recent study in Sweden found little or no incidence of *C. difficile* in ground meat (von Abercron et al., 2009). von Abercron et al. (2009) found *C. difficile* in 6.2% of ground beef samples, but were unable to detect *C. difficile* in ground pork, mixed beef and pork, sheep, reindeer, moose, veal, and poultry. Jobstl et al. (2010) found only 3 out of 100 of Austrian retail raw meat samples were contaminated with *C. difficile*. All of the *C. difficile* positive samples were mixed beef and pork. Only one of these samples contained *C. difficile* encoding toxins TcdA and TcdB. No *C. difficile* was recovered from beef or raw milk. A study in France found only 2 out of 109 ground beef samples were contaminated with *C. difficile* (Bouttier et al., 2010). Isolates from both samples were toxigenic. Both samples were vacuum packaged which may have facilitated the survival of the organism due to anaerobic conditions.

These findings suggest *C. difficile* may be considered a zoonotic agent disseminated from food animals through direct contact or human consumption of contaminated food animal products (Rupnik, 2007; Songer et al., 2009b). Researchers have established calves can be asymptomatic carriers of toxigenic *C. difficile* and may be a reservoir relevant to public health (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). It is possible that humans may be exposed to toxigenic *C. difficile* through consumption of veal. To date, no data exists on the possible dissemination of *C. difficile* from asymptomatic veal calves to humans through veal.

Although *C. difficile* is able to contaminate raw meat, characterizing *C. difficile* as a foodborne may be difficult. It is possible that *C. difficile* infection may not occur until sometime after ingestion of contaminated meat, making it difficult to determine a point of exposure. It is also possible that foodborne exposure is only significant if other risk factors are encountered, such
as immune suppression, subsequent antibiotic therapy, or use of proton pump inhibitors (Borriello, 1990; Dial et al., 2005; Weese, 2010b). Although no incidences of foodborne C. difficile infection have been reported, ground meat may be a source of exposure significant to community acquired CDAD (Weese, 2010b).

2.8 Summary

Although the literature reviewed here offers extensive knowledge of the epidemiology and pathology of C. difficile-associated disease in humans as it relates to nosocomial infection, many holes in the data exist. Little data exists pertaining to the significance of infection in certain animal species, particularly bovine calves, and the epidemiology of community acquired C. difficile infections in humans.

Gross pathology and histology data from naturally infected calves is needed to determine if C. difficile can be considered a significant etiologic agent of enteric disease in calves. In order to prevent C. difficile-associated enteric disease in calves, risk factors for disease must be established. Researchers have previously reported that exposure antibiotics used in human medicine leads to increased toxin production by certain C. difficile strains. Treating calves with antibiotics excreted in the gut may be a risk factor disease. Therefore, an investigation of the effects antibiotics used to treat calves on toxin production by C. difficile is needed.

The growing public health significance of community acquired C. difficile infections has become a great concern for physicians and public health workers. Although the risk factors hospital-acquired cases are well defined, these risk factors do not seem to apply to community acquired infections. It is known that animal species can be asymptomatic carriers of toxigenic C. difficile strains that have been associated with human infection. Researchers have inferred that C. difficile may be disseminated to humans through consumption of contaminated meat.
Contamination of ground meat meant for human consumption has been reported, but no data exists on the prevalence of *C. difficile* throughout the meat production continuum.

This review of literature reveals the epidemiology of *C. difficile*-associated disease in animal species as well as humans changed over the last decade. The emergence of hypervirulent strains of *C. difficile* has presented new challenges in controlling infections attributed to this well established pathogen. It is crucial to address gaps in knowledge regarding the epidemiology of toxigenic *C. difficile* to determine its significance in animal disease, to understand how the pathogen may disseminated through the food production continuum, and to determine the overall public health risks.
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Chapter 3

Real-time multiplex PCR assay for rapid detection of *Clostridium difficile*

*toxin producing strains*

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3.1 Abstract

*Clostridium difficile* is considered an important, well established pathogen capable of causing disease in humans and animal species. In our study, we developed and evaluated a real-time multiplex PCR assay for the rapid detection of *C. difficile* genes encoding toxin A (*tcdA*), toxin B (*tcdB*), and binary toxin (*cdtA* and *cdtB*). The detection limit of the standardized real-time multiplex PCR assay for toxin genes of *C. difficile* was $10^3$ cells/g and $10^1$ cells/g for non-enriched and enriched fecal and ground meat samples, respectively. The assay was used to screen for genetic elements of toxigenic *C. difficile* in fecal samples from 71 pre-weaned calves and 53 retail ground meat samples. All samples were also examined for *C. difficile* using traditional culture techniques to validate the PCR assay. A total of 24 fecal samples (33.80%) were positive for toxigenic *C. difficile* using either multiplex real-time PCR or culture. *Clostridium difficile* toxin genes were detected in 23 enriched fecal samples using the multiplex real-time PCR assay and only 15 samples using culture techniques. Sensitivity, specificity, positive predictive value, and negative predictive value for the multiplex real-time PCR assay were 0.93, 0.84, 0.61, and 0.98 for enriched fecal samples and 0.67, 0.86, 0.55, and 0.91 for non-enriched fecal samples. *C. difficile* was not detected in ground meat by traditional culture or real time PCR assay. Eleven fecal samples were positive for all 4 toxin genes suggesting pre-weaned calves may be a likely source for toxigenic *C. difficile*. Based on the findings of our study it can be concluded that real-time multiplex PCR carried out on samples enriched for *C. difficile* is a reliable, sensitive, and specific diagnostic tool for rapid screening and identification of samples contaminated with *C. difficile*. 
3.2 Introduction

*Clostridium difficile* is a well established human pathogen known to cause a disease state ranging from mild diarrhea to fulminant colitis, which may result in death. Infection has been associated with the use of gastric acid suppressing agents and antibiotics which allow survival of spores in the stomach and selection for *C. difficile* in the gut (Beaugerie et al., 2003; Dial et al., 2005. In the past, *C. difficile* was considered a nosocomial pathogen that mainly affected the elderly, the severely ill, and long term hospital inpatients (CDC, 2005; Hookman and Barkin, 2007). Recently, an increase in community acquired *C. difficile* associated disease (CDAD) has been reported in populations that were previously considered at low risk of infection (CDC, 2005).

*Clostridium difficile* has also been identified as a cause of enteritis in many animal species and has recently been identified as a possible etiologic agent of enteritis and diarrhea in calves (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). Food animals including cows, calves, pigs, and broiler chickens have been identified as carriers of toxigenic *C. difficile* (Indra et al., 2009, Jhong et al., 2008; Songer and Anderson, 2006). Toxigenic *C. difficile* (TXCD) has also been isolated from ground meats such as beef, veal, pork, and turkey intended for human consumption (Rodriguez-Palacios et al., 2007; Songer et al., 2009; Weese et al., 2009). These findings suggest *C. difficile* may be considered a zoonotic agent disseminated from food animals through direct contact or human consumption of contaminated food animal products (Rupnik, 2007; Songer, 2009).

*Clostridium difficile* associated disease is caused by 2 large clostridial toxins, TcdA and TcdB (Banno et al., 1984). Both toxins act on the Rho family of proteins through monoglucosylation (Just et al., 1995a; Just et al., 1995b). Disruption of F-actin regulation leads to loss of cytoskeletal integrity resulting in the loss of tight junctions between intestinal epithelial
cells (Keel and Songer, 2006). Some toxigenic strains also produce a binary toxin, CDT, which acts through ADP-ribosylation of actin (Popoff et al., 1988). This toxin is expressed in 2 components, an enzymatic subunit (CDTA) and a binding subunit (CDTB).

Increases in community acquired cases and of severity of CDAD and the emergence of hypervirulent strains have resulted in increasing interest in diagnosis and monitoring of pathogen prevalence in the environment (Hookman and Barkin, 2009). Diagnostics methods routinely include culture, cytotoxicity assay, and/or immunoassay for the detection of C. difficile toxins A and/or B. A low survival rate of the organism in feces over time can lead to a false negative culture result if there is a delay in sample processing (Weese et al., 2009). Culture may also result in false positive results because there is no specificity for toxin producing strains. Immunoassays provide a more rapid sample throughput and are widely used in diagnostic settings, although sensitivity of such assays has been questioned (Barbut et al., 1993; Shanholtzer et al., 1992). Also, the emergence of TcdA negative/TcdB positive strains as well as TcdA negative/ TcdB negative/CDT positive strains necessitates immunoassays capable of detecting more than one toxin, significantly increasing the cost of accurate diagnosis (Drudy et al., 2007; Geric et al., 2003).

PCR amplification of target genes is known to have very high specificity and sensitivity when compared to other pathogen detection methods (Louie et al., 2000). The advent of probe based real-time PCR has further increased the specificity and sensitivity of such assays. Probe based real-time PCR assays allow detection of targets much earlier during PCR cycling and negates the need for post-assay analysis of the PCR product. Therefore, probe based real-time PCR allows the acquisition of results in less time as compared to other diagnostic methods.

The objectives of this study were to develop, standardize, and validate a high throughput assay for detection of C. difficile toxin genes using multiplex real-time PCR directly from fecal and food samples.
3.3 Materials and methods

3.3.1 Real-time multiplex PCR design and standardization

Three well characterized reference strains of *C. difficile* were obtained from Dr. J. Glenn Songer of The University of Arizona (CDa: non-toxigenic; CDb: *tcdA* positive/*tcdB* negative/*cdtA* negative/*cdtB* negative; CDC: *tcdA* positive/*tcdB* positive/*cdtA* positive/*cdtB* positive). Non-*difficile* *Clostridium* isolates were obtained from The Pennsylvania State University Animal Diagnostic Laboratory (PSU-ADL) including: *C. glycolicum*, *C. novyi*, *C. perfringens*, and *C. septicum*. Other reference strains used for real-time multiplex PCR assay standardization in this study include *Escherichia coli*, *Salmonella enterica*, and *Staphylococcus aureus* (Table 3-2). DNA was isolated from all reference strains using standard phenol/chloroform extraction methods (Pospiech and Neumann, 1995).

Primers and Taqman probes (Operon, Huntsville, AL) for *tcdA*, *tcdB* (Genbank X92982), *cdtA*, and *cdtB* (Genbank EF581852) are listed in Table 3-1 and were designed using Beacon Designer 7 software (Premier Biosoft International, Palo Alto, CA). Real-time multiplex PCR for *C. difficile* toxin genes was carried out using 2 duplex reactions. Reaction 1 included *tcdA* (FAM labeled probe) and *tcdB* (HEX labeled probe) while reaction 2 included *cdtA* (FAM labeled probe) and *cdtB* (HEX labeled probe). Primer and probe concentrations and PCR conditions were optimized for 2 duplex reactions so that standard curves yielded $R^2$ values above 0.900. Template DNA was added to each duplex reaction containing two sets of forward and reverse primers each at a concentration of 50 nM, two respective probes each at a concentration of 100 nM, and 12.5 ul 2X Brilliant Multiplex QPCR Mastermix (Stratagene, La Jolla, CA) for a final volume of 25 ul. Seven dilutions (highest amount used in PCR reaction was 4.2ug CDc DNA, lowest amount used was 4.2 pg CDc DNA) were subjected to PCR reactions 1 and 2 to determine the lowest detectible amount of TXCD DNA (Figure 3-1). Both duplex PCR reactions were carried out.
using approximately 50 ng of DNA isolated from well characterized TXCD strains as well as non-
*C. difficile* strains to ensure specificity to respective toxin gene targets. An initial
denaturation cycle of 95ºC for 10 min was followed by 35 cycles of 95ºC for 1 min
(denaturation), 54ºC for 30 sec (annealing), and 72ºC for 1 min (primer extension). DNA from
TXCD (CDc) was serially diluted in 10X increments.

PCR product from standard *C. difficile* strains generated by multiplex real-time PCR
reactions 1 and 2 were run on a 2% agarose gel. The bands were excised and purified using gel
extraction spin columns (Bio-Rad, Hercules, CA). The purified PCR product was submitted for
sequencing at the Penn State Nucleic Acid Facility to ensure the intended gene fragment was
amplified.

### 3.3.2 Real-time multiplex PCR laboratory validation

Detection limits of the assay for detection of TXCD in fecal and ground meat samples
was determined (Figure 3-1). Three fecal and ground veal samples which tested negative for *C.
difficile* using both culture and multiplex real-time PCR were separated into 1 g aliquots. Aliquots
were inoculated with known 10 fold dilutions of CDc. Each aliquot was homogenized in 9 ml
cycloserine cefoxitin fructose broth (CCFB; per liter: 40 g proteose peptone, 6 g fructose, 5 g
disodium phosphate, 2 g sodium chloride, 1 g monopotassium sulfate 0.1 g magnesium sulfate,
500 mg cycloserine, 16 mg cefoxitin). One ml of the suspension was removed for DNA
extraction and 100 ul of the suspension was plated on cycloserine cefoxitin fructose agar (CCFA;
same formulation as CCFA with 15 g/l agarose and 30 mg/l neutral red). Extraction of DNA was
carried out using the QIAamp DNA Stool Mini Kit™ (Qiagen, Valencia, CA). This kit was
chosen based on its ability to remove PCR inhibitors from DNA samples. The remaining
suspension was incubated at 37ºC for 5 days in an anaerobic chamber. After incubation, DNA
was extracted from 1ml of broth culture and 100ul of the culture was plated on CCFA. All CCFA
plates were incubated at 37°C for 5 days and *C. difficile* colonies were enumerated. The DNA extracts were examined for TXCD using the multiplex real-time PCR assay to determine the lowest detectible copy number of *C. difficile* toxin genes in DNA extracted directly from feces and ground meat and from enriched cultures.

### 3.3.3 Real-time multiplex PCR field validation

A variety of samples (n=124) including calf feces (n=71) and food animal products meant for human consumption (ground beef (n=9), pork (n=6), veal (n=21), turkey (n=9), and chicken (n=8)) were examined for the presence of TXCD. Fecal samples used in the study were obtained from PSU-ADL. Pathologists collected fecal samples from calves less than 5 weeks of age submitted to PSU-ADL for routine necropsy over a period of 14 months. Ground meat samples were purchased from 3 different local grocery stores of State College, Pennsylvania on 3 occasions. Fecal and meat samples were split into two 1 g aliquots. DNA was extracted directly from one aliquot of each sample. The other aliquot was enriched for *C. difficile* using previously described methods (Rodrigues-Palacios et al., 2006). Briefly, aliquots were mixed with 9 ml cycloserine cefoxitin fructose broth (CCFB; per liter: 40 g proteose peptone, 6 g fructose, 5 g disodium phosphate, 2 g sodium chloride, 1 g monopotassium sulfate 0.1 g magnesium sulfate, 500 mg cycloserine, 16 mg cefoxitin) and incubated anaerobically at 37°C for 5-7 days and DNA was extracted from 1 ml of culture. Extraction of DNA was carried out using the QIAamp DNA Stool Mini Kit™ (Qiagen, Valencia, CA). Extracted DNA from both enriched and non-enriched samples was screened for *C. difficile* toxin genes using the multiplex real-time PCR assay described above. In order to further ensure specificity of the assay, all samples were also screened for *tpi*, a housekeeping gene specific to *C. difficile*, as described by Lemee et al. (2004).
In order to validate the results of the multiplex real-time assay, enriched fecal samples were also cultured for *C. difficile* (Figure 3-1). Five ml of 100% ethanol was added to 5ml of the CCFB culture and incubated at room temperature for 1 hour. Cultures were then pelleted and resuspended in PBS. The suspension was streaked onto cycloserine cefoxitin fructose agar (CCFA; includes same ingredients as CCFB with the addition of 0.03g neutral red and 15g agarose per liter) and incubated anaerobically at 37°C for 3-5 days. For sample plates with colonies showing characteristic morphology of *C. difficile*, 2-3 colonies were subcultured and stored for confirmation of *C. difficile* identity. DNA was extracted from pure culture using standard phenol/chloroform extraction methods (Pospiech and Neumann, 1995). Isolates were confirmed as *C. difficile* using a PCR assay for the housekeeping gene, tpi, described by Lemee et al. (2004). The DNA was then screened for toxin genes *tcdA*, *tcdB*, *cdtA*, and *cdtB* using the multiplex real-time assay described above. Results of the multiplex real-time PCR assay for enriched and non-enriched samples were compared to culture results in order to calculate sensitivity, specificity, positive predictive values, and negative predictive values.

### 3.4 Results

#### 3.4.1 Real-time multiplex PCR assay standardization

Standard curves for each reaction were generated by plotting the Ct values (the number of cycles needed for fluorescence to be detected above the fluorescence background threshold) for each 10-fold dilution of CDc DNA by the amount (ng) of DNA in each dilution. All four reactions showed a linear relationship between Ct and DNA amount. Reactions 1 and 2 yielded R² values of 0.988 and 0.978 for *tcdA* and *tcdB* (Figure 3-2), and 0.993 and 0.996 for *cdtA* and *cdtB* respectively (Figure 3-3). Similar sensitivities were observed for each of the four probe and primer sets. Sensitivity assays showed that a minimum of 42 pg, 420 pg, 420 pg, and 420 pg of
TXCD DNA was required to detect tcdA, tcdB, cdtA, and cdtB respectively. Cycle threshold values were as low as 14, 18, 16, and 14 for tcdA, tcdB, cdtA, and cdtB respectively when using 4.20 µg of TXCD DNA.

A fluorescent signal at least 10 times that of the standard deviation of the baseline emission mean after 35 cycles was considered as a positive result for both reactions 1 and 2. Negative results were observed for both non-toxigenic C. difficile and non-C. difficile strains demonstrating the specificity of the assay among isolates (Table 2). The assay also demonstrated specificity within isolates, where CDb gave a positive result for tcdA and negative result for tcdB, cdtA, and cdtB.

3.4.2 Real-time multiplex PCR laboratory validation

Sensitivity results generated by the laboratory validation showed the detection limit of the multiplex real-time PCR assay for all four toxin genes was $10^3$ cells/g in non-enriched samples of both feces and ground veal. The sensitivity for all four toxin genes was increased to $10^1$ cells/g when both fecal and ground veal samples were enriched before DNA extraction.

3.4.3. Real-time multiplex PCR field validation

Using the three detection methods described, toxin genes were detected in 24 (33.80%) out of 71 calf fecal samples and comprised 6 different toxin types (Table 3-3). Clostridium difficile was not detected in ground meat through culture or multiplex real-time PCR regardless of enrichment. Results showed C. difficile toxin genes tcdA, tcdB, cdtA, and cdtB were present in 19 (26.76%), 22 (30.98%), 13 (18.31%), and 13 (18.31%) calf fecal samples, respectively. Forty-six percent of TXCD positive samples (n= 11) were positive for all 4 toxin genes. Seven samples were positive for tcdA and tcdB, but negative for both binary toxin gene components. Seven samples were tcdA positive and negative of all other toxin genes. Four samples were tcdA
negative and *tcdB* positive, one being positive for binary toxin genes. One sample was positive for only *tcdA* and one sample was positive only for binary toxin genes.

Sensitivity, specificity, positive predictive value (PPV), and negative predictive value (NPV) for the multiplex real-time PCR assay were 0.93, 0.84, 0.61, and 0.98 for enriched fecal samples and 0.67, 0.86, 0.55, and 0.91 for non-enriched fecal samples, respectively (Table 3-4). The multiplex real-time PCR assay detected toxigenic *C. difficile* in 23 of 71 (32.39%) enriched fecal samples and 18 of 71 (25.35%) of the non-enriched fecal samples. Culture analysis resulted in identifying 17 of 71 (23.94%) enriched fecal samples as positive for *C. difficile*, isolates from 15 of 17 enriched fecal samples encoded for toxin genes of *C. difficile*. Ninety-three percent (14 out of 15) of samples culture positive for TXCD were also positive for TXCD using the multiplex real-time assay on enriched fecal samples. Toxigenic *C. difficile* was detected in 9 (16.07%) of the 56 culture negative fecal samples using the multiplex real-time PCR assay on enriched fecal samples. Only one sample was culture positive for toxigenic *C. difficile*, but negative using the multiplex real-time PCR assay on enriched samples. Only 10 out of 15 (66.67%) culture positive samples were also positive for TXCD using the real-time assay without enrichment. However, 8 (14.29%) out of 56 culture negative samples were positive for TXCD using the multiplex real-time PCR assay on non-enriched fecal samples.

Toxin type results differed slightly between *C. difficile* isolated through culture and those detected through multiplex real-time PCR on enriched and non-enriched samples. Multiplex real-time PCR showed a negative result for *tcdB* on five non-enriched samples from which *tcdB* positive *C. difficile* was isolated. Within these 5 samples, *cdtA* and *cdtB* also were not detected in 2 samples where binary toxin positive isolates were present. Toxin type results from only 2 samples differed between *C. difficile* isolated through culture and those detected through multiplex real-time PCR on enriched fecal samples. In both samples, *tcdA* was not detected in samples where *tcdA* positive *C. difficile* isolates were present.
3.5 Discussion

Nucleic acid amplification methods for the detection of microbes have been found to have unrivaled sensitivity and specificity and yield results much faster than culture-based methods (Louie et al., 2000). The specificity of primers to a DNA target weighs heavily on the accuracy of a PCR reaction. The amplification of non-specific products can yield false positive results. The use of a fluorescent labeled probe in real-time PCR assays minimizes the risk of amplification of non-specific targets since the binding of the probe as well as the primers to the target sequence is needed for a signal to be produced. Multiplex real-time PCR assays are gaining popularity over traditional PCR methods in diagnostic settings because of their increased sensitivity and specificity, increased sample throughput, and because the use of low resolution gel detection methods can be excluded.

The probe based real-time multiplex PCR assay designed and standardized in this study was able to distinguish TXCD from *C. perfringens, C. septicum, C. glycolicum, C. novyi*, as well as non-toxigenic *C. difficile*. These results confirm that the assay is able to detect genetic elements of TXCD without amplifying non-specific targets. The assay described here was able detect toxin genes in as low as 14, 18, 16, and 14 cycles for *tcdA, tcdB, cdtA*, and *cdtB*, respectively. The assay showed sensitivity in that it was able to detect toxin genes in enriched and non-enriched fecal and ground meat samples containing as little as $10^3$cells/g and $10^4$cells/g, respectively.

A recent study has shown that real-time PCR based assays are superior to traditional PCR, immunoassays, and culture based methods for detection of toxigenic *C. difficile* in fecal samples (van den Berg et al., 2007). We calculated PPVs of 0.61 and 0.55 for enriched and non-enriched fecal samples, respectively. This means PCR results agreed with culture results only 55-61% of the time. We calculated NPVs of 0.98 and 0.91 for enriched and non-enriched fecal
samples, respectively, showing the PCR assay rarely gave a false negative result. The results of this study agree with the van den Berg findings in that 14.29% and 16.07% of culture negative samples were positive for at least one toxin gene using the multiplex real-time PCR assay on non-enriched and enriched samples, respectively. This is most likely due to the ability of the multiplex real-time PCR assay to detect DNA extracted from *C. difficile* cells that did not survive in the sample and were, therefore, unculturable.

There were, however, 5 samples which were culture positive for TXCD and negative for all toxin genes using PCR assay on DNA extracted directly from fecal samples. Although, only one sample was culture positive for TXCD and negative for all toxin genes using PCR assay on DNA extracted from enriched fecal samples (Table 3-4). A likely explanation for this result is that TXCD was present in non-enriched samples at lower than detectable levels. These results show, when fecal samples are enriched prior to total DNA extraction, the sensitivity of the assay is increased from 0.67 to 0.93. Pre-enrichment serves to increase and concentrate low numbers of TXCD in fecal samples by allowing TXCD to grow and inhibiting the growth of competing bacteria. It is suggested that when the multiplex real-time PCR assay gives a negative result for DNA samples extracted directly from fecal samples, fecal samples should be enriched, total DNA extracted, and the multiplex real-time assay run again to ensure no TXCD is present in the sample.

Toxin type results differed for 5 and 2 samples when comparing multiplex real-time PCR results for non-enriched and enriched samples to toxin types of *C. difficile* isolated from fecal samples through culture. This is could be due to PCR reaction inhibitors present in fecal material which were unsuccessfully removed during DNA extraction. These inhibitors would be more concentrated in DNA samples which were extracted directly from feces, resulting in more toxin gene negative results for the non-enriched fecal samples when compared to the enriched fecal samples.
PCR based methods for the detection of *C. difficile* toxin genes have been reported in the past (Antikainen et al., 2009; Belanger et al., 2003; Lemee et al., 2004; Peterson et al., 2007). Most of these assays only include genes encoding toxins TcdA and TcdB or use traditional gel based detection methods. Until recently, symptoms related to *C. difficile* infection were mostly attributed to TcdA and TcdB (Barbut et al., 2005; McEllistrem et al., 2005). The role of CDT toxin in disease is not well understood, although researchers have reported strains that produce CDT but do not produce TcdA and TcdB. Therefore assays that detect TXCD based on the presence of only *tcdA* and *tcdB* may yield false negative results. The multiplex real-time PCR described here is capable of detecting genes encoding for TcdA, TcdB, as well as CDT.

Geric et al. (2003) reported that 15.5% of *tcdA* and *tcdB* negative *C. difficile* isolates examined were positive for both *cdtA* and *cdtB*. In this study, 4.17% of samples positive for TXCD harbored *cdtA* and *cdtB* genes but lacked *tcdA* and *tcdB* genes. Hammitt et al. (2008) found 25.3% of diarrheic calves were culture positive for *C. difficile* and all *C. difficile* isolated in the study was PCR positive for toxin genes *tcdA* and *tcdB*. In the data shown here, however, only 75% (18 out of 24) of samples where toxin genes of TXCD were detected were positive for both *tcdA* and *tcdB*. There are very few reports of the prevalence of *C. difficile* binary toxin in calves. Gonclaves et al. (2004) reported 6% of *C. difficile* isolated from humans with *C. difficile* related disease harbored both *cdtA* and *cdtB* genes. Another study reported *cdtB*, encoding for the binding portion of the toxin, was found in 2 out of 48 horse isolates (Arroyo et al., 2006). In this study, 54.17% (13 out of 24) of pre-weaned calf fecal samples harbored both genes encoding for the subunits of the binary toxin. The detection of binary toxin is becoming more important due to high prevalence rates in human outbreak TXCD strains. In fact, one study reported that almost two thirds of TXCD isolated during a hospital outbreak were positive for binary toxin genes (McEllistrem et al., 2005). It has also been reported that the production of binary toxin by TXCD may increase the severity of *C. difficile* associated disease (Barbut et al., 2005).
A study by Lemee et al. (2004) reported that 27 out of 622 of animal stool samples contained TXCD. This is a low prevalence when compared to the frequency of TXCD reported in calf feces. In this study, 24 out of 71 (33.80%) fecal samples contained TXCD genes. Similar findings were reported by Rodriguez-Palacios et al. (2006) who found toxins A and/or B in 30.6% of calf fecal samples. Another study by Hammitt et al. (2008) reported 22.9% of calf fecal samples contained TXCD as detected by immunoassay. The results of this study suggest that pre-weaned calves may be considered a reservoir for TXCD.

Weese et al. (2009) reported 12% of Canadian retail ground beef and pork samples contained TXCD, although upon enumeration, contamination levels were found to be low. In another study C. difficile was detected in 20.8% and 14.3% of Canadian retail ground beef and veal, respectively, and most isolates encoded for TcdA, TcdB, and CDT (Rodriguez-Palacios et al., 2007). Songer et al. (2009) found a much higher incidence of C. difficile in retail ground meat in Tucson, Arizona. In their study 42.4%, 41.3%, and 44.4% of beef, pork, and turkey were found to contain TXCD, respectively. In our study, we did not detect genetic elements of TXCD in any of the retail food animal products examined. Another recent study in Sweden also found little or no incidence of C. difficile in ground meat. von Abercron et al. (2009) found C. difficile in 6.2% of ground beef samples, but were unable to detect C. difficile in ground pork, mixed beef and pork, sheep, reindeer, moose, veal, and poultry.

3.6 Conclusions

Culture methods are time consuming, are not specific for TXCD, and are inferior in sensitivity. Immunochemistry assays lack the sensitivity needed for samples containing low levels of toxin and is a more costly diagnostic method when used to detect more than one toxin per sample. PCR based methods of diagnosis exist, but many of them do not include the gene target
for CDT, which research suggests may be a clinically significant toxin. The multiplex real-time PCR assay described here allows for sensitive and specific detection of genes encoding for all three toxins produced by TXCD. When DNA is extracted directly from samples, toxin genes may be detected in as little as 4 hours. Based on sensitivity results, it is suggested that when the multiplex real-time PCR assay gives a negative result for DNA samples extracted directly from fecal or ground meat samples, samples should be enriched, total DNA extracted, and the multiplex real-time assay run again to confirm the negative result. Results of the prevalence study conducted here suggest pre-weaned calves may be a source of TXCD.
3.7 References


37. Weese JS, Avery BP, Rousseau J, Reid-Smith RJ, 2009. Detection and Enumeration of

Table 3-1: Primer and probe sequences for *Clostridium difficile* toxin genes

<table>
<thead>
<tr>
<th>Target</th>
<th>Oligo</th>
<th>Sequence (5'- 3')</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdA F</td>
<td>TTCAAGCAGAAATAGAGCACTC</td>
<td></td>
</tr>
<tr>
<td>tcdA R</td>
<td>TATCAGCCCATTGTATTATGTATTC</td>
<td></td>
</tr>
<tr>
<td>tcdA probe</td>
<td>FAM-TCACTGACTTCTCCACCTATCCATAA-BHQ(^1)</td>
<td></td>
</tr>
<tr>
<td>tcdB F</td>
<td>GGTATTACCTAATGCTCCAAATAG</td>
<td></td>
</tr>
<tr>
<td>tcdB R</td>
<td>TTTGTGCCATCATTTTCTAAGC</td>
<td></td>
</tr>
<tr>
<td>tcdB probe</td>
<td>HEX-ACCTGGTGTCACATCCCTGTTCCBA-BHQ</td>
<td></td>
</tr>
<tr>
<td>cdtA F</td>
<td>GGGTAAAGCAAATTATAATGATTGG</td>
<td></td>
</tr>
<tr>
<td>cdtA R</td>
<td>CTATATACAGTTAAAATAGTTGGAATAGG</td>
<td></td>
</tr>
<tr>
<td>cdtA probe</td>
<td>FAM-AATTAACACCTAATGAACTTGCTGATGT-BHQ</td>
<td></td>
</tr>
<tr>
<td>cdtB F</td>
<td>TGGTGTGCTGTTAATGATAG</td>
<td></td>
</tr>
<tr>
<td>cdtB R</td>
<td>CTTGTGAATACATATTACAGTATCC</td>
<td></td>
</tr>
<tr>
<td>cdtB probe</td>
<td>HEX-CTCCATACTCTTGGAACACAGTGTTGA-BHQ</td>
<td></td>
</tr>
</tbody>
</table>

\(^1\)Black Hole Quencher
### Table 3-2: Reference strains used for real-time multiplex PCR standardization

<table>
<thead>
<tr>
<th>Strain</th>
<th>Source</th>
<th>tcdA</th>
<th>tcdB</th>
<th>cdtA</th>
<th>cdtB</th>
</tr>
</thead>
<tbody>
<tr>
<td><em>C. difficile</em> (CDa)</td>
<td>JG Songer</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. difficile</em> (CDb)</td>
<td>JG Songer</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. difficile</em> (CDc)</td>
<td>JG Songer</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td><em>C. glycolicum</em></td>
<td>PSU-ADL(^1)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. novyi</em></td>
<td>PSU-ADL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. sordelli</em></td>
<td>PSU-ADL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. septicum</em></td>
<td>PSU-ADL</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>C. perfringens</em></td>
<td>ATCC(^2) 13124</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Escherichia coli</em></td>
<td>ATCC 43895</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Staphylococcus aureus</em></td>
<td>ATCC 29213</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td><em>Salmonella enterica</em></td>
<td>ATCC 14028</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

1. Pennsylvania State University Animal Diagnostic Laboratory
2. American Type Culture Collection
Table 3-3: Prevalence of toxigenic *Clostridium difficile* in feces of preweaned calves

<table>
<thead>
<tr>
<th>Toxin genes</th>
<th>N</th>
<th>% TXCD+</th>
<th>% Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>1 tcdA</td>
<td>11</td>
<td>45.83</td>
<td>15.49</td>
</tr>
<tr>
<td>1 tcdB</td>
<td>7</td>
<td>29.17</td>
<td>9.86</td>
</tr>
<tr>
<td>1 cdtA</td>
<td>1</td>
<td>4.17</td>
<td>5.63</td>
</tr>
<tr>
<td>1 cdtB</td>
<td>3</td>
<td>12.50</td>
<td>1.41</td>
</tr>
<tr>
<td>1 tcdB + cdtB</td>
<td>1</td>
<td>4.17</td>
<td>1.41</td>
</tr>
<tr>
<td>1 cdtA + cdtB</td>
<td>1</td>
<td>4.17</td>
<td>1.41</td>
</tr>
<tr>
<td>Total TXCD+</td>
<td>24</td>
<td>100.00</td>
<td>33.80</td>
</tr>
</tbody>
</table>

1Includes genes detected though multiplex real-time PCR on enriched fecal samples, non-enriched fecal samples, and isolates from culture.
Table 3-4: Field validation of a multiplex real-time PCR assay for the detection of toxigenic *Clostridium difficile* in pre-weaned calf feces

A

<table>
<thead>
<tr>
<th>Culture Result</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>Predictive Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Result</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>14</td>
<td>9</td>
<td>23</td>
<td>0.61&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Negative</td>
<td>1</td>
<td>47</td>
<td>48</td>
<td>0.98&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>56</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

Sensitivity: 0.93  Specificity: 0.84

B

<table>
<thead>
<tr>
<th>Culture Result</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>Predictive Values</th>
</tr>
</thead>
<tbody>
<tr>
<td>PCR Result</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Positive</td>
<td>10</td>
<td>8</td>
<td>18</td>
<td>0.55&lt;sup&gt;1&lt;/sup&gt;</td>
</tr>
<tr>
<td>Negative</td>
<td>5</td>
<td>48</td>
<td>53</td>
<td>0.91&lt;sup&gt;2&lt;/sup&gt;</td>
</tr>
<tr>
<td>Total</td>
<td>15</td>
<td>56</td>
<td>71</td>
<td></td>
</tr>
</tbody>
</table>

Sensitivity: 0.67  Specificity: 0.86

A: Fecal samples enriched before DNA extraction; B: DNA extracted directly from fecal samples

<sup>1</sup>Positive predictive value

<sup>2</sup>Negative predictive value
Figure 3-1. Experimental design scheme for standardization and validation of the real-time multiplex PCR assay for the detection of toxigenic genetic elements elements of *C. difficile* in feces and ground meat.
**Figure 3-2:** Reaction 1 Standard Curve. Log scale standard curves of reaction 1 for the detection of *tcdA* (FAM) and *tcdB* (HEX) in CDc DNA template; x-axis: Linear regression of Ct, y-axis: quantity of CDc DNA.
Figure 3-3: Reaction 2 Standard Curve. Log scale standard curves of reaction 2 for the detection of *cdtA* (FAM) and *cdtB* (HEX) in CDc DNA template; x-axis: Linear regression of Ct, y-axis: quantity of CDc DNA.
Chapter 4

Prevalence and characterization of toxigenic *Clostridium difficile* and its association with enteric disease in young calves
4.1 Abstract

A study was conducted to determine if *C. difficile* is associated with enteric disease in bovine calves. Fecal and colon tissue samples were collected from calves less than 5 weeks of age submitted to The Pennsylvania State University Animal Diagnostic Laboratory for postmortem examination. All samples were cultured for *C. difficile* and fecal samples were screened for toxins A and B (TcdA/B) using a commercially available enzyme immunoassay (EIA). *Clostridium difficile* and TcdA/B were detected in 21 (29.6%) and 7 (9.8%) out of 71 total calves, respectively. Logistic regression analysis of culture, EIA, and postmortem examination data revealed the odds of observing enteric lesions in young calves are nearly 11 times greater when toxins A and/or B are detected in the feces. Isolates collected from calves with and without evidence of clinical enteric disease were characterized using genotypic and phenotypic methods. Characterization of *C. difficile* (n=67) isolated from calves showed 16 different PFGE profiles upon *Sma*I digestion. We observed a high prevalence of toxin genes (*tcdA, tcdB, cdtA, cdtB*), 39 bp *tcdC* deletions, and resistance to tetracycline and cefoxitin among isolates. The results of this study suggest *C. difficile* should be considered as an possible etiologic agent when diagnosing enteric disease in calves and that emphasis should be placed on results of EIA testing for TcdA/B for diagnosis of *C. difficile* related disease in calves. Calves should also be considered a reservoir for pathogenic *C. difficile*. 
4.2 Introduction

Calf mortality from enteric disease continues to be a source of significant losses to producers in the dairy industry. The National Animal Health Monitoring System reported in 2007 that 56% of preweaned dairy calf mortality is due to diarrhea (NAHMS, 2007). It has been estimated that the losses associated with calf diarrhea cost producers $33.46 per calf per year (Frank and Kaneene, 1993). Therefore, it is important to identify etiologic agents of enteric disease in order to generate effective prevention, diagnostic, and treatment plans to reduce calf losses.

*Clostridium difficile* has long been established as a human pathogen commonly involved in diseases such as antibiotic-associated diarrhea and pseudomembranous colitis (Pothoulakis, 1996; Razavi et al., 2007; Riccardi et al., 2007; Sunenshine and McDonald, 2006). Recently, *C. difficile* has been identified in cases of enteric disease in animals including calves (Songer, 2004). Many pathogenic strains produce an enterotoxin (TcdA) and/or a cytotoxin (TcdB) which are able to monoglucosylate proteins important in F-actin regulation. Loss of F-actin regulation leads to disruption of the cytoskeleton resulting in the compromise of intestinal epithelial cell tight junctions (Keel and Songer, 2006; von Eichel-Streiber et al., 1996). Some strains also produce an ADP-specific ribosyltransferase which also targets actin known as CDT (Popoff et al., 1988). Although the clinical significance of CDT has yet to be determined, it has been shown that CDT increases the adherence abilities of *C. difficile* suggesting CDT may be considered a virulence factor (Schwan et al., 2009). Also, TcdA negative/TcdB negative/CDT postive strains have been shown to be enterotoxic (Geric et al., 2006).

The prevalence of *C. difficile* in calves has been reported to be 9.5 to 23%, although data is limited (Avbersek et al., 2009; Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). Recent studies have shown an association between diarrhea in calves and the presence of *C. difficile*
toxins TcdA and TcdB (TcdA/B) while other studies fail to establish the clinical relevance of C. difficile in enteric disease in calves (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006; Rodriguez-Palacios et al., 2007). In order to determine the role C. difficile in enteric disease of calves, experimental infection with C. difficile has been attempted but a disease state has not been achieved (Rodriguez-Palacios et al., 2007). Researchers have shown TcdA/B are toxigenic to the small and large intestine in calves upon experimental intoxication showing TcdA/B are able to cause enteric lesions in calves (Hammitt et al., 2008). Very few studies have been conducted on natural infection and intoxication of the calf gut with C. difficile. The objectives of this study were to: 1) determine the odds of calves exhibiting gross pathologic and histologic evidence of enteric lesions and/or diarrhea when C. difficile and/or TcdA/B are present; and 2) characterize C. difficile isolates from calves using genotypic and phenotypic methods.

4.3 Materials and Methods

4.3.1 Sample collection

The study included all calves (n=71) less than 5 weeks of age submitted to The Pennsylvania State University-Animal Diagnostic Lab (PSU-ADL) for post mortem examination from October, 2007 through January, 2009. Upon post mortem examination, fecal and colon tissue samples were collected from all calves for culture of C. difficile and detection of TcdA/B (Figure 4-1). Major organs including small and large intestine were collected, fixed in 10% buffered formalin, routinely processed, and sectioned at 5 microns. Sections were stained with hematoxylin and eosin and examined microscopically. Necropsy reports were accessed and instances of enteric disease including enteritis, colitis, and intestinal necrosis were recorded for each case for the purposes of this study. The observation of fecal material of fluid consistency in the colon was also recorded and considered evidence of diarrhea. Routine laboratory results
generated by PSU-ADL including the presence of virulent *Escherichia coli*, *Salmonella*, and *Clostridium perfringens* in the feces, small intestine, and colon were also accessed and recorded for each case. Virulence of *E. coli* was determined through PCR and was carried out at The Pennsylvania State University *E. coli* Reference Center (PSU-ECRC). Bovine relevant virulence genes targeted in the PCR assays included: *sta* (heat stable enterotoxin), *stxI* and *stxII* (Shiga-like toxins I and II), *cnfI* and *cnfII* (cytotoxic necrotizing factors I and II), *eae* (intimin), *K99* (pilus), *CS31A* (adhesin) and *F1845* (fimbria).

### 4.3.2 Culture methods

Fecal samples (1 g or 1 mL) and colon tissue samples (1 cm², homogenized by tissue grinding) were enriched in 9 mL cycloserine cefoxitin fructose broth (CCFB) for one week under anaerobic conditions at 37°C. The selective enrichment broth contained proteose peptone (40 g/L), fructose (6 g/L), disodium phosphate (5 g/L), sodium chloride (2g/L), monopotassium phosphate (1 g/L), magnesium sulfate (100 mg/L), cycloserin (500 mg/L), and cefoxitin (16 mg/L). After enrichment, 5 mL of culture was added to 5 mL ethanol (98%) and incubated at room temperature for 60 min. The culture was then spun down and the pellet washed with phosphate buffered saline (PBS). The final pellet was resuspended with 1-2 mL PBS and streaked onto cycloserine cefoxitin fructose agar (CCFA). Medium used for plating was of the same formulation as CCFB, but contained agarose (15 g/L) and neutral red (30 mg/L). Plates were incubated anaerobically at 37°C for up to 5 days and observed for growth. Three to 4 colonies showing morphology characteristic of *C. difficile* were subcultured and DNA was extracted as described by Pospiech and Neumann (1995). Identity of *C. difficile* isolates was then confirmed with a PCR assay described by Lemee et al. (2004) targeting *tpi*, a species-specific housekeeping gene. Confirmed *C. difficile* isolates were stored in peptone yeast glucose broth containing 50% glycerol for further characterization.
4.3.3 Detection of toxins A and B

Fecal samples were screened for the presence of TcdA/B using a commercially available enzyme immunoassay (EIA), \textit{C. difficile} Tox A/B II kit (TechLab, Blacksburg, VA). This assay has been previously validated and is able to detect as little as 1 ng/ml and 2-5 ng/ml of TcdA and TcdB, respectively, with 100% specificity (Lyerly et al., 1998). The EIA contains antibodies against TcdA/B in the same assay well, therefore, a positive result can be due to the presence of either toxin or the presence of both. Isolates from culture positive calves were also screened for toxin production \textit{in vitro}. Isolates were revived on blood agar and one colony was transferred into 2 ml of TY medium containing 3% bacto tryptose, 2% yeast extract, and 0.1% thioglycolate. Cultures were incubated 24 h anaerobically at 37°C and then pelleted through centrifugation. Supernatant was then used for the EIA as described for fecal samples.

4.3.4 Isolate Characterization

4.3.4.1 Phenotypic typing methods

Antibiotic resistance profiles were determined using methods approved by the Clinical and Laboratory Standards Institute (CLSI) (2007). Agar dilutions (2-fold) were made using Brucella agar containing 5% laked sheep’s blood and supplemented with vitamin K (1mg/L) and hemin(5mg/L). Antibiotics were chosen based on their relevance to \textit{Clostridium difficile}-associated disease (CDAD) in humans and animals as well as animal agriculture and include ampicillin, oxytetracycline, tetracycline, neomycin, sulfamethazine, clindamycin, metranidazole, enrofloxacin, vancomycin, erythromycin, florfenicol, cefoxitin, and ciprofloxacin.

Suspensions of culture at a turbidity of 0.5 McFarland were stamped onto each antibiotic dilution plate as well as a control plate containing no antibiotic in triplicate. American Type Culture Collection (ATCC) \textit{C. difficile} strain 700057 was used as control, although, CLSI has not
published standard minimum inhibitory concentration (MIC) data for all antibiotics used here including Oxy, Smz, Neo, Enr, Van, Ery, Flo and Cip. Plates containing the lowest concentration of antibiotic where no growth was observed was considered the MIC.

4.3.4.2 Genotypic typing methods

DNA was extracted from all C. difficile isolated from calves using standard phenol-chloroform extraction methods (Pospiech and Neumann, 1995). Each isolate was confirmed to be C. difficile using a PCR assay described by Lemee et al. (2004) which targets tpi, a species specific housekeeping gene. The DNA extracts were then examined for genes encoding for toxins TcdA, TcdB, and CDT (binary toxin encoded in 2 subunit genes cdtA and cdtB) using a real-time multiplex PCR assay described by Houser et al. (2010). Isolates encoding for TcdA and/or TcdB were examined for deletions in the tcdC gene, a negative regulator of tcdA and tcdB expression. Deletions in the tcdC gene were detected using a traditional PCR assay described by Antikainen et al. (2009). The product was run on a 2.5% agarose gel, stained with ethidium bromide, and visualized under UV light. An intact tcdC gene yields a 139 bp PCR product. Isolates yielding a product size of 121 bp, 100 bp, or 85 bp in size were considered to have a tcdC deletion of 18 bp, 39 bp, or 54 bp, respectively.

Genotypic diversity among isolates was determined using pulsed field gel electrophoresis (PFGE). Isolates were subcultured from blood agar to peptone yeast glucose broth and incubated overnight at 37°C in an anaerobic chamber. One ml of each culture was pelleted and resuspended in 400 µl TE buffer, 25 µl lysozyme (20 mg/ml) was added, and suspensions were incubated at 55°C for 15 min. Proteinase K (25 µl of 20 mg/ml stock) and 400µl melted agarose was then added to each suspension. Suspensions were mixed thoroughly, dispensed into plug molds, and allowed to solidify. Plugs were incubated in a shaker bath with vigorous agitation at 54°C for 3 h in 5 ml Gram positive lysis buffer (6 mM Tris HCl, 1 M NaCl, 100 mM EDTA, 0.5% Brij, 0.2% sodium deoxycholate, 0.5% sodium lauryl sarcosine) containing proteinase K (25 µl of 20 mg/ml
Plugs were then washed twice with sterile water and 5 times with TE buffer. Plugs were stored at 4°C in TE buffer. A 1-2mm slice of each plug was digested with SmaI overnight and bands were resolved using a Chef Mapper System (BioRad, Hercules, CA). Lambda ladder PFG marker (New England BioLabs, Ipswich, MA) was included in each gel for normalization purposes. All gels were stained with ehtidium bromide and images were captured under UV light. The resulting PFGE banding patterns were analyzed using GelCompar II version 6.0 software (Applied Maths, Austin, Texas) in order to generate a dendoGram. The relatedness of PFGE profiles was determined using the unweighted pair group method with arithmetic mean clustering. All clustering was based on the Dice correlation coefficient.

4.3.5 Data Analysis

Logistic regression was used to determine if the odds of observing evidence of enteritis, colitis, intestinal necrosis, and diarrhea were higher in calves positive for C. difficile or TcdA/B than in calves that tested negative. This analysis may be confounded by the presence of other intestinal pathogens in enteric disease cases; therefore, cases had to meet certain criteria to be included in the logistic regression analysis for each of the four enteric conditions as listed in Table 4-1. Criteria are based on the presence of enteric pathogens other than C. difficile including virulent E. coli, pathogenic Salmonella serovars, and C. perfringens which may contribute to certain enteric disease symptoms.

Enteritis and colitis cases were not included in analysis if 1) pathogenic Salmonella was present, 2) C. perfringens was present and hemorrhage was observed, or 3) E. coli carrying Shiga-like toxin I and/or II genes (stxI and stxII) was present in the small intestine and colon respectively. Cases of intestinal necrosis were not included in the analysis if 1) pathogenic Salmonella was present at the site of necrosis, 2) C. perfringens was present and hemorrhage was observed at the site of necrosis, or 3) E. coli carrying genes encoding for cytotoxic necrotizing
factor (cnfl and cnfII) were present at the site of necrosis. Diarrhea cases were not included in the analysis if 1) pathogenic Salmonella was present, 2) C. perfringens was present and hemorrhage was observed, or 3) E. coli carrying genes encoding for virulence factors associated with diarrhea was present anywhere in the intestine. Virulence factors associated with bovine diarrhea as per PSU-ECRC include heat stable enterotoxin (sta), Shiga-like toxins I and II (stxI and stxII), cytotoxic necrotizing factor I and II (cnfl and cnfII), intimin (eae), pilus associated with virulence (K99), adhesin (CS31A) and fimbriae (F1845). The presence or absence of C. difficile and/or TcdA/B was not considered when excluding cases from analysis. All cases where no enteric pathology was observed were included in the analysis regardless of the presence or absence of all intestinal pathogens mentioned here.

4.4 Results

4.4.1 PSU-ADL findings

A total of 71 calves were examined ranging in age from 2 to 30 days. Enteric lesions noted upon postmortem examination included in the study revealed 26, 12, and 8 cases of enteritis, colitis, and intestinal necrosis, respectively. Thirty-nine calves had fecal material of fluid consistency in the colon and were considered diarrheic for the purposes of this study. Necrosis was reported in the small intestine and colon in 5 and 2 cases, respectively. In one case, necrosis was observed in both the small and large intestine. In 31 calves no enteric lesions or diarrhea were observed.

Salmonella sp. were present in 17 calves and detected in the small intestine and colon in 15 and 12 calves, respectively. Serovars included Dublin (n=2), Typhimurium (n=2), Newport (n=2), Anatum (n=1), Montevideo (n=3), Kentucky (n=1), and Cerro (n=6). Pathogenic Salmonella serovars were detected in 5, 3, 3, and 5 cases of enteritis, colitis, intestinal necrosis,
and diarrhea respectively. Pathogenic *Salmonella* serovars detected in the small intestine of enteritis cases included Newport (n=1), Kentucky (n=1) Montivideo (n=2) and Dublin (n=1). Pathogenic *Salmonella* serovars detected in the colon of colitis cases included Dublin (n=1) and Typhimurium (n=2). For cases where necrosis was observed in the small intestine, *Salmonella* serovars Dublin (n=1) and Kentucky (n=1) were detected. *Salmonella* serovar Anatum was detected in one case of necrosis of the colon. Diarrheic calves positive for pathogenic *Salmonella* included serovars Kentucky (n=1), Montevideo (n=2), and Dublin (n=2). *Clostridium perfringens* was present in 14 calves and detected in the small intestine and colon of 13 and 1 calves, respectively. *Clostridium perfringens* was detected and hemorrhage observed in 2 cases of enteritis and 1 diarrheic calf, but was not seen in colitis and intestinal necrosis cases.

*Escherichia coli* was detected in the intestines of 48 calves and isolates from 28 of these calves were negative for genes encoding for virulence factors. Isolates from 20 calves were positive for virulence genes including *sta* (n=2), *stxI* (n=4), *cnfII* (n=1), *eae* (n=5), *K99* (n=4), and *CS31A* (n=12). Virulence genes *stxII*, *cnfI*, and *F1845* were not detected in any of the isolates. Isolates from 7 calves encoded more than one virulence gene. Shiga-like toxin encoding *E. coli* were not detected in any enteritis or colitis cases, *E. coli* encoding for cytotoxic necrotizing factor were not detected in any intestinal necrosis cases. Virulent *E. coli* was detected in 12 diarrheic calves.

### 4.4.2 Prevalence of *Clostridium difficile* and toxins A and B in young calves

*Clostridium difficile* and TcdA/B were detected in 21 (29.6%) and 7 (9.8%) out of 71 total calves, respectively. Four calves were both culture and toxin positive (Table 4-2). *C. difficile* was cultured from 5, 4, and 2 cases of enteritis, colitis, and intestinal necrosis, respectively. Six diarrheic calves were culture positive for *C. difficile*. Toxins A and/or B were detected in 4, 3, and 2 cases of enteritis, colitis, and diarrhea, respectively. Toxin was not detected
in any cases of intestinal necrosis. *Clostridium difficile* and TcdA/B were detected in 9 and 1 calves, respectively, with no enteric lesions or diarrhea. All calves positive for both *C. difficile* and TcdA/B had at least one enteric lesion or evidence of diarrhea upon postmortem examination.

### 4.4.3 Clostridium difficile and enteric disease in calves

Data from 7, 3, 3, and 17 calves showing evidence of enteritis, colitis, intestinal necrosis, and diarrhea, respectively, were not included in the odds ratio analysis based on the criteria listed in Table 4-1. Logistic regression analysis included a total of 64, 68, 68, and 54 calves when analyzing the odds of observing evidence of enteritis, colitis, intestinal necrosis, and diarrhea in young calves positive for *C. difficile* or TcdA/B (Table 4-3). Analysis was also carried out to determine the odds of observing any enteric lesion when *C. difficile* or TcdA/B were present; 13 cases were excluded from analysis based on the criteria for exclusion of enteritis, colitis, and intestinal necrosis cases (evidence of diarrhea was not included because almost half of the cases would have been excluded from the analysis based on Table 4-1).

Data analysis revealed that the odds of observing evidence of colitis were almost 7X higher (p=0.03) for calves that were positive for TcdA/B than in calves that tested negative for the toxin (Table 4-3). In the study, TcdA/B was detected in the feces of 7 calves. Six of these calves had clinical signs of enteric disease. Statistically, this translated to almost 11X higher (p=0.03) odds of finding enteric lesions in calves that tested positive for TcdA/B than in calves that were negative for the toxin. A culture positive result did not increase the odds of observing enteric lesions or evidence of diarrhea.

### 4.4.4 Isolate characterization

Eighty-one *C. difficile* isolates from the 21 culture positive calves were collected over the course of the study. Sixty-seven of these isolates from 16 culture positive calves were examined
as 14 isolates from 5 culture positive calves were not able to be revived from frozen stock. Sixty-four (95%) isolates encoded for at least one toxin and 49 (73%) isolates encoded for all three toxins (Table 4-4). Of the 58 isolates encoding for TcdA and/or TcdB, 50 (86%) contained a 39 bp deletion in the \textit{tcdC} gene. \textit{In vitro} TcdA/B production was observed for 5 (7.5%) isolates after 24 h of incubation. Minimum inhibitory concentrations of antibiotics are listed for all isolates in Table 4-5. All isolates were susceptible to Metranidazole and Clindamycin. Fifty-two percent of the isolates tested were found to be resistant to Tetracycline and all were resistant to Cefoxitin.

Although none of the isolates were resistant to Ampicillin, 63% had intermediate MIC values. We are not able to infer about the significance of MICs found for all other antibiotics because resistance break points have not yet been established for \textit{C. difficile}, although MIC values for Sulfamethazine and Neomycin were relatively high. Among the antibiotics with set resistance MIC breakpoints for \textit{C. difficile}, six different antibiotic resistance profiles were observed (Table 4-6). Profile 2 (intermediate for Ampicillin, susceptible to Clindamycin and Metranidazole, resistant to Cefoxitin and Tetracycline) was the most common among isolates (41.8%).

Sixteen distinguishable PFGE patterns were observed among 61 \textit{C. difficile} isolates showing an overall similarity of 66% (Figure 4-2). After several attempts, 6 isolates were not able to be typed using PFGE due to DNA degradation. Two distinct clusters, A and B, were observed. Cluster A contained 52 isolates which showed 81% similarity. Cluster A was broken down into 3 sub-clusters, A1, A2, and A3. Sub-cluster A1 contained 6 isolates showing 88% similarity from 2 calves with evidence of colitis, enteritis, and diarrhea or necrosis. Sub-cluster A2 contained 21 isolates showing 93% similarity from 8 calves and was further broken down into sub-clusters A2-1 and A2-2. Sub-cluster A2-1 contained 5 isolates showing 100% similarity from 4 different calves. All but one isolate from sub-cluster A2-1 were from calves with evidence of either enteritis, diarrhea, or necrosis. Sub-cluster A2-2 contained 16 isolates showing 94% similarity from 6 calves. All but one isolate in this sub-cluster were from calves with no evidence of enteric
disease upon postmortem examination. Eleven isolates in sub-cluster A2-2 from calves with no evidence of enteric disease had indistinguishable PFGE patterns. Sub-cluster A3 contained 25 isolates showing 90% similarity from 9 calves. Within this sub-cluster were 2 groups containing 14 and 10 isolates with indistinguishable PFGE patterns. These isolates were from cases including enteritis, diarrhea, and necrosis as well as cases with no evidence of enteric disease. Cluster B accounted for the majority of the diversity observed among isolates and contained 9 isolates showing only 67% similarity. These nine isolates were from 4 different calves. Interestingly, all but one of the isolates were from cases where colitis, enteritis, and diarrhea were observed or cases where enteritis and diarrhea were observed. No obvious correlations were observed between genotypic nor phenotypic characteristics of isolates and SmaI digestion patterns.

There were only 2 instances where isolates belonging to clusters A and B were from the same calf. In six instances isolates from different sub-clusters of cluster A were from the same calf. This observation suggests more than one strain of *C. difficile* can be found in the calf gut at one time.

### 4.5 Discussion

In our study the prevalence of *C. difficile* (29.6%) in calves was much higher than the prevalence of TcdA/B (9.8%). Previous studies report TcdA/B to be more prevalent. Hammitt et al. (2008) detected *C. difficile* and TcdA/B in 14.9% and 20.9% of nondiarrheic calves and in 7.6% and 39.6% of diarrheic calves, respectively. Rodriguez-Palacios et al. (2006) found *C. difficile* and TcdA/B in 13.2% and 30.2% of nondiarrheic calves and in 25.3% and 22.9% of diarrheic calves, respectively. The presence of toxins produced by *C. difficile* is evidence that the organism was present in calves. It is possible that viable organisms in these samples did not
survive sample handling and transport. Therefore, it can be inferred that *C. difficile* was more prevalent than the culture results suggest in previous studies (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). The lower prevalence of TcdA/B reported here as compared to previous studies may be due to the time of sampling. Fecal samples were taken from calves during post mortem examination which, in some cases, was performed 12 hours or more after the death of the calf, allowing time for toxins to degrade. A recent study on *C. difficile* in horses, though, found similar prevalence rates to those shown here. Ruby et al. (2009) found 83 (29%) and 33 (11%) horses out of a total of 292 were positive for *C. difficile* and TcdA, respectively.

Due to a low number of cases where TcdA/B was present, logistic regression was chosen for data analysis. Logistic regression requires a sample size 50x larger than the number of independent variables being tested Grimm and Yarnold, 1995). Since our sample size was n=71, analysis was carried out separately for each independent variable (presence of *C. difficile* and presence of TcdA/B). Logistic regression analysis of data collected in this study showed the odds of observing evidence of colitis in young calves was 6.87X higher when TcdA/B is present in the feces. The odds of observing any enteric lesions in calves was nearly 11X higher when TcdA/B is present in the feces. Ruby et al. (2009) observed horses positive for TcdA showed more severe clinical disease than horses that were TcdA negative regardless of *C. difficile* culture results. Although we found the presence of TcdA/B to be associated with colitis, we found no such association with diarrhea in calves which is in disagreement with previous findings of other researchers (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). Similar findings, however, have been reported in pigs. Alvarez-Perez et al. (2009) were not able to link the presence of *C. difficile* in piglets to neonatal porcine diarrhea.

It should be noted that although the presence of other bacterial enteric pathogens were accounted for in this study, it was not possible to completely rule out other etiologic agents in cases of enteric disease. This study relied on the routine PSU-ADL diagnostic screening for
enteric pathogens where appropriate. The possible presence of other pathogens including viruses and protozoa were not accounted for in this study.

Effects of TcdA and TcdB have been assessed in the small intestine and colon of anesthetized calves (Hammitt et al., 2008). The investigators found intoxication with both toxins lead to tissue damage, fluid accumulation, and neutrophil infiltration in both the large and small intestines. In this study, the presence of *C. difficile* in the calf gut did not increase the odds of observing enteric lesions or diarrhea. Rodriguez-Palacios et al. (2006) found that experimental inoculation of *C. difficile* resulted in colonization, but not toxin production, and did not result in enteric disease in neonatal calves. In this study we observed asymptomatic carriage of *C. difficile* by calves. No enteric lesions or diarrhea was observed in 9 (43%) out of 21 calves culture positive for *C. difficile*. Therefore, the absence of evidence of enteric disease suggests toxin production may be influenced by environmental factors in the calf gut. Based on our findings, it is suggested that diagnosis of CDAD in calves should be based on the presence of TcdA/B in the feces and culture results alone are not sufficient for diagnosis. Similar conclusions have been drawn for diagnosis of CDAD in humans (Gogate et al., 2005; Weese et al., 1999).

Asymptomatic carriage of toxigenic *C. difficile* and young calves as seen in this study suggests young calves may be an important reservoir for the pathogen. We report a high prevalence of tcdA (86.6%) and tcdB (85.1%) among calf isolates. We also observed a high prevalence (83.6%) of isolates encoding for CDT. Most isolates (73.1%) harbored genes for all three toxins. A high prevalence of toxin genes among animal isolates is not uncommon. Alvarez-Perez et al. reported 94.3% of *C. difficile* isolated from piglets encoded for both TcdA and TcdB (Alverez-Perez et al., 2009). Norman et al. (2009) examined 131 swine isolates and found 122 (93.1%) encoded for TcdA and TcdB. Rodriguez-Palacios et al., (2006) reported 30 out of 31 calf isolates encoded for at least one toxin while 35.5% encoded for all three. They also reported 29% of isolates were tcdA-/tcdB+. In this study we did not observe any tcdA-/tcdB+ strains. Previous
research has shown genotypic similarity between *C. difficile* isolates of human and animal origin, suggesting animals should be considered an important source of exposure to *C. difficile* relevant to public health (Jhung et al., 2008; Rodriguez-Palacios et al., 2006; Rupnik, 2007). Reports of outbreaks of enteric disease associated with fairs and petting zoos have increased in the US and common sources of infection include cattle (Steinmuller et al., 2006).

As previously observed by other researchers, we observed a high prevalence of 39 bp tcdC deletion-carrying *C. difficile* strains. Norman et al. (2009) reports 129 (98.5%) of 131 swine isolates contained a 39 bp deletion in the tcdC gene. Jhung et al. (2008) reported 100% of 14 human isolates collected from 1989-2006 had a 39 bp deletion. Verdoorn et al. (2009) also reported isolates from 42 out of 99 human patients carried a tcdC deletion. The tcdC gene product is a negative regulator of TcdA/B expression (Matamouros et al., 2007). Deletions in this gene are associated with human epidemic strains (Loo et al., 2005; Warny et al., 2005). It has been suggested that the presence of deletions in the tcdC gene may indicate hypervirulence due to unregulated toxin production. However, Verdoorn et al. (2009) did not find tcdC deletions to correlate with severity of disease suggesting these deletions may not result in full loss of function of the TcdC protein.

In this study we observed 63%, 100% and 75% of calf isolates were intermediate or resistant to ampicillin, cefoxitin, and tetracycline, respectively, and all isolates were susceptible to clindamycin and metranidazole (Table 4-6). In another study, 89%, 100%, 100%, and 10% of 131 porcine isolates were intermediate or resistant to ampicillin, cefoxitin, clindamycin, and tetracycline, respectively (Norman et al., 2009). Jhung et al. (2008) reported 86%, 100%, and 12% of human, porcine, and bovine isolates were intermediate or resistant to clindamycin, respectively. These results suggest bovine *C. difficile* isolates are more susceptible to clindamycin and more resistant to tetracycline than isolates from other species. The susceptibility of isolates to clindamycin may be explained by the fact that this antibiotic is not used in dairy calves.
Tetracyclines, however, are used very commonly in dairy calves for prevention of disease and enteritis treatment which may lead to selection for tetracycline resistant strains (Frost, 1991; Sawant et al., 2005).

There are few reports on the diversity of *C. difficile* isolates from calves based on PFGE typing and no data to our knowledge has been published on how PFGE profiles of calf isolates may relate to enteric disease in calves. Avbersek et al. (2009) observed 17 distinguishable PFGE patterns among 131 isolates from pigs, calves, and horses. The results shown by Avbersek et al. (2009) are similar to those seen here in that the isolates showed approximately 50% similarity overall, with two distinct clusters, one containing many PFGE profiles, the other only two. In this study, the majority of isolates from calves with evidence of enteric disease at necropsy grouped together in clusters A1, A2-1, A3 and B whereas most isolates from calves with no evidence of enteric disease grouped in clusters A2-2 and A3, suggesting genetic similarity may be shared between strains associated with disease.

### 4.6 Conclusions

The results of this study suggest calves less than 5 weeks of age may be an important reservoir for a diverse population of toxigenic *C. difficile*. Logistic regression analysis of the data generated by this study reveal that the presence of TcdA/B, but not the presence of *C. difficile*, increases the odds of observing evidence of colitis in young calves. Therefore it is suggested that *C. difficile* be considered as a possible etiologic agent when diagnosing enteric disease in calves. Results suggest that *C. difficile* culture results are not sufficient for diagnosis of CDAD in young calves and an emphasis should be placed on results of EIA testing for TcdA/B for diagnosis.
4.7 References


Table 4-1: Case exclusion criteria for logistic regression analysis

<table>
<thead>
<tr>
<th>Condition</th>
<th>N²</th>
<th>Criteria for exclusion from analysis</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enteritis (7)</td>
<td>5</td>
<td>Pathogenic <em>Salmonella</em> serovars present in the small intestine</td>
</tr>
<tr>
<td></td>
<td>2</td>
<td>Presence of <em>C. perfringens</em> in the small intestine and observation of hemorrhage of the small intestine</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Presence of <em>stxI</em> or <em>stxII</em> positive <em>E. coli</em> in the small intestine</td>
</tr>
<tr>
<td>Colitis (3)</td>
<td>3</td>
<td>Pathogenic <em>Salmonella</em> serovars present in the colon</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Presence of <em>C. perfringens</em> in the small intestine and observation of hemorrhage of the colon</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Presence of <em>stxI</em> or <em>stxII</em> positive <em>E. coli</em> in the colon</td>
</tr>
<tr>
<td>Intestinal Necrosis (3)</td>
<td>3</td>
<td>Pathogenic <em>Salmonella</em> serovars present at the site of necrosis</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Presence of <em>C. perfringens</em> and observation of hemorrhage at the site of necrosis</td>
</tr>
<tr>
<td></td>
<td>0</td>
<td>Presence of <em>E. coli</em> positive for <em>cnfI</em> or <em>cnfII</em> at the site of necrosis</td>
</tr>
<tr>
<td>Diarrhea (17)</td>
<td>5</td>
<td>Pathogenic <em>Salmonella</em> serovars present anywhere in the intestine</td>
</tr>
<tr>
<td></td>
<td>1</td>
<td>Presence of <em>C. perfringens</em> and observation of hemorrhage anywhere in the intestine</td>
</tr>
<tr>
<td></td>
<td>12</td>
<td>Presence of <em>E. coli</em> positive for any diarrhea associated virulence gene (<em>sta, stxI, stxII, cnfI, cnfII, eae, K99, CS31A, or F1845</em>) anywhere in the intestine</td>
</tr>
</tbody>
</table>

¹Value in parenthesis is total number of excluded cases

²Number of cases excluded for given criteria

³Both pathogenic *Salmonella* and *C. perfringens* with hemorrhage present in one case
Table 4-2: The frequency *Clostridium difficile* and its toxins in enteric diseases of young calves

<table>
<thead>
<tr>
<th>Condition</th>
<th>N²</th>
<th>Culture + only</th>
<th>TcdA/B + only</th>
<th>Culture + and TcdA/B</th>
<th>Culture - and TcdA/B</th>
</tr>
</thead>
<tbody>
<tr>
<td>Enteritis</td>
<td>19 (29.7)</td>
<td>2 (10.5)</td>
<td>1 (5.3)</td>
<td>3 (15.8)</td>
<td>13 (68.4)</td>
</tr>
<tr>
<td>Colitis</td>
<td>9 (13.2)</td>
<td>2 (22.2)</td>
<td>1 (11.1)</td>
<td>2 (22.2)</td>
<td>4 (44.4)</td>
</tr>
<tr>
<td>Intestinal Necrosis</td>
<td>5 (7.3)</td>
<td>2 (40.0)</td>
<td>0</td>
<td>0</td>
<td>3 (60.0)</td>
</tr>
<tr>
<td>Diarrhea</td>
<td>22 (40.7)</td>
<td>4 (18.2)</td>
<td>0</td>
<td>2 (9.1)</td>
<td>16 (72.7)</td>
</tr>
<tr>
<td>Any³</td>
<td>25 (41.7)</td>
<td>4 (16.0)</td>
<td>2 (8.0)</td>
<td>4 (16.0)</td>
<td>15 (60.0)</td>
</tr>
<tr>
<td>None</td>
<td>35 (49.3)</td>
<td>9 (25.7)</td>
<td>1 (2.9)</td>
<td>0</td>
<td>25 (71.4)</td>
</tr>
<tr>
<td>Total</td>
<td>71 (100)</td>
<td>17 (23.9)</td>
<td>3 (4.2)</td>
<td>4 (4.2)</td>
<td>47 (66.2)</td>
</tr>
</tbody>
</table>

¹Values in parenthesis are percentages of the total number of respective cases
²N= number of enteritis cases observed, value in parenthesis is percentage of total cases used for odds ratio analysis
³Any condition does not include diarrhea
Table 4-3: The odds of observing evidence of enteric disease in young calves when *Clostridium difficile* or TcdA/B are present

<table>
<thead>
<tr>
<th>Condition</th>
<th>Culture +</th>
<th>TcdA/B +</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Odds Ratio</td>
<td>95% CI</td>
</tr>
<tr>
<td>Enteritis (64)</td>
<td>1.25</td>
<td>3.73</td>
</tr>
<tr>
<td></td>
<td>0.31-4.01</td>
<td>0.75-18.66</td>
</tr>
<tr>
<td>Colitis (68)</td>
<td>2.15</td>
<td>6.87</td>
</tr>
<tr>
<td></td>
<td>0.51-9.03</td>
<td>1.23-38.31</td>
</tr>
<tr>
<td>Intestinal Necrosis (68)</td>
<td>1.67</td>
<td>0^3</td>
</tr>
<tr>
<td></td>
<td>0.26-10.82</td>
<td>N/A</td>
</tr>
<tr>
<td>Diarrhea (54)</td>
<td>0.96</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>0.28-3.23</td>
<td>0.09-3.07</td>
</tr>
<tr>
<td>Any Enteric Lesion^4 (60)</td>
<td>1.36</td>
<td>10.74</td>
</tr>
<tr>
<td></td>
<td>0.44-4.22</td>
<td>1.20-95.95</td>
</tr>
</tbody>
</table>

^1P-values are based on $\chi^2$ analysis
^2Value in parenthesis=71 total calves - excluded cases
^3There were no cases of necrosis where toxin was present
^4Collective data for cases where evidence of enteritis, colitis, and/or intestinal necrosis was observed
Table 4-4: Genotypic characteristics of *Clostridium difficile* isolated from calves

<table>
<thead>
<tr>
<th>No. isolates</th>
<th>tcdA</th>
<th>tcdB</th>
<th>cdtA</th>
<th>cdtB</th>
<th>tcdC deletion¹</th>
<th>PFGE types²</th>
<th>Calves</th>
</tr>
</thead>
<tbody>
<tr>
<td>49</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>10³</td>
<td>11</td>
</tr>
<tr>
<td>8</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>1</td>
<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>1</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>N/A</td>
<td>4</td>
<td>1</td>
</tr>
<tr>
<td>3</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>N/A</td>
<td>1⁴</td>
<td>1</td>
</tr>
</tbody>
</table>

¹ Only isolates that encode for TcdA and TcdB were screened for deletions in *tcdC*, all deletions detected were 39 bp in size
² Value indicates the number of different PFGE patterns observed among isolates for each respective category.
³ Four isolates from this category were not able to be typed using PFGE
⁴ Two isolates from this category were not able to be typed using PFGE
Table 4-5: Minimum inhibitory concentration values for *C. difficile* isolates (n=67) from calves

| Antibiotic      | MIC (mg/L)<sup>1</sup> | <2 | 0.0625 | 0.125 | 0.25 | 0.5 | 1 | 2 | 4 | 8 | 16 | 32 | 64 | 128 | 256 | 512 | 1024 | 2048 | ><sup>3</sup> |
|-----------------|-------------------------|----|--------|-------|------|-----|----|---|---|---|----|----|----|-----|-----|-----|-----|-----|---|---|
| ampicillin      |                         | 0  | 0      | 0     | 25   | 42  | 0  | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| sulfamethazine  |                         | 0  |        | 0     | 0    | 32  | 16 | 0 | 0 | 0 | 54 | 12 | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| oxytetracycline |                         | 16 | 0      | 0     | 0    | 32  | 16 | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| tetracycline    |                         | 17 | 0      | 0     | 0    | 15  | 35 | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| enrofloxacin    |                         | 0  | 0      | 0     | 0    | 0   | 2  | 32| 33| 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| clindamycin     |                         | 51 | 0      | 16    | 0    | 0   | 0  | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| vancomycin      |                         | 66 | 0      | 1     | 0    | 0   | 0  | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| metronidazole   |                         | 67 | 0      | 0     | 0    | 0   | 0  | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| erythromycin    |                         | 6  | 61     | 0     | 0    | 0   | 0  | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| neomycin        |                         | 0  | 0      | 0     | 0    | 0   | 0  | 0 | 6 | 0 | 0  | 60 | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| florfenicol     |                         | 6  | 55     | 0     | 0    | 0   | 0  | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |
| cefoxitin       |                         | 0  | 0      | 0     | 0    | 0   | 0  | 0 | 0 | 0 | 0  | 51 | 16 | 0   | 0   | 0   | 0   | 0   | 0  |   |
| ciprofloxacin   |                         | 0  | 0      | 13    | 54   | 0   | 0  | 0 | 0 | 0 | 0  | 0  | 0  | 0   | 0   | 0   | 0   | 0   | 0  |   |

<sup>1</sup> Values listed in table represent the number of isolates with MIC values respective to each antibiotic concentration, no number is listed for antibiotic concentrations that were not tested.

<sup>2</sup> MIC less than the lowest concentration tested, no growth observed at all concentrations tested.

<sup>3</sup> MIC greater than the highest concentration tested, growth observed at all concentrations tested.

Boxes indicate MIC resistance cut-offs specified by CLSI for *C. difficile* for respective antibiotics, isolates with MICs at these concentrations or higher are considered resistant. Underlines indicate susceptibility cut-offs for *C. difficile* for respective antibiotics, isolates with MICs at these concentrations or lower are considered susceptible. MIC values between the underlined and boxed values are considered intermediate.

Resistance cut-off MIC values relevant to *C. difficile* have not been specified by CLSI for all antibiotics listed, no boxes are present in these instances.
Table 4-6: Antibiotic resistance profiles of *Clostridium difficile* isolated from calves.

<table>
<thead>
<tr>
<th>Antibiotic Resistance Profile(^1)</th>
<th>No. isolates (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Profile Number</td>
<td>Amp(^2)</td>
</tr>
<tr>
<td>1</td>
<td>I</td>
</tr>
<tr>
<td>2</td>
<td>I</td>
</tr>
<tr>
<td>3</td>
<td>I</td>
</tr>
<tr>
<td>4</td>
<td>S</td>
</tr>
<tr>
<td>5</td>
<td>S</td>
</tr>
<tr>
<td>6</td>
<td>S</td>
</tr>
</tbody>
</table>

\(^1\)I-intermediate, S-susceptible, R-resistant

\(^2\)Amp-ampicillin, Cld-clindamycin, Cxt-cefoxitin, Met-metranidazole, Tet-tetracycline
Figure 4-1. Experimental design used to determine the association of toxigenic *Clostridium difficile* with enteric disease in naturally infected calves.
Figure 4-2: PFGE profiles of *C. difficile* isolated from calves. Similarity within clusters: A-81.5%, A1-87.7%, A2-85.6%, A2-1-100%, A2-2-93.8%, A3-89.6%, B-67.6%. Condition: C-colitis, D-diarrhea, E-enteritis, N-necrosis. AbR profile- antibiotic resistance profile. *In vitro* TcdA/B as assessed at 24h. Calf-isolates with the same number were isolated from the same calf.
Chapter 5

Effects of sub-inhibitory concentrations of oxytetracycline on toxins A and B production by *Clostridium difficile* isolated from calves
5.1 Abstract

Antibiotics used in human medicine have been shown to increase or induce production of toxins A and B by *Clostridium difficile*, an important enteric pathogen in humans and animals. In a previous study we found an association of enteric disease in calves with the presence of *C. difficile* toxins TcdA and/or TcdB in the feces. Oxytetracycline (Oxy) is commonly incorporated in milk-replacer fed to calves for treatment and prevention of illness, improvement of digestion, and growth promotion. Administering this antibiotic in milk replacer reduces bioavailability, but also increases fecal excretion due to calcium ion binding. In this study, the objective was to determine the shift of minimum inhibitory concentrations (MIC) of Oxy due to the presence of calcium ions and to determine the effects of Oxy on *in vitro* TcdA and TcdB production under conditions relevant to the calf colon. Nine representative toxigenic *C. difficile* isolates collected from calves were examined. The Oxy MIC value doubled for 7, 1, and 1 isolates in cultures containing Ca$^{2+}$ concentrations of 50 mg/l, 100 mg/l, and 200 mg/l, respectively. The MIC value was quadrupled for 2 and 1 isolates in cultures containing Ca$^{2+}$ concentrations of 100 mg/ml and 200 mg/ml, respectively. Oxytetracycline was able to increase toxin production of one isolate and induced toxin production in another. Both of these isolates have a 100 bp deletion in the tcdC gene, a negative regulator of TcdA and TcdB expression. No increase or induction of toxin expression was observed for isolates with intact tcdC genes. We also observed higher toxin production at pH 8 (median pH of milk-fed calf feces) for three isolates. Data collected in this study show the administration of Oxy to calves may promote toxin production by some *C. difficile* strains and the presence of calcium ions increases Oxy MIC. Further *in vivo* investigation is needed to fully understand how use of Oxy in milk replacer may affect *C. difficile*-associated enteric disease in calves.
5.2 Introduction

*Clostridium difficile* is an established human pathogen commonly involved in diseases such as antibiotic-associated diarrhea and pseudomembranous colitis (Pothoulakis et al., 1996; Razavi et al., 2007; Riccardi et al., 2007). Toxins produced by *C. difficile* include TcdA, an enterotoxin, and TcdB, a cytotoxin. In the previous chapter, we observed an association of enteric disease in calves with the presence of toxins TcdA and/or TcdB (TcdA/B) in the feces. Further, it has been previously reported that experimental intoxication with both toxins leads to tissue damage, fluid accumulation, and neutrophil infiltration in both the large and small intestines of calves (Hammitt et al., 2008). Both toxins monoglucosylate proteins important in F-actin regulation (von Eichel-Streiber et al., 1996). Loss of F-actin regulation leads to disruption of the cytoskeleton resulting in the compromise of intestinal epithelial cell tight junctions. Severity of *Clostridium difficile*-associated disease (CDAD) is attributed to the level of toxins released in the gut (Akerlund et al., 2006; Keel and Songer, 2006; von Eichel-Streiber et al., 1996).

It is well known that the use of broad spectrum antibiotics contributes to *C. difficile*-associated disease by inhibiting growth of normal gut flora and allowing colonization of resistant pathogenic strains of *C. difficile* (Borriello and Barclay, 1986). Current research has provided evidence that antibiotics may also influence *C. difficile* toxin production. Antibiotics used in human medicine have been shown to induce and/or increase production of TcdA/B by *C. difficile in vitro* when added to culture media at sub-inhibitory concentrations (Drummond et al., 2003; Gerber et al., 2008; Onderdonk et al., 1979). Understanding how antibiotics may affect the pathogenesis of *C. difficile* disease is critical in calves. Young calves are commonly administered antibiotics for treatment and prevention of illness, improvement of digestion, and growth promotion. The most common antibiotics used in calves are tetracyclines and sulfonamides (Frost, 1991; Sawant et al., 2005). Oxytetracycline (Oxy) is incorporated in milk replacer at doses of 0.05-0.1 mg/lb body weight per day to promote growth and improve digestion. A dose of 10
mg/lb body weight per day (for 7-14 days) is administered in milk replacer for treatment of enteritis (USDA, 2008).

Fractions of orally administered Oxy are excreted in the feces (Bryskier, 2005; Kelly and Buyske, 1960; Kunin and Finland, 1961). When administered with foods containing calcium, such as milk, absorption of tetracyclines is reduced due to ion binding (Bryskier, 2005). The result is the gut microflora are exposed to a larger fraction of the administered dose, although antimicrobial activity may be reduced due to ion binding (Chiang, 1981; Nouws, 1992). If Oxy is able to increase or induce toxin production of *C. difficile*, feeding milk replacer supplemented with Oxy may contribute to the severity of *C. difficile* associated enteric disease in calves.

Oxytetracycline is a bacteriostatic antibiotic which inhibits growth of both Gram positive and Gram negative bacteria by targeting the 30S subunit of the ribosome. Protein synthesis is inhibited by preventing the binding of aminoacyl-tRNA to the ribosome (Bryskier, 2005). Sub-inhibitory concentrations of clinamycin, an antibiotic used in human medicine which also interferes with protein synthesis by acting on the ribosome, has been shown to increase TcdA/B expression (Drummond et al. 2003). Evidence provided by previous researchers suggests up-regulation of TcdA/B expression is a non-specific stress related response to sub-optimal environmental conditions (Haslam et al., 1986; Hennequin et al., 2001; Karlsson et al., 2003; Onderdonk et al., 1979; Yamakawa et al., 1996). In fact, Underwood et al. showed genes of the sporulation pathway, known to be regulated by environmental cues, may be involved in expression of toxin genes (Underwood et al., 2009). The objectives of the study were to: 1) determine the effect of Ca\(^{2+}\) on the minimum inhibitory concentration (MIC) of Oxy for *C. difficile* isolated from calves; and 2) investigate the effects of Oxy and non-specific cell stress on TcdA/B production of *C. difficile* isolated from calves with respect to the environmental conditions of the calf gut.
5.3 Materials and Methods

5.3.1 Isolates

Nine representative isolates of *C. difficile* were chosen from a collection of calf isolates collected in the previous chapter to examine the effects of Oxy on expression of clinically relevant toxins, TcdA/B. Isolates were selected based on three characteristics: 1) production of TcdA/B in a 24 h culture, 2) presence or absence of a 39 bp deletion in the *tcdC* gene, and 3) the enteric pathology of the calves from which the isolates originated. Isolates were first placed in one of four categories based on the presence of a 39 bp deletion in the *tcdC* gene, a negative regulator of TcdA/B production (Matamouros, 2007), and expression of TcdA/B *in vitro* (Table 5-1). The four categories were: 1) *in vitro* TcdA/B production at 24 h and *tcdC* deletion, 2) *in vitro* TcdA/B production at 24 h and no *tcdC* deletion, 3) no *in vitro* TcdA/B production at 24 h and *tcdC* deletion, 4) no TcdA/B production at 24 h and no *tcdC* deletion. All five isolates in the first category were included in the study. Two isolates each were then chosen from categories 3 and 4 (no isolates belonged to category 2) based on pathology data for a total of nine isolates described in Table 5-2. Isolates were from nine different calves and had a wide range of Oxy MIC values (0.5 mg/L to 16 mg/L). Isolates originated from calves with and without evidence of enteric disease upon postmortem examination. Isolates a and c had indistinguishable PFGE patterns upon digestion with *Sma*I while all other isolates had distinguishable patterns. Isolates a-e were used to determine if sub-inhibitory concentrations of Oxy can increase TcdA/B production at 24 h of incubation. Isolates f through i were used to determine if toxin production is induced in the presence of sub-inhibitory concentrations of Oxy.

5.3.2 Effect of Ca$^{2+}$ on oxytetracycline MIC values

Cations and pH are known to affect the activity of Oxy (Bryskier, 2005; Lorian, 2005; Nouws, 1992). In this study it was our intent to assess the activity of Oxy at cation concentrations
and pH levels relevant to the calf colon. Susceptibility assays were carried out using a range of different concentrations of Ca\(^{2+}\) because cation concentrations in the gut may differ from calf to calf. In order to determine the appropriate media pH to use, the pH of 30 random fecal samples from milk-replacer fed calves 4-6 weeks of age was measured. The pH of media used in susceptibility assays was 8.0, based on the median and mode fecal pH readings.

Although agar dilution is the method approved by the Clinical and Laboratory Standards Institute (CLSI) for determining antibiotic susceptibility of *C. difficile*, we chose to use a broth dilution assay for the purposes of this study (CLSI, 2007). This decision was made based on fact that the pH may change in agar plates under anaerobic conditions due to absorption of CO\(_2\) (Lorian, 2005). We used a broth dilution method so that the pH could be adjusted to 8.0 following prereduction of media in the anaerobic chamber.

Four different concentrations of Ca\(^{2+}\) (in the form of CaCl\(_2\)) ranging from 50 mg/L to 400 mg/L were added to 4 aliquots of Brucella broth supplemented with hemin (5 mg/L) and vitamin K (1 mg/L). A fifth Brucella broth aliquot was prepared containing no Ca\(^{2+}\). Broth preparations were pre-reduced in an anaerobic chamber for at 2 h and the pH of each aliquot was adjusted to 8.0. Each of the five aliquots was then separated into 12 sub- aliquots. The sub- aliquots were used to make two-fold dilutions Oxy (ranging from 2 mg/L to 1024 mg/L). The twelfth sub- aliquot of each of the five Brucella broth preparations contained no Oxy and were used as positive controls. A suspension of each isolate was made in pre-reduced Brucella broth (supplemented with vitamin K (1 mg/L) and hemin (5 mg/L), pH adjusted to 8.0) and turbidity was adjusted to 0.5 MacFarland as described by CLSI (2007). An inoculum of 50 µl was added to each control and experimental well, resulting in final experimental concentrations of Ca\(^{2+}\) ranging from 25 mg/L to 200 mg/L and Oxy ranging from 1 mg/L to 512 mg/L for each Ca\(^{2+}\) concentration as shown in Appendix A. A negative control of uninoculated broth was added to one well of the plate as described by CLSI (2007) to ensure the experiment was free from contamination.
Plates were incubated anaerobically for 24 h at 37°C. Plates were then examined for growth. For each isolate, the lowest concentration of Oxy where growth was inhibited was considered the MIC and recorded for each Ca²⁺ concentration. The data collected, then, included five MIC values for each isolate relating to the Ca²⁺ concentrations used. This data was used to determine the appropriate experimental conditions to be used in toxin production experiments.

5.3.3 Effects of sub-inhibitory concentrations of oxytetracycline and media pH on toxins A and B production levels

Isolates a through e were grown in 10 ml of pre-reduced Brucella broth supplemented with hemin (5 mg/L) and Vitamin K (1 mg/L) (pH adjusted to 8.0). Brucella broth aliquots (5 ml) were prepared as described in section 5.3.2 with concentrations of Oxy ranging from the MIC to 0.06xMIC determined for each isolate at respective concentrations of Ca²⁺ (0 mg/L, 25 mg/L, 50 mg/L, 100 mg/L, 200 mg/L). No Oxy controls were also prepared for each Ca²⁺ concentration as well as a broth only control. The experimental matrix used for each isolate is illustrated in Appendix B. Approximately 10⁶ cells were added to each aliquot and incubated anaerobically for 24 h at 37°C. A tube of un-inoculated broth was also incubated to ensure no contamination had occurred.

All cultures were examined for growth and TcdA/B levels after 24h incubation. Growth of experimental and control cultures was determined by measuring the optical density (OD) of 200 ul of culture at 600 nm using a spectrometer for the purposes of data normalization. One ml of culture was centrifuged at 14000 rpm for 5 min. Supernatant (50 µl) was examined for TcdA/B levels using a commercially available enzyme immunoassay (EIA), *C. difficile* Tox A/B II kit (TechLab, Blacksburg, VA). This assay has been previously validated and is able to detect as little as 1 ng/ml and 2-5 ng/ml of TcdA and TcdB, respectively, with 100% specificity (Lyerly et al., 1998). The EIA contains antibodies against TcdA/B in the same assay well, therefore, a positive result can be due to the presence of either toxin or the presence of both. The optical
density resulting from the EIA reactions was measured at 450 nm (OD_{450}) using a spectrometer as described by the EIA manufacturer for quantification of TcdA/B.

In order to assess the effects of non-specific cell stress on toxin production, isolates a through e were also grown in 10 different 5 ml broth aliquots of Brucella broth supplemented with hemin (5 mg/L) and Vitamin K (1 mg/L) with a pH range of 3 to 12 (pH adjusted following pre-reduction of media) as shown in Appendix D. Cultures were grown for 24 h and growth and toxin levels measured as described above.

5.3.4 Induction of toxins A and B production by sub-inhibitory levels of oxytetracycline and media pH

Isolates f through i were grown in 10 ml of pre-reduced Brucella broth supplemented with hemin (5 mg/L) and Vitamin K (1 mg/L) (pH adjusted to 8.0). Brucella broth aliquots (5 ml) were prepared with concentrations of Oxy ranging from the MIC to 0.06X MIC determined for each isolate as illustrated in Appendix E. In order to determine if toxin expression is indeed by non-specific cell stress, isolates f through i were also grown in 10 different 5 ml broth aliquots with a pH range of 3 to 12 as described in section 5.3.3 (Appendix E). Cultures incubated in an anaerobic chamber at 37°C and examined for growth and TcdA/B levels at several time points: 24 h, 48 h and 72 h. The growth of control cultures and cultures under experimental conditions was monitored by measuring the OD_{600}. Toxin was detected using methods described in section 5.3.3. Growth readings were plotted over time to generate growth curves for each isolate and each culture condition. The time point at which TcdA/B was first detected for each culture condition was denoted on the growth curve of each isolate.

5.3.5. Data Analysis

Growth rates may be slowed due to the presence of Oxy which can result in lower overall toxin level readings. Because our intention was to determine the effects of Oxy on toxin
production, we normalized the data with respect to growth levels at 24 hours of incubation. Data was normalized by dividing the OD$_{450}$ toxin reading by the OD$_{600}$ growth reading for each experimental and control culture tube. The mean of the 5 normalized toxin readings at each sub-MIC concentration of Oxy was calculated for a total of 5 mean toxin level readings for isolates a through e (Appendix B): Mean 1- normalized toxin level of no-Oxy control, Mean 2- normalized toxin level at 0.06xMIC of Oxy, Mean 3- normalized toxin level at 0.12xMIC of Oxy, Mean 4- normalized toxin level at 0.25xMIC of Oxy, Mean 5- normalized toxin level at 0.50xMIC of Oxy.

In order to determine if sub-inhibitory concentrations of Oxy significantly increased toxin production of *C. difficile* isolated from calves, 2-sample paired t-tests were performed comparing TcdA/B production levels under experimental conditions to TcdA/B production levels under control conditions for isolates a through e. The 2-sample paired t-test was chosen due to its ability to determine if mean TcdA/B production under 2 different culture conditions are significantly different (Zar, 1999). The 2-sample t-tests were paired so that TcdA/B production under experimental conditions was related to TcdA/B production in controls containing respective concentrations of Ca$^{2+}$. Four separate 2-sample paired t-tests were used to compare the mean toxin level under control conditions to mean toxin levels under experimental conditions:

$H_0^1$: Mean 1 = Mean 2

$H_0^2$: Mean 1 = Mean 3

$H_0^3$: Mean 1 = Mean 4

$H_0^4$: Mean 1 = Mean 5

The above statistical analysis was carried out for each isolate and also for collective data from all isolates. Differences in toxin production were considered significant (null hypothesis was rejected) where $p<0.05$ and only slightly significant where $p<0.50$. 
In order to rule out a possible influence of Ca$^{2+}$ on toxin production levels, 4 separate 2-sample paired t-tests were performed comparing TcdA/B production levels at different Ca$^{2+}$ concentrations for each sub-MIC experimental condition (Appendix C). Samples were paired so that TcdA/B production levels at each Ca$^{2+}$ concentration were respective to each isolate:

\[ H_0^5: \text{Mean 6} = \text{Mean 7} \]
\[ H_0^6: \text{Mean 6} = \text{Mean 8} \]
\[ H_0^7: \text{Mean 6} = \text{Mean 9} \]
\[ H_0^8: \text{Mean 6} = \text{Mean 10} \]

To determine the difference in toxin production due to non-specific cell stress, data was normalized as described above. A series of 2-sample paired t-tests was used to compare the mean toxin production of all isolates cultured at basic and acid pH levels to that of culture grown at neutral pH (Appendix D). The t-tests were paired so that TcdA/B production under experimental conditions was compared to TcdA/B production in controls respective to each isolate. Differences in toxin production were considered significant where $p<0.05$ and only slightly significant where $p<0.50$.

In order to determine if sub-inhibitory concentrations of Oxy or media pH (acidic or basic) are able to induce TcdA/B expression, the OD$_{600}$ growth readings of isolates f through i respective to the time point when toxin was first detected (if detected at all) were compared. To determine if sub-inhibitory concentrations of Oxy are able to induce TcdA/B expression, the time point and growth level (OD$_{600}$) of first TcdA/B detection under experimental conditions was compared with the time point and growth level (OD$_{600}$) of first TcdA/B detection under control conditions for each isolate. To determine if media pH is able to induce toxin A and B expression, the time point and growth level (OD$_{600}$) of first TcdA/B detection at basic and acidic pH levels was compared with the time point and growth level (OD$_{600}$) of first TcdA/B detection at pH 7 for each
isolate. We concluded sub-inhibitory concentrations of Oxy and/or media pH is able to induce production of TcdA/B if toxin was detected at an earlier time point or at a lower OD_{600} growth reading in cultures under experimental conditions than in control cultures.

5.4 Results

5.4.1 Effect of Ca^{2+} on oxytetracycline MIC

The shifts in MIC values resulting from the presence of Ca^{2+} for all isolates are listed in Table 5-3. No shift in MIC was observed for any of the isolates cultured at a Ca^{2+} concentration of 25mg/l. The MIC value doubled for 7, 1, and 1 isolates cultured at Ca^{2+} concentrations of 50mg/l, 100mg/l, and 200mg/l, respectively. The MIC value was quadrupled for 2 and 1 isolates cultured at Ca^{2+} concentrations of 100mg/ml and 200mg/ml, respectively.

5.4.2 Effects of sub-inhibitory concentrations of oxytetracycline and media pH on toxins A and B production levels

Differences in TcdA/B production resulting from sub-inhibitory concentrations of Oxy are listed in Table 5-4. Data analysis showed no significant difference in TcdA/B production due to the presence of Ca^{2+}. Although toxin was detected in control cultures, isolates b and e did not produce detectable levels of toxin at any sub-inhibitory concentration of Oxy. A significant increase in TcdA/B production was seen for isolate d at 0.50xMIC of Oxy. A normalized mean toxin level of 2.70 was recorded for 0.50xMIC of Oxy when compared to the no-Oxy control (p=0.04). A slightly significant increase in toxin was also seen for isolate d at 0.25xMIC of Oxy (p=0.28). Toxin production at 0.06xMIC and 0.12xMIC of Oxy for isolate d was less and statistically no different, respectively, as compared to no-Oxy controls.

Isolates a and c produced less TcdA/B at sub-inhibitory concentrations of Oxy than that detected in the no-Oxy controls. For isolate a, TcdA/B levels at 0.06xMIC of Oxy were not
significantly different than TcdA/B levels measured in the controls. Toxin production was significantly lower (p=0.007) for isolate a at 0.25xMIC of Oxy when compared to the no-Oxy control. For isolate c, the difference in TcdA/B production as compared to the controls was only slightly significant (p<0.5). When collectively analyzing data from all isolates, significantly less TcdA/B production was observed in the presence of 0.06xMIC (p=0.008), 0.12xMIC (p=0.0003), and 0.25xMIC (p=0.02) of Oxy when compared to the no-Oxy controls. Toxin production was also less overall at 0.50xMIC of Oxy, but the difference as compared to the no-Oxy control was only slightly significant (p=0.22).

Isolates were able to grow at a pH range of 6 to 9. Isolates a, b, and c produced toxin at a pH range of 7 to 8, isolate d produced toxin at a pH range of 7 to 9, and isolate e produced TcdA/B at a pH range of 6 to 9. Isolates a, b, and d all produced more TcdA/B at pH 8 than at neutral pH (Table 5-5). Isolate d produced more TcdA/B at pH 9 as compared to pH 7 as well. Isolates c and e produced more TcdA/B at neutral pH than under acidic or basic conditions. Overall, more TcdA/B production (1.08) was seen at pH 8 than at pH 7 (0.88), although statistical analysis showed the result to be only slightly significant (p=0.32). Significantly less TcdA/B was produced at pH 6 as compared to neutral pH (p=0.02).

5.4.3 Induction of toxins A and B production by sub-inhibitory levels of oxytetracycline and media pH

Production of TcdA/B was induced by Oxy (0.25xMIC) for isolate g (Table 5-6). Toxin was detected in the culture containing 0.25xMIC of Oxy at 48h (OD$_{600}$=0.38). The no-Oxy control had a higher growth reading at 72 h than the culture grown with 0.25xMIC of Oxy, yet no TcdA/B was detected in the control culture (Figure 5-3). Toxin was first detected in no-Oxy control cultures of isolates f and i at 48 h with OD$_{600}$ growth readings of 0.51 and 0.52, respectively. Toxin was also produced by isolate f at 0.06xMIC and 0.12xMIC of Oxy at 72 h and 48 h with OD$_{600}$ growth readings of 0.50 and 0.26, respectively (Figure 5-1). Although TcdA/B
was first detected at 48 h for both the no-Oxy control and the 0.12xMIC of Oxy cultures of isolate f. Culture grown at 0.12xMIC of Oxy had a lower OD$_{600}$ growth reading (0.23) than the no-Oxy control (0.51). Toxin levels at 72 h were higher in the culture containing 0.06xMIC of Oxy for isolate f than the no-Oxy control even though OD$_{600}$ growth readings were similar. No toxin was detected in cultures of isolate i containing sub-inhibitory concentrations of Oxy. No TcdA/B was produced after 72 h by isolate h in no-Oxy control cultures as well as cultures containing Oxy.

None of the isolates were able to grow at pH<6 or pH >9. Toxin was first detected for isolates f, g, and i after 48h (Table 5-7). Acidic and basic culture conditions did not result in earlier detection of toxin for any isolate. Although isolate f produced toxin at a later time point under acidic conditions than at pH7, the isolate produced TcdA/B at a lower OD$_{600}$ growth reading and produced more TcdA/B overall than the control culture at 72 h (Figure 5-2). Isolate f also produced more TcdA/B overall at pH 8 and pH 9 when compared to the control even though OD$_{600}$ growth readings were similar. Isolate g did not produce toxin at pH 6 or pH 8 and produced more toxin overall at pH 7 than at pH 9. Isolate i did not produce toxin at pH 6 or pH 9 and produced more toxin overall at pH 7 than at pH 8. Toxin was not detected in experimental or control cultures for isolate h.

### 5.5 Discussion

This is the first study investigating the effects of Oxy on *in vitro* production of TcdA/B by *C. difficile* isolated from calves. Isolate d, from a diarrheic calf, produced significantly more toxin in the presence of 0.50xMIC of Oxy than the in the no-Oxy control. Although toxin production by this isolate was not statistically different at lower concentrations of Oxy as compared to the no-Oxy control, toxin production increased as the concentration of Oxy increased (Table 5-4). As expected, the antimicrobial activity of Oxy was reduced by the presence of Ca$^{2+}$ (Table 5-3). Using the experimental matrix for isolate d (Appendix B) we have
determined that in order for Oxy to increase toxin production, a concentration of 8 mg/l is needed in the presence of 25 mg/l Ca\(^{2+}\). When Ca\(^{2+}\) is present in concentrations of 50-200 mg/l an Oxy concentration of 16 mg/l is needed to significantly increase isolate d toxin production.

Oxytetracycline was also able to induce toxin production by an isolate (g) from a calf with enteric evidence of colitis, enteritis, and diarrhea. Toxin production was induced in isolate g at 0.25xMIC of Oxy. The presence of Oxy also resulted in toxin production at lower growth levels for isolate f as compared to no-Oxy controls (Table 5-6, Figure 5-1, Figure 5-2). Isolates d and g both carry a 100 bp deletion in the \textit{tcdC} gene. The TcdC protein negatively regulates TcdA/B production and deletions in this gene have been associated with hypervirulent strains (Loo et al., 2005; Lyerly et al., 1998; Warny et al., 2005). Interestingly, we did not observe induction of toxin expression by sub-inhibitory concentrations of Oxy for isolates h and i, both of which have intact \textit{tcdC} genes. This suggests strains with \textit{tcdC} deletions may have increased virulence capabilities, but toxin production in many more strains must be evaluated before this conclusion can be made. Other researchers have found no connection between \textit{tcdC} deletions and severity of CDAD (Verdoorn et al., 2009).

The results of this study are surprising due to the mechanism of action of Oxy. Because Oxy inhibits growth of bacteria through disabling protein elongation by the ribosome (Bryskier, 2005), it is expected that growth and toxin production would decrease as the concentration of Oxy increases. Interestingly, other researchers have reported increases in toxin production by \textit{C. difficile} in the presence of clindamycin, an antibiotic with a mechanism of action similar to Oxy (Drummond et al., 2003). Drummond et al. (2003) reported that sub-inhibitory concentrations of clindamycin resulted in toxin production earlier in the growth curves for 3 standard \textit{C. difficile} strains. They also observed higher toxin production in the presence of clindamycin in one strain. Gerber et al. (2008) observed toxin production earlier in the growth curves of four isolates grown with 0.50xMIC of Met, Van, or linezolid.
A likely explanation for this phenomenon is that as the concentration of antibiotic rises, cell stress levels also rise. Under stressful environmental conditions, bacteria alter protein expression patterns to promote survival (Vorob’eva, 2003). Hennequin et al. (2001) found expression of GroEL, a heat shock protein involved in adhesion, was upregulated by heat shock, acid shock, osmotic shock, iron deprivation, and subinhibitory levels of ampicillin. Underwood et al. (2009) found that sporulation, an event known to occur in *C. difficile* under stressful conditions, is linked to *C. difficile* toxin production. They showed mutation of the *spo0A* gene, involved in the sporulation initiation pathway and controlled by environmental cues, resulted in a 90% decrease in toxin A production, suggesting *spo0A* may also play a role in the regulation of toxin expression. The data collected here on toxin production levels under basic conditions further suggests toxin production increases due to stressful environmental conditions. Although the overall data was only slightly significant, 3 out of 5 isolates produced more toxin on a per cell basis at pH 8 than at pH 7 (Table 5-5). This is a relevant observation because the pH of feces from 30 random calves measured here had a median and mode of pH 8 suggesting conditions of the calf gut may promote toxin production in certain *C. difficile* strains.

The effect of antibiotics on toxin production has been reported to be strain dependent (Barc et al., 1992; Drummond et al., 2003). This study showed similar findings in that some isolates showed no toxin production, significantly lower toxin production, or no significant difference in toxin production in the presence of sub-inhibitory concentrations of Oxy or under acidic or basic conditions as compared to controls. Also, isolate d, which showed increases in TcdA/B production under experimental conditions did so in the presence of Oxy and in media with basic pH levels. Toxin expression by isolate d increased with increasing concentrations of Oxy and also showed increasing TcdA/B production as pH increased.

The observed association of toxin production with Oxy concentration suggests the level of Oxy resistance may dictate the significance of the effect Oxy will have on *C. difficile* toxin production. Resistance to tetracyclines in most *C. difficile* strains is attributed to the tet(M) gene
carried on the conjugative transposon Tn5397 (Mullany et al., 1990). It has been reported that a small percentage of isolates resistant to tetracyclines carry the tet(M) gene on the Tn916 transposon (Spigaglia et al., 2007). It has been shown that Tn916 can be transferred to from strain to another and may promote dissemination of tetracycline resistance among C. difficile (Rice, 1998; Rice, 2002; Spigaglia et al, 2007). It should be noted, though, that plasmids are not commonly detected in C. difficile isolates suggesting resistance elements have been incorporated in the chromosomal DNA of most resistant strains (Spigaglia et al., 2007). Tetracycline resistance may allow for C. difficile colonization of Oxy treated calves. More research is needed, however, to determine if an association exists between the genetic elements contributing to tetracycline resistance and the effects of Oxy on toxin production in C. difficile.

Understanding how Oxy may affect the pathogenesis of C. difficile disease is critical in calves. Oxytetracycline is incorporated in milk replacer at doses of 0.05-0.1 mg/lb body weight per day to promote growth and improve digestion. A dose 10mg/lb body weight per day (for 7-14 days) is administered in milk replacer for treatment of enteritis (USDA, 2008). It has been reported that fecal toxin levels correlate to severity of disease. Akerlund et al. (2006) found fecal toxin levels were correlated with diarrhea frequency and severity as well as abdominal cramping in humans. Similar results have been reported in gnotobiotic mice, where C. difficile strains producing high toxin levels in vitro were associated with severe ulceration of cecal epithelium and 100% mortality (Vernet et al., 1989). The data presented here suggest the presence of sub-inhibitory concentrations of Oxy in the calf gut may result in increased toxin production by certain C. difficile strains and in doing so may result in greater severity of disease.

5.6 Conclusions

We are the first to report the effects of subinhibitory concentrations of Oxy on toxin expression in C. difficile isolated from calves. The results of this study suggest use of
oxtetracycline in calves may promote toxin production by certain *C. difficile* strains found in the calf gut, although it is difficult to use the data reported here to infer on how Oxy may effect *C. difficile* toxin production *in vivo*. As we have shown here, calcium ions found in the calf diet reduce the activity of Oxy (Table 5-3). Therefore, in order for Oxy to effect toxin production of *C. difficile* in the calf gut, Oxy must be present in the gut at a sufficient concentration (determined to be 8-16mg/l for isolate d) relevant to the concentration of Ca\(^{2+}\). Although Oxy in known to be excreted in the feces, it is unclear if it is excreted at sufficient concentrations to effect toxin production by *C. difficile* (Bryskier et al., 2005; Kelly and Buyske, 1960; Kunin and Finland, 1961). There are also other factors in the gut that may effect toxin expression including the presence of other bacterial species and available nutrition (Banerjee et al., 2009; Haslam et al., 1986; Karlsson et al., 2003; Yamakawa et al., 1996).
5.7 References


Table 5-1: Distribution of \( tcdC \) deletions among isolates expressing TcdA/B \textit{in vitro} at 24h

<table>
<thead>
<tr>
<th>TcdA/B + at 24 hours</th>
<th>( tcdC ) deletion</th>
<th>total</th>
</tr>
</thead>
<tbody>
<tr>
<td>+</td>
<td>5</td>
<td>5</td>
</tr>
<tr>
<td>-</td>
<td>43</td>
<td>53</td>
</tr>
<tr>
<td>total</td>
<td>48</td>
<td>58</td>
</tr>
</tbody>
</table>
### Table 5-2: Characteristics of isolates to be examined

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Toxin Genes</th>
<th>Characteristics for selection</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>tcdA</td>
<td>tcdB</td>
</tr>
<tr>
<td>a</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>b</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>c</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>d</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>e</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>f</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>g</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>h</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>i</td>
<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

1. Isolate designation
2. All deletions detected were 39 bp in size
3. *In vitro* expression of TcdA/B after 24 h incubation
4. Evidence of enteric disease observed at necropsy; C: colitis; D: diarrhea; E: enteritis; N: intestinal necrosis
5. Minimum concentration of oxytetracycline required to inhibit 90% of growth of isolate (mg/L)
Table 5-3: Shift of oxytetracycline MICs of *Clostridium difficile* isolated from calves in the presence of Ca\(^{2+}\)

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Ca(^{2+}) (mg/l)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0</td>
</tr>
<tr>
<td>a</td>
<td>8(^1)</td>
</tr>
<tr>
<td>b</td>
<td>16</td>
</tr>
<tr>
<td>c</td>
<td>16</td>
</tr>
<tr>
<td>d</td>
<td>16</td>
</tr>
<tr>
<td>e</td>
<td>16</td>
</tr>
<tr>
<td>f</td>
<td>16</td>
</tr>
<tr>
<td>g</td>
<td>0.5</td>
</tr>
<tr>
<td>h</td>
<td>0.5</td>
</tr>
<tr>
<td>i</td>
<td>0.5</td>
</tr>
</tbody>
</table>

\(^1\) Concentration of oxytetracycline (mg/l) required to inhibit growth
Table 5-4: Effect of sub-inhibitory concentrations of oxytetracycline on toxin production levels of *Clostridium difficile* isolated from calves

<table>
<thead>
<tr>
<th>Isolate</th>
<th>C²</th>
<th>0.06xMIC³</th>
<th>0.12xMIC</th>
<th>0.25xMIC</th>
<th>0.50xMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>1.47</td>
<td>1.12</td>
<td>0</td>
<td>0.10⁴</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.53</td>
<td>-</td>
<td>5.06</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.62</td>
<td>-</td>
<td>0.007</td>
<td>-</td>
</tr>
<tr>
<td>b</td>
<td>0.63</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>c</td>
<td>0.77</td>
<td>0.24</td>
<td>0.21</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>1.76</td>
<td>1.91</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.15</td>
<td>0.13</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>d</td>
<td>0.97</td>
<td>0.54</td>
<td>0.98</td>
<td>1.71⁶</td>
<td>2.70⁵</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>1.04</td>
<td>0.38</td>
<td>1.25</td>
<td>3.03</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.36</td>
<td>0.97</td>
<td>0.28</td>
<td>0.04</td>
</tr>
<tr>
<td>e</td>
<td>0.56</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Mean</td>
<td>0.88</td>
<td>0.38⁴</td>
<td>0.24⁴</td>
<td>0.36⁵</td>
<td>0.54</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>2.89</td>
<td>4.23</td>
<td>2.43</td>
<td>1.26</td>
</tr>
<tr>
<td></td>
<td>N/A</td>
<td>0.008</td>
<td>0.0003</td>
<td>0.02</td>
<td>0.22</td>
</tr>
</tbody>
</table>

¹ Normalized mean data  
² Control- no oxytetracycline  
³ Sub-inhibitory concentration of oxytetracycline with respect to MIC for each isolate  
⁴ Significant (p<0.05) result, less toxin produced in the presence of oxytetracycline  
⁵ Significant (p<0.05) result, more toxin produced in the presence of oxytetracycline  
⁶ More toxin produced in the presence of oxytetracycline, slight significance (p<0.5)
Table 5-5: Effect of pH on toxin production levels of *Clostridium difficile* isolated from calves

<table>
<thead>
<tr>
<th>Isolate</th>
<th>3</th>
<th>4</th>
<th>5</th>
<th>6</th>
<th>7</th>
<th>8</th>
<th>9</th>
<th>10</th>
<th>11</th>
<th>12</th>
</tr>
</thead>
<tbody>
<tr>
<td>a</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.01</td>
<td>1.34</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>b</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>1.33</td>
<td>2.00</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>c</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.33</td>
<td>0.16</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>d</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.34</td>
<td>0.76</td>
<td>0.88</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>e</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0.17</td>
<td>1.39</td>
<td>1.14</td>
<td>1.20</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

| Mean    | 0 | 0 | 0 | 0.03\(^4\) | 0.88 | 1.08\(^3\) | 0.42 | 0 | 0 | 0 |
| t-statistic\(^2\) | - | - | - | 3.93 | - | 1.14 | 1.42 | - | - | - |
| p-value\(^2\) | - | - | - | 0.02 | - | 0.32 | 0.23 | - | - | - |

1 Normalized mean toxin level in media at respective pH
2 Statistics based on comparison to toxin production at pH 7
3 Slightly significant (p<0.5) result, more toxin produced at pH 8 than pH 7
4 Significant (p<0.05) result, less toxin produced at pH 6 than pH 7
Table 5-6: Induction of toxin production in the presence of sub-inhibitory concentrations of oxytetracycline in *Clostridium difficile* isolated from calves

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Control</th>
<th>0.06xMIC⁴</th>
<th>0.12xMIC</th>
<th>0.25xMIC</th>
<th>0.50xMIC</th>
</tr>
</thead>
<tbody>
<tr>
<td>f</td>
<td>0.51⁵</td>
<td>0.50</td>
<td>0.26</td>
<td>0.35</td>
<td>0.13</td>
</tr>
<tr>
<td></td>
<td>1.38 (48)</td>
<td>6.18 (72)</td>
<td>0.48 (48)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>g</td>
<td>0.40</td>
<td>0.32</td>
<td>0.36</td>
<td>0.38</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>1.04 (48)</td>
<td>-</td>
</tr>
<tr>
<td>h</td>
<td>0.51</td>
<td>0.25</td>
<td>0.30</td>
<td>0.46</td>
<td>0.12</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>i</td>
<td>0.52⁵</td>
<td>0.26</td>
<td>0.13</td>
<td>0.14</td>
<td>0.16</td>
</tr>
<tr>
<td></td>
<td>0.57 (48)</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

¹ OD₆₀₀ at 72 h is listed for cultures where toxin was not detected, OD₆₀₀ ≤ 0.05 was considered no growth

² Normalized toxin level after 72 hours of incubation, number in parenthesis indicates the time point when toxin was first detected

³ Control - no oxytetracycline

⁴ Sub-inhibitory concentration of oxytetracycline with respect to MIC for each isolate

⁵ Bold print indicates toxin production
Table 5-7: Induction of toxin production by media pH in *Clostridium difficile* isolated from calves

<table>
<thead>
<tr>
<th>Isolate</th>
<th>pH 6 $^3$</th>
<th>pH 7</th>
<th>pH 8</th>
<th>pH 9</th>
</tr>
</thead>
<tbody>
<tr>
<td>f</td>
<td>0.34$^4$</td>
<td>0.47</td>
<td>0.51</td>
<td>0.51</td>
</tr>
<tr>
<td></td>
<td>1.41 (72)</td>
<td>0.54 (48)</td>
<td>1.38 (48)</td>
<td>0.64 (48)</td>
</tr>
<tr>
<td>g</td>
<td>0.22</td>
<td>-</td>
<td>0.40</td>
<td>0.58</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1.20 (48)</td>
<td>-</td>
<td>0.45 (48)</td>
</tr>
<tr>
<td>h</td>
<td>0.35</td>
<td>0.53</td>
<td>0.51</td>
<td>0.46</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>i</td>
<td>0.01</td>
<td>0.51</td>
<td>0.52</td>
<td>0.002</td>
</tr>
<tr>
<td></td>
<td>-</td>
<td>1.28 (48)</td>
<td>0.57 (48)</td>
<td>-</td>
</tr>
</tbody>
</table>

$^1$ OD$_{600}$ at 72h is listed for cultures where toxin was not detected, OD$_{600} \leq 0.05$ was considered no growth

$^2$ Normalized toxin level after 72 hours of incubation, number in parenthesis indicates the time point when toxin was first detected

$^3$ Media pH, no growth was observed at pH<6 or pH>9

$^4$ Bold print indicates toxin production
Figure 5-1: Induction of isolate f toxin production by sub-inhibitory concentrations of oxytetracycline. A- growth curves for cultures of isolate f at sub-inhibitory concentrations of oxytetracycline, data points with no fill indicate time points where toxin was detected. B- normalized toxin production by isolate f at sub-inhibitory concentrations of oxytetracycline over time.
Figure 5-2: Induction of isolate f toxin production by media pH. A- growth curves for cultures of isolate f at different media pH levels, data points with no fill indicate times points where toxin was detected. B- normalized toxin production by isolate f at different media pH levels over time.
Figure 5-3: Induction of isolate g toxin production by sub-inhibitory concentrations of oxytetracycline. A- growth curves for cultures of isolate f at sub-inhibitory concentrations of oxytetracycline, data points with no fill indicate toxin production. B- normalized toxin production by isolate g at sub-inhibitory concentrations of oxytetracycline over time.
Chapter 6

Prevalence of *Clostridium difficile* toxin genes in the feces of veal calves and incidence of ground veal contamination
6.1 Abstract

A study was conducted to determine the prevalence of toxigenic *C. difficile* at different points in the veal production chain. Four veal herds of 100-200 calves (N=600) owned by a Pennsylvania veal production company were included in the study. Fifty veal calves from each herd (n=200) were followed for 18-22 weeks from the time of arrival on the veal farm to the time of slaughter. Fecal samples were collected from calves every 4-6 weeks for a total of 4-5 samples per calf and screened for the presence of *C. difficile* toxin genes. Data was analyzed to determine if the presence of toxin genes of *C. difficile* in feces influenced calf weight gain and to determine if factors including age, season, and farm influenced the prevalence *C. difficile* toxin genes in veal calf feces. Half of the calves included in the study (n=100) were followed to slaughter to examine toxigenic *C. difficile* contamination of carcasses. Finished ground veal product (n=50 samples) distributed by the veal producer participating in the study was purchased from local grocery stores over a period of 4 months and examined for toxigenic *C. difficile* contamination. All samples were examined for genes encoding *C. difficile* toxins TcdA, TcdB, and CDT using multiplex real-time PCR. Carcass swabs and ground veal samples were also examined for *C. difficile* using traditional culture methods. During the sampling period, fecal samples from 58 (29%) calves tested positive for *C. difficile* toxin genes at least once over the course of the study. Weight gain was not affected by the presence of *C. difficile* toxin genes. Calf age (p=0.011) and season (p=0.28e-5) influenced prevalence of *C. difficile* toxin genes in calf feces. Toxin genes of *C. difficile* were detected in 1 carcass swab by multiplex real-time PCR only. Toxigenic *C. difficile* was detected by PCR and culture in 4 (8%) and 2 (4%) ground veal samples, respectively. The data collected shows toxin genes of *C. difficile* can be detected throughout the veal production chain and contaminated ground veal may be a significant source of toxigenic *C. difficile* exposure relevant to public health.
6.2 Introduction

*Clostridium difficile* is a well established human pathogen known to cause a disease state ranging from mild diarrhea to fulminant colitis which may result in death. In the past, *C. difficile* was considered a nosocomial pathogen that mainly affected the elderly, the severely ill, and long term hospital inpatients (CDC, 2005; Hookman and Barkin, 2007). Recently, an increase in community acquired *C. difficile* associated disease has been reported in populations that were previously considered at low risk of infection (CDC, 2005; Pituch, 2009). *Clostridium difficile*-associated disease is caused by 2 large clostridial toxins, TcdA and TcdB (Banno et al., 1984). Both toxins act on the Rho family of proteins through monoglucosylation (Just et al., 1995a; Just et al., 1995b). Disruption of F-actin regulation leads to loss of cytoskeletal integrity resulting in the loss of tight junctions between intestinal epithelial cells (Keel and Songer, 2006). Some toxigenic strains also produce a binary toxin, CDT, which acts though ADP-ribosylation of actin (Popoff et al., 1988). This toxin is expressed in 2 components, an enzymatic subunit (CDTA) and a binding subunit (CDTB).

Food animals including cows, pigs, and broiler chickens have been identified as carriers of toxigenic *C. difficile* (TXCD) (Avbersek et al., 2009; Indra et al., 2009; Jhung et al., 2008; Songer and Anderson, 2006). A study by Lemee et al. reported 27 out of 622 of animal stool samples contained TXCD (Lemee et al., 2004). Recent studies have shown calves can act as asymptomatic carriers of TXCD (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). Researchers have observed genotypic similarity between *C. difficile* isolates of human and animal origin, suggesting animals should be considered an important source of exposure to *C. difficile* relevant to public health (Jhung et al., 2008; Rodriguez-Palacios et al., 2006; Rupnik, 2007).

*Clostridium difficile* has been isolated from ground beef, veal, pork, and turkey meant for human consumption. These findings suggest *C. difficile* may be considered a zoonotic agent
disseminated from food animals to humans through direct contact or consumption of contaminated food animal products (Rupnik, 2007). It is inferred that TXCD shed in the feces of food animals contaminates the products at some point during production and processing of meat. Researchers have established that calves can be asymptomatic carriers of TXCD and may be a reservoir relevant to public health (Hammitt et al., 2008; Rodriguez-Palacios et al., 2006). It is possible that humans may be exposed to TXCD through consumption of veal.

No research has been conducted on dissemination of TXCD from veal calves to finished veal product meant for human consumption. An understanding of the prevalence of TXCD at different points of production will provide vital information needed for implementing contamination control measures to limit human exposure to TXCD through ground meat. The objectives of this study were to examine factors influencing the prevalence of TXCD in veal calves and to monitor the prevalence of TXCD in veal calves over time, on carcasses at slaughter, and in the finished ground veal product on the grocery shelf.

6.3 Materials and Methods

6.3.1 Veal calves

A large Pennsylvania veal production company was recruited for participation in the study. Calves were routinely purchased throughout the northeast by the production company and sent to veal growers at approximately one week of age. A diet of milk replacer and routine veterinary care were provided by the production company and were identical on all veal grower farms. Four veal herds raised by three different veal growers were included in the study. Calves in three of the four herds (herds A, B and D) were housed in separate stalls. Calves in herd C were housed 2 per stall, dividers in each stall separated the calves until approximately 8 weeks of age when dividers were removed. Herds A and B were raised during the time period of October to
March and April to August and by the same veal grower, respectively. Herd C was raised during the period of September to January. Herd D was raised during the time period of April to July. When the calves reached sale weight (approximately 20-22 weeks of age), they were shipped to a common facility for slaughter. Finished veal product of the veal company used in this study is sold in retail grocery stores throughout Pennsylvania.

6.3.2 Sample collection

The prevalence of TXCD in veal calves was monitored from the time of arrival at the veal grower until slaughter. The four herds included in the study contained a total of 600 calves. A sample size of n=194 calves was determined using power analysis with an expected prevalence of 11%. Therefore, 50 calves from each herd, for a total of 200, were included in the study. Fecal samples were collected rectally from calves every 4-6 weeks for a total of 4-5 samples from each calf (Figure 6-1). Calves (n=100) from herds B and D were followed to slaughter. The outside surface of carcasses were swabbed with a sterile 4X4 gauze pad at the slaughter house immediately following slaughter before and after the carcasses were washed with 2.5% citric acid as described by Dorsa et al. (1996). An effort was made to swab as much area of the outside of the carcass as possible. One pound ground veal samples (n=50) produced by the veal operation participating in the study were purchased from 2 local grocery stores over a period of 4 months. One pound samples of ground meat distributed by other producers were purchased over the same time period for comparison and included beef (n=30), pork (n=7), and meatloaf mix (n=5; mix of beef, pork, and veal).

6.3.3 Sample analysis

All fecal samples (1 g) and carcass swabs were incubated anaerobically in 9 ml and 20 ml cycloserine cefoxitin fructose broth, respectively, for one week at 37°C. All ground meat samples
were cultured for *C. difficile* as described by Songer et al. (2009). Briefly, two 1 g aliquots of ground veal sample were homogenized in two different tubes containing 9 ml of pre-reduced BHI supplemented with 0.5% yeast extract, 0.1% taurocholate, and 0.05% cysteine. One tube was heated to 80°C for 10 min for detection of spores. Both tubes were incubated anaerobically at 37°C for 96 hours. DNA was extracted from all fecal cultures using the QIAamp DNA Stool Mini Kit and from carcass swab and ground meat cultured using the DNeasy Blood and Tissue Kit (Qiagen, Valencia, CA).

All DNA extracts from fecal samples and swabs were examined for *C. difficile* using PCR targeting *tpi*, a housekeeping gene, as described by Lemee et al. (2004). Samples positive for *tpi* were then screened for toxin genes using the multiplex real-time PCR assay described by Houser et al. (2010). Samples positive for *tpi* and negative for all toxin genes were considered positive for non-toxigenic *C. difficile* (NTCD).

In order to determine if viable TXCD was present on carcasses and in ground meat, carcass swab and ground meat broth cultures were also streaked on cycloserine cefoxitin fructose agar containing 0.1% taurocholate and incubated up to 7 days or until growth was observed. Isolates showing characteristic morphology of *C. difficile* were sub-cultured and DNA was extracted from pure culture using methods described by Pospiech and Neumann (1995). To confirm an identification of *C.difficile*, DNA from all isolates was screened for *tpi* as described above. Isolates carrying the *tpi* gene were then screened for toxin genes using multiplex real-time PCR (Houser et al., 2010).

### 6.3.4 Data analysis

Descriptive statistics were used to calculate the overall prevalence of *C. difficile* toxin genes or the organism itself in veal calf feces over time, on veal carcasses, and in finished veal product. Sale weight and dress weight of all calves included in the study were recorded by the
producer and provided to us for the purposes of this study. Weight gain was calculated by subtracting the sale weight from the dress weight. A t-test was used to determine if mean weight gain of calves with fecal samples that tested positive for \textit{C. difficile} toxin genes differed significantly from mean weight gain of calves with feces negative for toxin genes. The chi-square test of independence was used to determine if the prevalence of \textit{C. difficile} toxin genes in feces was influenced by calf age, season, or farm.

6.4 Results

6.4.1 Prevalence of \textit{Clostridium difficile} in veal calves

In this study, the prevalence of \textit{C. difficile} toxin genes was monitored over 18-22 weeks in fecal samples from 200 calves from 4 different herds (50 calves from each herd). A total of 850 fecal samples (4-5 from each calf) were examined. Eighty-five (10.0%) fecal samples from 73 (36.5%) calves were positive for \textit{tpi} and toxin genes were detected in 63 (7.4%) \textit{tpi} positive samples from 58 (29%) calves. During the sampling period, 5 (10%), 31 (62%), 19 (38%), and 18 (36%) calves from herds A, B, C, and D tested positive for \textit{C. difficile (tpi positive)} at least once and toxin genes were detected in 2(40%), 23 (74%), 19 (100%), and 14 (78%) \textit{tpi positive} samples, respectively (Table 5-1).

The prevalence of \textit{C. difficile} toxin genes in calf feces increased over time and the highest prevalence was observed at the last sampling before slaughter for herds A, B, and D. For herd C, the highest prevalence was observed at the second sampling when calves were approximately 4-6 weeks of age (Figure 6-2). The highest overall prevalence was observed at the last sampling, approximately 2 weeks before the calves were sent to slaughter. Very few calves (n=4) tested positive for toxin genes more than once over the course of the study, suggesting carriage of TXCD is transient.
Seven different toxin gene profiles were observed among fecal samples positive for C. difficile toxin genes (Table 5-2). Among the 63 positive fecal samples, toxin genes tcdA, tcdB, cdtA, and cdtB were detected in 37 (58.7%), 36 (57.1%), 46 (73.0%), and 46 (73.0%) samples, respectively.

Chi-square test of independence showed the prevalence of C. difficile toxin genes in herds B ($\chi^2 = 14.20, p=0.007$), C ($\chi^2 = 22.49, p=0.51e^{-4}$), and D ($\chi^2 = 8.91, p=0.03$) was influenced by calf age. Calf age was also found to influence fecal toxin gene prevalence when analyzing all herds collectively ($\chi^2 = 14.91, p=0.011$), with the highest overall prevalence observed for calves 20-22 weeks of age (Figure 6-2). The influence of season on C. difficile toxin gene prevalence showed the greatest significance ($\chi^2 = 33.30, p=0.28e^{-6}$), with the highest overall prevalence seen in the summer months (17.0%) and lowest in winter months (1.3%) (Figure 6-3). Weight gain did not differ significantly between calves with fecal samples positive for C. difficile toxin genes and calves with PCR negative sample, further suggesting asymptomatic carriage of TXCD as opposed to colonization.

### 6.4.2 Incidence of veal carcass contamination

The C. difficile housekeeping gene, tpi, was detected by PCR on 4 of 100 pre-citric acid wash carcass swabs, 2 from herd B and 2 from herd D. One of the tpi positive carcass swabs from herd B was positive for the tcdA gene. No genetic elements of C. difficile were detected on post-citric acid wash carcass swabs. Clostridium difficile was isolated from any carcass samples, pre or post-wash, using enriched culture techniques.

### 6.4.3 Incidence of ground veal contamination

Toxin genes of Clostridium difficile were also detected by multiplex real-time PCR in 4 out of 50 (8%) ground veal samples from the production company that participated in the study.
Genes encoding toxins TcdA, TcdB, and CDT were detected in 3 samples, while only genes encoding CDT were detected in the fourth positive sample (Table 5-3).

We observed a TXCD prevalence rate in ground beef from other distributors similar to that found in ground veal (Table 5-3). Toxin genes of *C. difficile* were detected in 10% of ground beef samples using the PCR assay. Genes encoding for all three toxin genes were detected in one sample, while the other two samples were positive for *cdtA* and *cdtB* only. Viable *C. difficile* was isolated from 2 of the PCR positive ground beef samples using enriched culture techniques. Isolates from one sample encoded for all three *C. difficile* toxins while isolates from the other sample encoded for TcdA and TcdB only. No TXCD was detected in ground pork or meatloaf mix using PCR nor traditional culture techniques.

### 6.5 Discussion

We report an overall prevalence rate of 29% for toxin genes of *C. difficile* in the feces of a healthy veal calf population. The prevalence rate reported here is greater than previously observed by other researchers. Rodriguez-Palacios et al. (2006) reported a *C. difficile* prevalence rate of 14.9% in healthy calves. Hammitt et al. (2008) found a similar prevalence rate of 13.2% in nondiarrheic calves. Costa et al. (2009) reports a higher prevalence rate of 34-61% in veal calves, but did not determine toxigenicicy of isolated strains. In this study toxin genes were detected in most fecal samples (74.1%) positive for *C. difficile* housekeeping gene, *tpi*. A high prevalence of genes encoding TcdA and TcdB has been previously reported in *C. difficile* isolated from calves. Hammitt et al. (2008) observed that all *C. difficile* isolated from diarrheic calves was PCR positive for *tcdA* and *tcdB*. In this study, 15 TXCD positive fecal samples belonged to toxin profiles that included both *tcdA* and *tcdB* genes. Geric et al. (2003) reported that 15.5% of *tcdA* and *tcdB* negative *C. difficile* isolates examined were positive for both *cdtA* and *cdtB*. Similar
results were observed in this study where only cdtA and cdtB were detected in 12 (19.0%) toxin gene positive fecal samples.

Costa et al. (2009) reported an increase from 34% to 61% and 33% to 49% in the prevalence of C. difficile in individually housed and group housed veal calves 6 days after arrival on the farm, respectively. We also observed an increase in prevalence over time. Calves in this study were confined to individual stalls except on farm C where calves were housed 2 per stall after the calves reached approximately 8 weeks of age. Physical contact between calves in neighboring pens, however, was not completely restricted on any farm. The increase in prevalence over time may be due to housing of calves in close proximity which facilitates fecal-oral transmission of bacteria. Although calves were housed on grates, fecal material noticeably accumulated in barns on all farms over time. Another possible explanation for the increase in prevalence of toxin genes of C. difficile fecal samples may be changes in diet. As veal calves near slaughter, their diet is changed from a starter formulation to a finishing formulation. This change in diet may alter the resident fecal flora so that C. difficile colonization is favored, although further investigation is needed to determine if this is the case. The highest prevalence was seen in calves at 20-22 weeks of age ($\chi^2 = 14.91, p=0.011$). This finding is significant because this is the approximate age of veal calves when they are sent for slaughter.

Statistical analysis showed season had an effect on the prevalence of C. difficile toxin genes in veal calf feces. It is possible that the spike in prevalence observed at the second sampling of herd C influenced the results of this statistical test, yet when the test of independence of season was run excluding the herd C data, the result was still significant ($\chi^2 = 24.04, p=0.57e^{-5}$). We are unable to explain this phenomenon. It is possible that C. difficile spores are better able to survive in the environment during warmer months, but the veal barns are heated during the winter and the temperature did not fall below 50°F and any time during the study. It is also important to distinguish between seasonal prevalence and an outbreak within the herd. Because calves were
not monitored symptoms of disease, we are not able to comment on the overall disease state of the herd over time. Also, not all herds were sampled over an entire year. This makes it difficult to determine if prevalence of toxin genes in calf feces is significantly different across seasons or is simply herd dependent. Prevalence of toxin genes in feces was not influenced by farm ($\chi^2 = 2.224$, $p=0.33$). The findings of this study are similar to that reported by Rodriguez-Palacios et al. (2006) who found calf age and month of sampling, but not farm, influenced the presence of *C. difficile* toxins TcdA/B in calf feces.

Borriello et al. (1986 and 1987) have shown *C. difficile* is unable to colonize the gut and cause disease unless the gut microflora is disrupted. Normal gut flora does not allow sporulation and colonization of *C. difficile* by out-competing the pathogen for nutrients (Borriello et al., 1990). Wilson et al. (1985) observed that *C. difficile* is quickly eliminated from the digestive tract of hamsters due to colonization resistance. They showed that in the absence of antibiotic treatment, spores and vegetative cells did not multiply, but were rapidly moved to the cecum and colon and were eliminated. It is likely that *C. difficile* cells also move rapidly through the digestive tract of healthy calves due to colonization resistance. Here we observed transient carriage of *C. difficile* toxin genes in calf feces and rarely found feces from the same calf to be positive for toxin genes more than once. Weight gain did not differ significantly between calves with feces positive for toxin genes and calves with feces negative for toxin genes, further suggesting transient asymptomatic carriage as opposed to colonization or infection.

We observed a low prevalence of *C. difficile* toxin genes in pre-wash carcass swabs and were unable to detect any toxin genes in carcass swabs collected after the citric acid wash. No TXCD was detected using traditional culture of pre and post-wash carcass swabs. Acid washes have been validated as an effective method for reducing carcass contamination (Dormedy et al., 2000). Because we were not able to isolate *C. difficile* from any carcass swabs using traditional culture techniques we are therefore unable to establish a genetic relationship between TXCD shed
by calves and TXCD contaminating veal carcasses. Toxigenic *C. difficile* was isolated from ground veal, though, suggesting either carcass washing does not completely eliminate TXCD contamination or TXCD contamination occurs in processing sometime after the carcass wash. We found 8% of ground veal samples from the production company that participated in the study were contaminated with TXCD as well as 10% of ground beef produced elsewhere. Unfortunately, we are not able to confirm that ground veal examined here was product of the herds included in the study. Therefore, we are not able to determine the influence of TXCD carcass contamination on ground veal contamination or show genetic relatedness between TXCD strains shed in calf feces and strains found in ground meat.

*Clostridium difficile* has been isolated from raw meats such as beef, veal, pork, chicken, and turkey in the past (Rodriguez-Palacios et al., 2007; Songer et al., 2009; Weese et al., 2009; Weese et al., 2010a). The prevalence of TXCD in ground meat observed in this study was lower than that reported in previous studies. Weese et al. (2009) reported 12% of Canadian retail ground beef and pork samples contained TXCD, although upon enumeration, contamination levels were found to be low. In another study *C. difficile* was detected in 20.8% and 14.3% of Canadian retail ground beef and veal, respectively, and most recovered isolates encoded for TcdA, TcdB, and CDT (Rodriguez-Palacios et al., 2007). Weese et al. (2010a) detected *C. difficile* in 9.0%, 18.0%, and 15.0% of chicken thighs, wings, and legs, respectively. Songer et al. (2009) found a much higher incidence of *C. difficile* in retail ground meat in Tucson, Arizona. In their study 42.4%, 41.3%, and 44.4% of beef, pork, and turkey were found to contain TXCD, respectively. Other studies report a much lower prevalence of *C. difficile* in ground meat.

A recent study in Sweden found little or no incidence of *C. difficile* in ground meat (Bouttier et al., 2010; Indra et al., 2009; Jobstl et al., 2010; von Abercron et al., 2009). von Abercron et al. (2009) found *C. difficile* in 6.2% of ground beef samples, but were unable to detect *C. difficile* in ground pork, mixed beef and pork, sheep, reindeer, moose, veal, and poultry.
A study in Austria showed only 3% of ground meat samples were contaminated with *C. difficile*, only 1% of samples were contaminated with toxigenic strains (2010). Indra et al. (2009) screened 51, 27, and 6 beef, pork, and chicken meat samples from retail stores in Austria, respectively. *Clostridium difficile* was not detected in any of the samples. A study in France found only 2 out of 109 ground beef samples were contaminated with *C. difficile* (Bouttier et al., 2010). Isolates from both samples were toxigenic. Both samples were vacuum packaged which may have facilitated the survival of the organism due to anaerobic conditions. The differences in reported prevalence rates of *C. difficile* raw meat contamination may due to differences geographic location. It has been suggested that in order to establish the true prevalence of toxigenic *C. difficile* in raw meat, systematic national sampling is required (Weese, 2010b).

The recent reported increase of community acquired *C. difficile* associated disease in populations that were previously considered at low risk has prompted researchers to investigate risk factors and possible sources of TXCD exposure (CDC, 2005; Pituch et al., 2009; Wilcox et al., 2008). Possible causes of the increase in community acquired cases include contact with asymptomatic carriers including animals as well as consumption of contaminated foods (Pituch et al., 2009; Songer and Anderson, 2006). Genotypic similarity has been observed in *C. difficile* isolates of human and animal origin, suggesting animals should be considered an important source of exposure to *C. difficile* relevant to public health (Jhung et al., 2008; Rodriguez-Palacios et al., 2006; Rupnik et al., 2007).

Although it is clear that raw meat is a source of community exposure to toxigenic *C. difficile*, the resulting risk to public health has not yet been established. Heat resistance of *C. difficile* spores survive in meat even after heating to proper cooking temperature. Rodriguez-Palacios et al. (2007) reported survival of *C. difficile* spores for up to two hours in ground beef heated to 71°C (recommended cooking temperature). Still, no incidences of foodborne *C. difficile* infection have been reported (Weese, 2010b). Weese (2010b) points out that a foodborne
association may be difficult to establish, if one does exist. It is possible that \textit{C. difficile} infection may not occur until sometime after ingestion of the organism, making it difficult to determine a point of exposure. It is also possible that foodborne exposure is only significant if other risk factors are encountered, such as subsequent antibiotic therapy or use of proton pump inhibitors (Borriello, 1990; Dial et al., 2005; Weese, 2010b).

6.6 Conclusions

The results reported here confirm veal calves are asymptomatic carriers of TXCD and the prevalence of \textit{C. difficile} toxin genes in calf feces may be affected by season and age. Toxigenic strains of \textit{C. difficile}, possibly originating from veal calves, are able to contaminate veal carcasses at slaughter and can be found in finished ground meat product meant for human consumption. The practice of citric acid carcass wash seems to reduce carcass contamination, although the presence of TXCD in ground veal suggests other routes of contamination need to be assessed. Although the public health risk of toxigenic \textit{C. difficile} in raw meat has not yet been established, ground meat should be considered a source of human community exposure to the pathogen.
6.7 References


Table 6-1: Prevalence of *Clostridium difficile* in veal calves

<table>
<thead>
<tr>
<th>Herd</th>
<th>N</th>
<th>TXCD(%)&lt;sup&gt;1&lt;/sup&gt;</th>
<th>NTCD(%)&lt;sup&gt;2&lt;/sup&gt;</th>
<th>Total (%)</th>
</tr>
</thead>
<tbody>
<tr>
<td>A</td>
<td>50</td>
<td>2 (4.0)</td>
<td>3 (6.0)</td>
<td>5 (10%)</td>
</tr>
<tr>
<td>B</td>
<td>50</td>
<td>23 (46.0)</td>
<td>8 (16.0)</td>
<td>31 (62.0)</td>
</tr>
<tr>
<td>C</td>
<td>50</td>
<td>19 (38.0)</td>
<td>0 (0.0)</td>
<td>19 (38.0)</td>
</tr>
<tr>
<td>D</td>
<td>50</td>
<td>14 (28.0)</td>
<td>4 (8.0)</td>
<td>18 (36.0)</td>
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<tr>
<td>Total</td>
<td>200</td>
<td>58 (29.0)</td>
<td>15 (7.5)</td>
<td>73 (36.5)</td>
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</table>

Data includes calves that tested positive for *C. difficile* at any time during the study

<sup>1</sup>TXCD: toxigenic *C. difficile*, includes *tpi* positive fecal samples that tested positive for at least one toxin gene

<sup>2</sup>NTCD: non-toxigenic *C. difficile*, includes *tpi* positive samples that tested negative for all toxin genes
Table 6-2: *Clostridium difficile* toxin profiles observed in veal calf feces

<table>
<thead>
<tr>
<th>Profile No.</th>
<th><em>tcdA</em></th>
<th><em>tcdB</em></th>
<th><em>cdtA</em></th>
<th><em>cdtB</em></th>
<th>No. Herds</th>
<th>No. Samples</th>
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<td>+</td>
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<td>-</td>
<td>-</td>
<td>4</td>
<td>12</td>
</tr>
<tr>
<td>2</td>
<td>+</td>
<td>+</td>
<td>-</td>
<td>-</td>
<td>3</td>
<td>3</td>
</tr>
<tr>
<td>3</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td>2</td>
<td>12</td>
</tr>
<tr>
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<td>+</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>10</td>
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<tr>
<td>5</td>
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<td>+</td>
<td>-</td>
<td>-</td>
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<tr>
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<td>+</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td>7</td>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td>3</td>
<td>12</td>
</tr>
<tr>
<td><strong>Total</strong></td>
<td>37</td>
<td>36</td>
<td>46</td>
<td>46</td>
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<td>63</td>
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Table 6-3: *Clostridium difficile* toxin profiles observed in ground meat

<table>
<thead>
<tr>
<th>Toxin Profile</th>
<th>Veal (n=50)</th>
<th>Beef (n=30)</th>
<th>Pork (n=7)</th>
<th>Meatloaf (n=5)</th>
<th>Total (n=92)</th>
</tr>
</thead>
<tbody>
<tr>
<td>tcdA</td>
<td>tcdB</td>
<td>cdtA</td>
<td>cdtB</td>
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<tr>
<td>+</td>
<td>+</td>
<td>+</td>
<td>+</td>
<td></td>
<td>3 (6.0)&lt;sup&gt;1&lt;/sup&gt; 1 (3.3) 0 (0.0) 0 (0.0) 4 (4.3)</td>
</tr>
<tr>
<td>-</td>
<td>-</td>
<td>+</td>
<td>+</td>
<td></td>
<td>1 (2.0) 2 (6.6) 0 (0.0) 0 (0.0) 3 (3.3)</td>
</tr>
</tbody>
</table>

Total samples containing TXCD<sup>2</sup> 4 (8.0) 3 (10.0) 0 (0.0) 0 (0.0) 7 (7.6)

<sup>1</sup>Values in parenthesis represent the percentage of respective ground meat samples

<sup>2</sup>TXCD: toxigenic *C. difficile*, includes tpi positive fecal samples that tested positive for at least one toxin gene
Figure 6-1. Sampling scheme used to determine the prevalence of toxigenic *Clostridium difficile* throughout the veal production continuum.
Figure 6-2: Prevalence of toxigenic *Clostridium difficile* in four veal herds over time.

TXCD: toxigenic *C. difficile*, includes *tpi* positive fecal samples that tested positive for at least one toxin gene. Diamond: herd A, triangle: herd B, square: herd C, circle: herd D, dotted line: collective data from all herds.
Figure 6-3: Seasonal prevalence of toxigenic *Clostridium difficile* in veal calves

TXCD: toxigenic *C. difficile*, includes *tpi* positive fecal samples that tested positive for at least one toxin gene
Chapter 7

Summary and Conclusions
7.1 Summary and Conclusions

Laboratory and field validation of the real-time multiplex PCR assay for the detection of toxigenic *C. difficile* developed here showed the assay is both sensitive and specific and can be employed for both diagnosis of *C. difficile*-related infection and for monitoring the food supply for incidence of *C. difficile* contamination. The detection limit of the standardized real-time multiplex PCR assay for toxin genes of *C. difficile* was $10^3$ cells/g and $10^1$ cells/g for non-enriched and enriched fecal and ground meat samples, respectively. When DNA is extracted directly from samples TXCD may be detected in as little as 4 hours, although, enrichment of samples in recommended to increase assay sensitivity.

A study of the association of *C. difficile* with enteric disease in calves showed the odds of observing evidence of colitis in young calves are nearly 7 times greater when toxins A and/or B are detected in the feces. Based on this data we conclude *C. difficile* should be considered an enteric pathogen in calves and emphasis should be placed on detection of TcdA/B when diagnosing *C. difficile*-associated disease in calves. This study also showed 43% carried toxigenic *C. difficile* asyptomatically suggesting calves are a reservoir for the pathogen and toxin expression may be influenced by factors in the calf gut. This is the first report of the effects of oxytetracycline on toxin production by *C. difficile*. Upon examining toxin production of *C. difficile* isolated form calves in the presence of sub-inhibitory concentrations of oxytetracycline, we observed increased toxin production in one isolate and induced toxin production in another. We also observed the highest toxin production among isolates occurred in cultures a pH 8, the median fecal pH measured among milk fed calves. These results suggest conditions of the calf gut may favor toxin production by *C. difficile* and administration of oxytetracycline may promote pathogenesis associated with *C. difficile* in the calf gut.
A survey conducted to determine the prevalence of toxigenic *C. difficile* at different points in the veal production chain showed veal calves should be considered a reservoir for toxigenic *C. difficile*. Although many researchers have identified raw meat as a source of community *C. difficile* exposure, this was the first study that monitored veal contamination throughout the entire production continuum. It was concluded that the prevalence of *C. difficile* in veal calves is influenced by age and season. Prevalence increased over time and the highest prevalence was seen just before slaughter suggesting housing of calves in close proximity facilitates fecal-oral transmission of bacteria. This increase in prevalence may increase the likelyhood of contamination of carcasses with *C. difficile* at slaughter. Although *C. difficile* was detected on carcasses at slaughter, viable bacteria were not recovered. Based on these findings we conclude *C. difficile* is able to contaminate carcasses at slaughter, but the incidence is low. We also established *C. difficile* can contaminate ground veal and beef and human consumption of these meats serves as a route of exposure. Although humans are exposed to toxigenic *C. difficile* through consumption of ground beef, more research needs to be conducted to determine if exposure through food increases the risk of developing community acquired *C. difficile*-associated infections.

In conclusion, this dissertation has provided evidence that *C. difficile* should be considered a pathogen in calves and may be disseminated from calves to humans through dissemination in the food chain. The work presented here has provided a platform for further investigation into the changing epidemiology of *C. difficile*-associated disease in both humans and animal species. Although we have identified *C. difficile* as an enteric pathogen in calves, further longitudinal studies are required to determine the prevalence of *C. difficile*-associated disease in calves and to determine the impact this pathogen has in agricultural industry. We have identified ground meat as a source of community exposure to *C. difficile*. It is important to recognize that this is only one possible route of exposure in the community and the relative risk
associated with this exposure has not yet been established. Comprehensive epidemiological studies on community acquired *C. difficile*-associated disease are needed to characterize exposure routes including person to person contact, person to animal contact, and exposure to spores in the general environment. Further, characterization of *C. difficile* in the community is needed to determine the factors and mechanisms contributing to the increasing severity of disease observed over the last decade. A complete understanding of the virulence and epidemiology of *C. difficile*-associated disease as it affects society today will allow for effective surveillance, prevention, and treatment strategies to reduce both the veterinary and public health burden associated with this pathogen.
Appendix A

Experimental Matrix for determination of oxytetracycline MIC shifts due to the presence of Ca$^{2+}$

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<th>4</th>
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C-no oxytetracycline (controls)
E-experimental conditions
Appendix B

Experimental matrices for determination of the effect of subinhibitory concentrations of oxytetracycline on toxins A and B production

Concentrations of oxytetracycline (mg/l) used to achieve sub-inhibitory levels with respect to Ca2+ concentration (mg/l) for each isolate examined:

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<thead>
<tr>
<th>Isolate a</th>
<th>Ca^{2+}</th>
<th>0</th>
<th>0.06xMIC</th>
<th>0.12xMIC</th>
<th>0.25xMIC</th>
<th>0.5xMIC</th>
<th>MIC</th>
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2-sample t-tests for data analysis were paired across rows
Appendix C

Experimental matrices for determination of the effect of Ca$^{2+}$ on toxins A and B production in the presence of subinhibitory concentrations of oxytetracycline

Concentrations of oxytetracycline (mg/l) used to achieve sub-inhibitory levels with respect to Ca2+ concentration (mg/l) for each isolate examined:

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2-sample t-tests for data analysis were paired across rows
Appendix D

Experimental matrix for determination of the effect of media pH on toxins A and B production

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C-controls
E-experimental conditions
Appendix E

Experimental matrices for determination of induction of toxins A and B production by sub-inhibitory concentrations of oxytetracycline and media pH

Concentrations of oxytetracycline (mg/l) used to achieve sub-inhibitory levels with respect to MIC for each isolate examined:

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C-controls
E-experimental conditions
Appendix F

IACUC Approval #28296

Date: May 1, 2008

From: William G. Greer, IACUC Administrator

To: David R. Wolfgang

Subject: Results of IACUC Protocol Review – New Protocol (IACUC# 28296)

Approval Expiration Date: April 27, 2009

“Evaluation of Risk Factors of Mycoplasma pneumonia, Protective Effects of Commercially Available Vaccines, and Impacts on Growth Rate”

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your protocol for the use of animals in your research. This approval has been granted for a one-year period.

Approval for the use of animals in this research project is given for a period covering one year from the date of this memo. If your study extends beyond this approval period, you must contact this office to request an annual review of this research.
This Institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance number is A 3141-01. As of February 13, 2001, The Pennsylvania State University was awarded Full Accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

By accepting this decision, you agree to notify the Office for Research Protections of (1) any additions or procedural changes and (2) any unanticipated study results that impact the animals. Prior approval must be obtained for any planned changes to the approved protocol. Any unanticipated pain or distress, morbidity or mortality must be reported to the attending veterinarian and the IACUC.

On behalf of the IACUC and the University, I thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of animals.

If you are interested in subscribing or being removed from ORP listserv, send an email to: L-ORP-Research-L-subscribe-request@lists.psu.edu to subscribe or L-ORP-Research-L-unsubscribe-request@lists.psu.edu to unsubscribe. There is no need to add any text in the subject line or in the message body of the email.

WGG/alb
Attachment
cc: Bhushan M. Jayarao
To the Investigator:

Please forward the enclosed original approval letter to your funding agency, if applicable. This approval is effective for one year. During this time, you should notify this office of any changes in the protocol that will affect the care and use of the approved animals or that will result in the use of additional animals.

In a continuing effort to comply with federal regulations, this office reviews IACUC approvals on an annual basis. On the anniversary of this approval, you should expect to receive a letter soliciting your request for an "annual review" by the IACUC. It is my hope that this process aids researchers in maintaining active IACUC approvals and avoids the use of animals without the proper approval.

Also, in order for records of your animal usage at ARP and ORP to remain current, please review the information below. If you feel there is any discrepancy between this information and your request, please contact our office (ORP) immediately at 865-1775. Thank you.
IACUC#: 28296  

PI: David R. Wolfgang

Title: "Evaluation of Risk Factors of Mycoplasma pneumonia, Protective Effects of Commercially Available Vaccines, and Impacts on Growth Rate"

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Appendix G

IACUC Modification Request Approval

Date: January 27, 2009

From: William G. Greer, IACUC Administrator

To: David R. Wolfgang

Subject: Results of IACUC Protocol Review – Modification (IACUC# 28296)

Approval Expiration Date: April 27, 2009
(Not: This date reflects the anniversary date of the actual submission approval date.)

“Evaluation of Risk Factors of Mycoplasma pneumonia, Protective Effects of Commercially Available Vaccines, and Impacts on Growth Rate”

The Institutional Animal Care and Use Committee (IACUC) has reviewed and approved your modification regarding the above referenced protocol.

COMMENT: Adding collecting via rectal fecal swabs. No additional animals requested.

This Institution has an Animal Welfare Assurance on file with the Office for Laboratory Animal Welfare. The Assurance number is A 3141-01. As of February 13, 2001, The Pennsylvania State
University was awarded Full Accreditation by the Association for Assessment and Accreditation of Laboratory Animal Care International (AAALAC).

By accepting this decision, you agree to notify the Office for Research Protections of (1) any additions or procedural changes that modify the animals' risks in any way and (2) any unanticipated study results that impact the animals. Prior approval must be obtained for any planned changes to the approved protocol. Any unanticipated pain or distress, morbidity or mortality must be reported to the attending veterinarian and the IACUC.

On behalf of the IACUC and the University, I thank you for your efforts to conduct your research in compliance with the federal regulations that have been established for the protection of animals.

If you are interested in subscribing or being removed from ORP listserv, send an email to: L-ORP-Research-L-subscribe-request@lists.psu.edu to subscribe or L-ORP-Research-L-unsubscribe-request@lists.psu.edu to unsubscribe. There is no need to add any text in the subject line or in the message body of the email.

WGG/alb

cc: Bhushan M. Jayarao
VITA

Beth A Houser

EDUCATION

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<td>Dec 2002</td>
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<td>Animal Bioscience</td>
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HONORS AND AWARDS

2010  Third Place- Health and Life Sciences
      The Pennsylvania State University Graduate Exhibition

2010  Third Place- Biological Sciences
      College of Agricultural Sciences/Gamma Sigma Delta Research Expo

2008  Third Place- Biological Sciences
      College of Agricultural Sciences/Gamma Sigma Delta Research Expo

PUBLICATIONS


*maiden name