Development of a conventional pig model for Clostridium difficile infection and associated disease in neonatal pigs

Joshua Thomas Lizer
Iowa State University

Follow this and additional works at: https://lib.dr.iastate.edu/etd
Part of the Veterinary Preventive Medicine, Epidemiology, and Public Health Commons

Recommended Citation
Lizer, Joshua Thomas, "Development of a conventional pig model for Clostridium difficile infection and associated disease in neonatal pigs" (2010). Graduate Theses and Dissertations. 11634.
https://lib.dr.iastate.edu/etd/11634
Development of a conventional pig model for Clostridium difficile infection and associated disease in neonatal pigs

by

Joshua Thomas Lizer

A thesis submitted to the graduate faculty
in partial fulfillment of the requirements for the degree of
MASTER OF SCIENCE

Major: Veterinary Microbiology
Program of Study Committee:
Ronald W. Griffith, Major Professor
Tim Day
Brad Blitvich
Alejandro Ramirez

Iowa State University
Ames, Iowa
2010

Copyright © Joshua Thomas Lizer, 2010. All rights reserved.
TABLE OF CONTENTS

Acknowledgements ................................................................................................................ iii
Abstract................................................................................................................................... iv
I. Introduction .......................................................................................................................... 1
II. Definitions of Genotypic Methods .................................................................................... 2
III. Research Questions .......................................................................................................... 4
IV. Review of the Literature .................................................................................................. 5
V. Research .............................................................................................................................. 18
   V.1. Materials and methods ............................................................................................. 18
       Animals and housing.......................... ........................................................................... 18
       Experimental Design.......................... ........................................................................ 19
       Inoculum .................................................. ................................................................... 19
       Inoculation ........................................... ........................................................................ 20
       Sample collection................................. .................................................................... 20
       Necropsy .................................................. .................................................................. 21
       Culture and toxin detection.................. ....................................................................... 21
       Histopathology ........................................ ................................................................... 22
       Scoring .................................................. ........................................................................ 23
       Statistical analysis................................. ...................................................................... 23
   V.2. Results ........................................................................................................................ 24
       Bacterial Culture ....................................... ..................................................................... 24
       Clinical signs ............................................. ..................................................................... 24
       Gross lesions ............................................ ................................................................... 25
       Microscopic lesions ...................................... .................................................................. 25
       Toxin .................................................. ........................................................................... 26
   V.3. Discussion ................................................................................................................... 27
VI. Conclusions ....................................................................................................................... 33
Research Acknowledgements .............................................................................................. 34
Appendix A. Figures ............................................................................................................... 35
Appendix B. Tables ................................................................................................................ 37
Works Cited ............................................................................................................................ 43
iii

Acknowledgements

Special thanks to:

Dr. Ron Griffith, my major professor, for being such a kind, generous, and patient mentor. I owe my passion for veterinary microbiology to him

and

The rest of my program of study committee, Drs. Tim Day, Brad Blitvich, and Alex Ramirez

also to

Drs. Jerry McVicker and Eric Rowe, for their encouraging support and motivation
Drs. Alex Ramirez and Darin Madson, for being such a great team to do research with

and last and most important

My family and fiance, Rosemary, my biggest supporters of all.
The purpose of this study was to reproduce *Clostridium difficile*-associated disease (CDAD) using conventional pigs as an animal model and to determine whether different disease outcomes occur with isolates of human and porcine origin. Piglets were snatch farrowed from a conventional sow farm where they were given pooled colostrum and then transported to a research facility. At four hours of age, 26 conventional pigs were oral-gastrically inoculated with one of four different isolates of *C. difficile* and 6 control pigs were sham-inoculated. All challenge isolates were toxinoenzyme V; three were isolated from clinical field cases of diarrhea in neonatal pigs in Illinois or Missouri, and the fourth isolate was of human origin. All pigs were individually housed and randomly selected for necropsy at 24, 48, or 72 hours post infection (hpi). The results of this study show that *C. difficile* was isolated from all pigs, inoculated and uninoculated. Commonly observed lesions and indications of CDAD, including mesocolonic edema, toxin detection, diarrhea, neutrophilic infiltration of the colonic and cecal lamina propria, as well as mucosal ulceration or erosion of the colon and cecum were observed in challenged pigs. No difference in disease between the human and porcine isolates was observed. Clinical signs and lesions were observed in some controls and these pigs may have been inadvertently contaminated. The data generated in this study provide evidence that this can be an effective challenge model.
I. Introduction

*Clostridium difficile* was originally described in 1935 as *Bacillus difficilis* when it was cultured from human infants (Hall and O’Toole 1935). It earned its name—appropriately—as it was a strictly anaerobic, gram positive spore-forming bacillus that was difficult to culture. Since it was first associated with antibiotic-associated diarrhea and pseudomembranous colitis in man in 1978 (George et al 1978, Larson et al 1978) and infection was confirmed in accidentally-exposed gnotobiotic pigs in 1980 (Lysons et al 1980), the number of diagnoses and severity of disease caused by this organism in man and animals have increased significantly (McDonald et al 2006, Sunenshine and McDonald 2006, Songer et al 2006, Redelings et al 2007, Jhung et al 2008). Despite this increasing significance, the origin of virulent strains remains unknown, necessitating the need to further understand disease in animals and humans, devise novel strategies and therapies for the treatment and prevention of disease, explore means to test these, and to bridge the gap between the two, if such a link exists (Jhung et al 2008). The research described in this manuscript attemps to answer some of these needs by describing a disease model in pigs for which studies in pathogenesis, therapies, and preventive measures can be investigated.
II. Definitions of Genotypic Methods

While several methods exist, two primary methods are used for genotyping *C. difficile* which will be frequently referred to throughout this manuscript. These methods are known as ribotyping and toxinotyping.

II.1. Ribotyping

Ribotyping was first described for *C. difficile* in 1993 by Gurtler who amplified the rRNA spacer region of 24 clinical *C. difficile* isolates (Gurtler 1993). The procedure uses PCR to detect polymorphisms that can occur in the intergenic spacer region between the 16S and 23S rRNA genes of the ribosomal RNA gene complex (Gurtler 1993, O’Neill et al 1996, Keel and Songer 2007, Stubbs et al 1999). While rRNA genes are highly conserved, this spacer region has been shown to be very heterogeneous (O’Neill et al 1996). As different numbers of rRNA genes are found in different strains of *C. difficile*, the resulting variations in the size of intergenic sequences yield distinct PCR products for each designated ribotype (Keel et al 2007). Currently 120 different ribotypes have been described (Songer, personal communication).

II.2. Toxinotyping

In reference to *C. difficile*, toxinotype is defined as “a group of strains with identical changes in the PaLoc [pathogenicity locus] when compared with other strains” (Rupnik 2008). Toxinotyping is a PCR method devised by Rupnik to identify restriction fragment length polymorphisms (RFLP) in toxin genes of *C. difficile* (Rupnik et al 1997, 1998, 2001). Strains with different polymorphisms are assigned to different toxinotypes, with strains similar to the reference strain VPI 10463 assigned to toxinotype 0 (Rupnik et al 1998). Toxinotyping is perhaps the more attractive method for initially genotyping *C. difficile* as 24 toxinotypes have been described compared to the 120 ribotypes (Songer, personal communication). Good correlation exists between results for toxinotyping and ribotyping as most strains within a single PCR ribotype belong to a single toxinotype. PCR ribotyping can
be used to subtype toxinotypes when a large number of strains exist within a single
toxinotype. Accordingly, toxinotypes are more readily subdivided by PCR ribotyping than
PCR ribotypes are subdivided by toxinotyping (Rupnik et al 2001).
III. Research Questions

Three basic questions were central to the hypothesis of the described research. Firstly, can disease be experimentally induced in conventional pigs from a production setting? Prior to the conception of this research, an experimental challenge model had only been described in an academic setting once, although the study was never published in the peer-reviewed literature (Post et al 2000). This controlled study was small \( n=10 \) pigs and tested two different isolates. Gross and microscopic lesions and clinical signs typical for \textit{C. difficile}, as well as recovery of \textit{C. difficile} organism and its toxin were observed. The current study was designed to see if similar results could be attained, but on a slightly larger scale with more pigs and isolates. Naturally one might have an initial preference for gnotobiotic pigs due to the ability to minimize confounding variables, but gnotobiotic pigs can be costly, time-intensive, and require special housing and handling. However, if a reliable model can be described in conventional piglets from production settings, the low cost and high availability of such animals make them an attractive alternative.

The second question asked ‘Is disease induced by this model repeatable when using different isolates?’ If a model such as the one described in this manuscript is to be broadly applicable to different types of investigations, flexibility can be afforded by the fact that future investigators can use their isolate of choice with the likelihood of successfully inducing disease. The current study attempted to address this question by using the same procedures to test four different isolates.

Can a human isolate be used to induce disease in a pig? This third and final question expands the practicality of the research by describing a testing platform that can be used to test hypotheses relating to \textit{C. difficile} disease in both pigs and humans. This can also open opportunities to study disease transmission links between animals and humans.
IV. Review of the Literature

*Clostridium difficile* in pigs

*Clostridium difficile* has been stated to be possibly the most important uncontrolled cause of neonatal diarrhea in pigs and has been documented as a major cause of enteritis in neonatal pigs since 2000 (Songer et al 2006). *Clostridium difficile*-associated disease (CDAD) has become the most commonly diagnosed cause of neonatal enteritis throughout swine producing areas of the United States. In a sample of ~1000 live piglets with enteritis, toxins A and B, the primary virulence factors of *C. difficile*, were the sole agent in 34%, and these toxins were detected in an additional 24% in which another agent was found (Songer et al 2006). Other studies report similar results:

- In a sample of over 600 live piglets submitted for diagnosis of enteritis, 35% were TcdA/B positive (Songer 2004).
- CDAD is involved in the majority of cases of neonatal enteritis in North Carolina. *C. difficile* was involved in or directly responsible for 58.4% and 34.1% of enteritis cases, respectively (Songer 2004).
- In 10 randomly sampled herds from a 13 herd commercial farrow-to-wean system, 47.6% of the pigs were toxin positive across herds. Prior to testing, more than half of these herds were thought to be free of *C. difficile* (Songer 2004).
- Of 100 1-7 day old piglets with a history of diarrhea submitted to the Iowa State University Veterinary Diagnostic Lab (ISU VDL) in 2000, 29% were toxin positive. *C. difficile* was the sole pathogen detected in 19% of the piglets. It was the most frequently identified agent on a case basis and of cases with multiple agents, *C. difficile* was the most common co-pathogen identified (Yaeger et al 2002).
- In another study of 129 piglets at ISU VDL in 2001, toxin was detected in 50% of the piglets, which included 23 of 29 control pigs and 42 of 100 case piglets. *C. difficile* was isolated from the small and large intestine of 47% and 51% of piglets, respectively (Yaeger et al 2007).
In Spain, *C. difficile* was isolated from 140 of 541 (25.9%) newborn piglets, which included diarrheic and non-diarrheic animals. *C. difficile* was not isolated from any of the 239 samples taken from 1-2 month old pigs (Alvarez-Perez et al 2009).

The prevalence of *C. difficile* in suckling piglets at a vertically-integrated swine operation in Texas in 2006 was 50% (Norman et al 2009).

Afflicted pigs are most commonly between the ages of 1-7 days old. Clinical signs may include pasty or watery yellow feces, mild abdominal distention, dyspnea, and scrotal edema. Macro and microscopic lesions commonly include mesocolonic edema, accumulation of neutrophils in the lamina propria, and exudation of mucus, fibrin, and neutrophils in the gut lumen resulting in pseudomembranes and characteristic ‘volcano lesions’ (Songer et al 2000). Mucosal lesions are generally limited to the cecum and colon (Keel and Songer 2006). Diarrhea is not a consistent clinical sign and as such, should not be used for exclusionary criteria for testing pigs (Waters et al 1998, Songer et al 2006, Yaeger et al 2007). Mesocolonic edema is not pathognomonic for *C. difficile* and only carries a positive predictive value as an indicator of *C. difficile* colitis of 42% (Waters et al 1998, Yaeger et al 2002). Morbidity and mortality both average 20%, while mortality has been reported as high as 50% in some cases (Songer et al 2000).

Diversity in swine isolates is less than that of other species; 83% of swine isolates were found to belong to PCR ribotype 078, toxinotype V (Keel et al 2007, Goorhuis et al 2008). This strain possesses genes for TcdA, TcdB, and binary toxin, and has a 39 bp deletion and a point mutation in the negative toxin regulating tcdC gene resulting in a premature stop codon (Goorhuis et al 2008).

**Clostridium difficile** in humans

*Clostridium difficile* is now implicated as the principal infectious cause of antibiotic-associated diarrhea in human adults, as well as clinically similar conditions in numerous other mammals. As such it is recognized as one of the most important nosocomial pathogens of humans (Keel and Songer 2006). The magnitude of human *C. difficile* infections is so
great that it may exceed that of methicillin-resistant Staphylococcus aureus (MRSA); during 2001-2003, numbers of discharges from US short-stay hospitals with a diagnosis of CDAD exceeded the annual figures of MRSA (120,000) infections for 1999-2000 (McDonald et al 2006). While C. difficile can be found in the feces of 3-6% of healthy adults, the figure can be two to five times greater for hospitalized adults (Kuhl et al 1993). More than 3.5 million cases of CDAD occur in hospitalized patients every year in the United States with an additional estimated cost to the patient of $3669 to $7234 (Songer 2004, Sunenshine and McDonald 2006).

While the incubation period from the time spores are ingested to disease development is not actually known, symptoms can appear in as little as one to six days and possibly longer following antibiotic exposure. Symptoms typically include diarrhea, abdominal pain, nausea, fever, anorexia, and malaise (Poutanen and Simor 2004). Diarrhea is not always associated with infection and may not be present in up to 20% of those patients who became colonized with C. difficile while on a course of antibiotics. As many as two-thirds of patients with nosocomial C. difficile infection can be asymptomatic (Mulligan et al 1993). Little to no diarrhea may be present in severely ill patients, resulting from toxic megacolon and paralytic ileus that can result from the loss of colonic muscle tone (Poutanen and Simor 2004). Those infected are prone to relapse, which occurs in 5-55% of patients (Kuhl et al 1993, Poutanen and Simor 2004, Sunenshine and McDonald 2006). Mortality rates range from 0.6-1.5% in hospital and nursing home patients, but if the patient develops toxic megacolon, these figures increase dramatically to 24-38% (Poutanen and Simor 2004, Sunenshine and McDonald 2006).

The increased prevalence and severity of C. difficile in humans may be due in part to a new hypervirulent epidemic strain of unknown origin. This strain belongs to restriction enzyme analysis (REA) group BI, pulsed-field gel electrophoresis (PFGE) type NAP1, toxinotype III, ribotype 027, and is designated BI/NAP1/027 (McDonald et al 2005, Jhung et al 2008). This strain, much like ribotype 078, has genes for both toxins as well as binary toxin. The high toxin production of this strain was originally thought to be due to an 18 bp deletion in the tcdC gene, but recent work has shown that the encoded TcdC protein with the deletion is still active in vivo and in vitro. The diminished function of the TcdC protein is
now thought to be due to a frameshift mutation located near the 5’ end of the tcdC gene (Dupuy et al 2008)

**Clostridium difficile in other animals**

*C. difficile* has been described by either isolation or disease in a large number of animals including camels, cattle, horses, donkeys, cats, dogs, hamsters, a duck, a snake, a Weddell seal, ostriches, prairie dogs, a Kodiak bear, and a penguin (Bartlett et al 1978, Dubos et al 1984, Orchard et al 1984, Levett 1986, Muller et al 1987, Frazier et al 1993, Weese et al 2001a, Weese et al 2001b). It has been implicated as a cause of diarrhea in calves and has been associated with enteric disease in cats (Weese et al 2001a, Hammitt et al 2008). Much like hospitals, veterinary clinics are probably a common source of infection. A dated study determined that 39% of cats and dogs were carriers of *C. difficile*, and that 68% of veterinary clinics were contaminated with the organism. Restriction enzyme analysis patterns showed good correlation between pet and clinic environmental isolates (O’Neill et al 1993).

**Toxin**

*C. difficile* has several virulence factors which include pilus expression, mucosal adherence, capsule production, and production of degradative enzymes such as collagenase in connective tissue, but the predominant virulence attributes are those of two toxins, toxin A (TcdA) and B (TcdB) (Borriello 1995). Nontoxigenic strains are avirulent and 5-25% of *C. difficile* strains lack the genes to produce TcdA and TcdB (Borriello 1995, Weese et al 2001b, Yaeger et al 2002). Toxin A (308 kDa) and Toxin B (270 kDa) are among the largest known toxins and are biochemically, immunologically, and structurally related to toxins produced by *Clostridium sordellii* and *Clostridium novyi*. Those toxins are known as the large Clostridial toxins (Voth and Ballard 2005, Rupnik et al 1997). There is some degree of homology to TcdA and B as they are 45% identical to each other at the amino acid level (Songer 2004). While both toxins are cytotoxic for a number of cell types, TcdA is primarily
enterotoxic because it causes fluid accumulation in the intestine and is thought to play the dominant role in disease pathogenesis (Bartlett 1994, Weese et al 2001b, Poutanen and Simor 2004, Yaeger et al 2007). However, an increasing emergence of virulent TcdA-/B+ strains and recent evidence indicating that TcdB, not TcdA, is responsible for virulence both indicate that more work is needed for a more complete understanding of toxin pathogenesis (Drudy et al 2007, Lyras et al 2009). TcdB is up to 1000 times more cytotoxic than TcdA but does not cause fluid accumulation in vivo (Bartlett 1994, Weese et al 2001b). Many strains also produce a third toxin known as binary toxin (CDT). This toxin is an ADP-ribosylating toxin but its role in the pathogenesis of CDAD is not yet understood (Genth et al 2008). Recent work indicates it may have a role in increasing bacterial adherence by inducing microtubule protrusions on the surface of intestinal epithelial cells which forms a meshwork to secure bacterial cells (Schwan et al 2009).

Both TcdA and TcdB induce inflammatory effects, which include increased epithelial permeability, cytokine and chemokine production, neutrophil infiltration, activation of submucosal neurons, production of reactive oxygen intermediates, activation of mast cells, production of substance P and TNF-α, and direct damage to the intestinal mucosa (Poutanen and Simor 2004, Voth and Ballard 2005).

The toxin genes, \textit{tcdA} (8,133 bp) and \textit{tcdB} (7,098 bp) are both encoded on a 19.6-kb pathogenicity locus (PaLoc). The PaLoc is composed of three additional open reading frames (ORF): \textit{tcdR} (also identified as \textit{tcdD}), a positive toxin regulator; \textit{tcdE}, a putative holin-like pore forming protein; and \textit{tcdC}, a negative toxin regulator (Voth and Ballard 2005, Dupuy et al 2008, Giesemann et al 2008). Transcription of toxin genes \textit{tcdA} and \textit{tcdB} is activated by the autoregulated sigma factor TcdR, also encoded in the PaLoc. Factors such as an excess of nutrients may downregulate \textit{tcdR} transcription and keep toxin production low when growth is unrestricted (Karlsson et al 2008). TcdC has an inverse regulating function as it is expressed during log growth phase and shut off during stationary growth phase. TcdC is thought to interfere with the TcdR RNA polymerase recognition of \textit{tcdA} and \textit{tcdB} promoters (Dupuy et al 2008). The PaLoc is essential for virulence; in non-toxigenic strains the PaLoc is replaced by 115 bp with no ORF but a hairpin loop (Braun et al 1996).
The toxin structure is composed of four domains that have been proposed to collectively form two larger domains (Genth et al 2008). A C-terminal binding domain, composed of a number of repetitive peptides called “combined repetitive oligopeptides” (CROPS), binds to a receptor which facilitates toxin entry into the cell by receptor-mediated endocytosis (Genth et al 2008, Voth and Ballard 2005, Giesemann et al 2008). After cell entry, acidification of the endosomal lumen by vesicular H⁺-ATPase has been postulated to induce toxin refolding, exposing a hydrophobic region of the toxin—the transmembrane domain (TMD)—followed by its subsequent insertion into the endosomal membrane. Once the TMD is inserted, a pore is formed (Genth et al 2008, Giesemann et al 2008). A catalytic or glucosyltransferase domain (GTD) located at the N-terminus translocates through the pore into the cytosol and is cleaved by an adjacent proteolytic third domain, the cysteine protease domain (Genth et al 2008, Giesemann et al 2008).

Once free in the cytosol, the biologically-active GTD mono-glucosylates the small GTPases Rho, Rac, and Cdc42 (Voth and Ballard 2005, Genth et al 2008). As the Rho family of GTPases are regulators of the actin cytoskeleton, Rho glucosylation results in a loss of filopodia, actin networks, and peripheral membrane ruffling as well as disorganization of focal complexes. The final effect is a loss of cell shape, more commonly referred to as cell-rounding or cytopathic effects (CPE) (Genth et al 2008). TcdA and B also induce apoptosis and necrosis. Toxin-induced necrosis is probably what alerts the immune system to initiate inflammatory events (Genth et al 2008).

As a result of TcdA and B having four unique domains, several modifications to their classification have been proposed. Genth and colleagues introduced the term ‘delivery domain’ and grouped the cysteine protease domain, transmembrane domain, and receptor binding domain into this master domain. The second master domain remained as the glucosyltransferase domain (Genth et al 2008). Staying with the four domains classification, Giesemann and colleagues suggested amending the classical ‘AB’ toxin denomination to ‘ABCD’ toxin, with ‘A’ for biological activity (or active site), ‘B’ for binding, ‘C’ for cutting, and ‘D’ for delivery. In this classification, the delivery domain only includes the hydrophobic transmembrane domain (Giesemann et al 2008).
Pathogenesis

The pathogenesis of *C. difficile* involves three key events: disruption of normal flora, ingestion and colonization of toxigenic *C. difficile*, and growth of the organism with toxin production (Poutanen and Simor 2004). The organism is transmitted through the fecal-oral route and is especially hardy since it can form persisting spores resistant to environmental adversity. Unlike vegetative cells, spores are resistant to the acidic stomach environment and can pass to the small intestine where exposure to bile acids induces germination (Poutanen and Simor 2004). Pigs are unique in that they have seemingly spontaneous disease and require no antibiotic pre-treatment; this could be explained by the hypothesis that pigs are born with a multitude of toxin receptors--one of them thought to be α-galactose--in contrast to other infant mammalian species (Waters et al 1998, Songer et al 2000, Yaeger et al 2002, Yaeger et al 2007, Keel and Songer 2007). This allows the organism to fully exploit the fact that a mature gut flora will not yet be developed as pigs are born with a sterile alimentary tract (Yaeger et al 2002).

As described in the previous section, much of the pathology of *C. difficile* can be attributed to toxin. TcdA can also induce endothelial retraction, permitting the extravasation of albumin among other plasma proteins, and fluid (Wolfhagen et al 1994). As albumin leaks into tissue spaces, plasma colloidal osmotic pressure decreases and tissue colloidal osmotic pressure increases, which can explain hydrothorax, ascites, and edema that can be observed in pigs (Waters et al 1998). Substantial tissue damage results from inflammatory mediators recruiting granulocytes such as neutrophils, which play a significant role in the pathophysiology of CDAD (Wolfhagen et al 1994, Keel and Songer 2006). Damage is not always induced by inflammation; stimulation of intestinal neurons by toxin also leads to degranulation of mucosal mast cells (Castagliuolo et al 1994). Pseudomembranes composed of neutrophils, fibrin, mucin, and cellular debris are frequently a result of CDAD (Poutanen and Simor 2004). Paralytic ileus and toxic megacolon can develop in patients with severe colitis, conditions for which surgery is often required. However, after surgery mortality rates increase to 32-50% (Sunenshine and McDonald 2006).
Colonization demographics

The neonatal pig’s susceptibility to CDAD is unique as resistance to it is found in neonates of most species, even though they are commonly colonized with the organism. Availability of toxin receptors has been hypothesized to explain this phenomenon, as neonatal pigs have abundant toxin receptors while most other species lack them until later in life (Waters et al 1998, Keel et al 2007). Young animals and infant humans with incomplete flora are readily colonized, but the organism disappears as gut flora develops and matures. Fifty to 70% of human infants are estimated to be colonized but remain asymptomatic, even though their stool toxin levels may be as high as those found in symptomatic adults (Wilson 1993, Waters et al 1998).

Risk factors and antibiotic susceptibility

*Clostridium difficile* is estimated to be responsible for approximately 25% of antibiotic-associated diarrhea cases and is found in the stool of over 95% of patients with pseudomembranous colitis (Songer 2004, Bartlett 1994). Over 90% of these cases occur during or after a course of antibiotic therapy. With the exception of aminoglycosides, nearly all other antimicrobial agents have been associated with CDAD (Sunenshine and McDonald 2006). A strong association with human *C. difficile* infection exists among the cephalosporins, second-generation penicillins, and clindamycin (Yaeger et al 2002). Resistance to widely-used fluoroquinolones may have given rise to the current human epidemic strain (Songer et al 2009). Low serum antibody response to TcdA and age >65 years are additional risk factors. Those of old age or exposure to additional antibiotics have a 50% increased risk of post-treatment recurrent *C. difficile* diarrhea (Poutanen and Simor 2004).

Development of disease in humans has also been linked to enemas and stool softeners, laxatives, anatacids, increased age, and severe underlying illness (McFarland et al 1990). In pigs, the lack of a mature colonic flora may be a possible risk factor. As pigs are born with a sterile alimentary tract, 5-6 days are required to establish normal intestinal
microflora, and even more time is needed to establish normal colonic flora (Yaeger et al 2002). A major predisposing factor also includes exposure to environmental contamination with *C. difficile* spores (Waters et al 1998). A major source of this is the hospital. *C. difficile* is commonly isolated from floors, bedpans, and bathrooms of those who are carriers, as well as hands of care givers (Wilson 1993).

**Treatment**

Currently, no immunoprophylactic products are commercially available. Competitive exclusion (inoculation with a nontoxigenic isolate) shows promise as toxigenic strains were excluded from colonization and toxin production was inhibited (Songer et al 2007). Feedback of intestines to gestating gilts has also been found to be an effective preventive measure (Waters et al 1998). Results of antimicrobial and probiotic treatment have been unsatisfactory and lack consistency (Waters et al 1998, Songer et al 2000). Metronidazole, bacitracin zinc, and vancomycin are used for treatment in horses and humans, but extend no benefit to swine as both vancomycin and metronidazole are not approved for use in food animals (Songer et al 2000, Sunenshine and McDonald 2006).

Antibodies are effective in maintaining immunity to or treating CDAD and can supplement the action of antibiotics. Songer (2004) cites eleven studies in which antibodies against TcdA prevented toxin binding, eliminated secretion and inflammation, or prevented clinical disease in mouse and hamster models. In human cases of CDAD antibodies are commonly used for treatment. The use of antibiotics alone has a relapse rate of 10-20%, of which half is due to re-infection (Fekety et al 1993, Pothoulakis et al 1993, Kelly et al 1994, Barbut et al 2000). Systemic antibody is important in these patients with recurring CDAD. It has been shown that antibiotics used in conjunction with intravenous infusion of immunoglobulin or orally administered hyperimmune bovine colostrum allowed these patients to overcome disease (Leung et al 1991, Salcedo et al 1997, Kyne et al 2000, Lyerly et al 1990, Schellenberg et al 1994, Kelly et al 1996, Kelly et al 1997). Furthermore, rising anti-TcdA antibody titers correlate with resolution of disease and antibody concentration in
serum is inversely proportional to the severity of disease and the risk of relapse (Warny et al 1994).

**Screening**

Several screening methods, including PCR and toxin detection are used to aid the diagnosis of CDAD. Isolation of *C. difficile* alone is not indicative of the cause of diarrhea as the organism may reside as part of the normal flora in some humans and animals, nor does isolation discriminate against nontoxigenic strains which are not thought to cause disease (Levett 1986, Brazier 1993, O’Neill et al 1993). As a result, clinical isolation of *C. difficile* is not commonly practiced (Marks et al 2002). Recent work has challenged this paradigm regarding toxigenic culture as the true gold standard, since detection of toxin alone may not be as sensitive as culture verified to be toxigenic by a tissue cell cytotoxic assay (Sloan et al 2008). Any advantage gained in specificity by this route may very well be lost in practicality, as the combination of culture and toxigenicity verification is time consuming and specialized.

PCR can be 100 times more sensitive than anaerobic culture, with capabilities of detecting as few as ten bacilli in one gram of stool (Gumerlock et al 1991). Another study found PCR to have 86% sensitivity and 97% specificity for diagnosis of *C. difficile* infection (Sloan et al 2008). Regardless of its advantages, PCR can be time intensive, reagents can be expensive, and not all clinics may possess the equipment or expertise to make PCR a practical diagnostic tool.

Enzyme immunoassays (EIA) are the most popular diagnostic tool. In 2003, 93% of hospital laboratories used an EIA to help in the diagnosis of CDAD (McDonald et al 2006). These assays are attractive for clinical laboratories as they are inexpensive compared to alternative testing means, but most importantly offer fast results and are easy to perform. Testing to determine the sensitivity of these assays has been variable; different investigators have found sensitivities to range from a low of 32% to over 90%. Specificities range from 84-100% (Sloan et al 2008). Four different EIA’s used on diarrheic dog specimens had sensitivities of 7-33% and specificities of 65-100%, indicating that their use for diagnosis of
C. difficile in dogs based on fecal toxin is inadequate (Chouicha and Marks 2006). The manufacturer of the toxin EIA used in the research described in this manuscript claims a 92% sensitivity, 100% specificity, and 98.8% correlation to the toxin tissue culture assay (Techlab product insert). A predecessor assay of the same manufacturer was tested with swine samples and was found to have a sensitivity and specificity of 91% and 86%, respectively. Its correlation to the tissue culture assay was 88% (Post et al 2002).

**Culture methods**

Because it is a strict anaerobe, C. difficile can be difficult to grow. Recovery of C. difficile is best when the sample is shocked by heat (70°C for 20 min or 80°C for 10 min) or alcohol (equal volumes 95-100% ethanol and sample for 30 minutes). These processes enhance recovery of C. difficile spores by destroying vegetative cells and reducing the bacterial burden of the sample (Brazier 1993, Hanff et al 1993, Sloan et al 2008, Kinyon personal communication, Songer et al 2007). Growth is optimal in environments at or near a ratio of 5% CO2:5% H2:90% N2 (Songer et al 2007, Sloan et al 2008). The addition of sodium taurocholate to media enhances the recovery and germination of spores (Wilson et al 1982). Taurocholate-cycloserine-cefoxitin-fructose agar is a recommended and commonly used selective medium for the detection and isolation of C. difficile (Fedorko and Williams 1997, Sloan et al 2008). Reduced brain heart infusion broth (BHI), sometimes supplemented with 0.1% sodium taurocholate or 0.5% yeast extract and 0.05% cysteine is an excellent medium for the cultivation of C. difficile cultures (Brazier 1993, Keel and Songer 2007).

Identification is noted by gram stain revealing long gram positive rods that may have terminal spores, a characteristic stable-like odor, fluorescence under long-wave UV light, a positive L-proline aminopeptidase disc test, gray umbonate nonhemolytic colonies with filamentous edges and a ground glass appearance, and PCR (Brazier 1993, Kuhl et al 1993, Braun et al 1996, Fedorko and Williams 1997, Yaeger et al 2007, Sloan et al 2008).
**Clostridium difficile** in commercial retail meat

To date, three studies have evaluated the presence of *C. difficile* in commercially available meat obtained through retail outlets. In a US study, 47.8% of ready to eat meat products and 40% of uncooked meats were culture positive. Among those meat products in which the highest percentage of *C. difficile* isolates were recovered were ground beef (50%) and pork braunschweiger (62.5%) (Songer et al 2009).

In Canada, *C. difficile* was isolated from 12%-20% of commercial ground beef, pork, and veal samples (Rodriguez-Palacios et al 2007, Weese et al 2009). Twenty-five percent of the pork-derived isolates were indistinguishable from human isolates, of which included human pathogen ribotypes 077 and 014 (Rodriguez-Palacios et al 2007). The most common ribotype found in 66% of the samples was different than previous ribotypes identified by the investigators and had genes for TcdA, TcdB, and CDT, an 18 bp deletion in the *tcdC* gene, and a toxinotype III designation (Rodriguez-Palacios et al 2007). PCR ribotypes indistinguishable from bovine and canine ribotypes were also found. Meat-sourced PCR ribotypes indistinguishable from these three species ribotypes suggests the possibility of interspecies cross contamination.

While the isolation of *C. difficile* in meat does not necessarily indicate foodborne transmission, it has the characteristics to make it a successful foodborne pathogen as spores can survive recommended cooking temperatures of 71°C for 2 hours (Rodriguez-Palacios et al 2007). While still unknown, the source of the *C. difficile* could be from fecal or environmental contamination from persisting spores in the processing or retail meat market environments, or from antemortem deposition of spores in animal tissue (Songer et al 2009).

**Disinfection**

Controlled trials of efficacious disinfectants for *C. difficile* are lacking. While spores are resistant to alcohol, bleach has been shown to lower rates of *C. difficile* contamination and may also lower rates of CDAD (Sunenshine and McDonald 2006).
**Interspecies transmission**

Research has yet to show that interspecies transmission of *C. difficile* occurs between humans and animals, but mounting evidence suggestive of such a relationship warrants continued investigation (O’Neill et al 1993, Lefebvre et al 2006, Goorhuis et al 2008). Ribotype 078/Toxinotype V, which is the predominant ribotype in neonatal pigs and calves, has become increasingly involved in human cases of CDAD. In the Netherlands, the incidence of ribotype 078 in humans has increased from three to 13% while the incidence of ribotype 027 decreased from 27 to one percent, making ribotype 078 the second-most prevalent type (Goorhuis et al 2008).

Toxinotype V isolates are highly associated with community-associated *C. difficile*-associated disease (CA-CDAD), which is defined by an onset of symptoms or a positive toxin test within 48 hours after patient admission to the healthcare facility, whereas the patient had no previous discharges from a healthcare facility in the previous three months (Goorhuis et al 2008, Jhung et al 2008). One study found 46% of their human toxinotype V cases were community-associated, even though CA-CDAD accounts for only 20% of all human cases of CDAD (Jhung et al 2008). As a new emerging hypervirulent strain, ribotype 078 causes severe disease in a younger population and is genetically indistinguishable from porcine ribotype 078 strains (Goorhuis et al 2008, Debast et al 2009). Potential causes of human toxinotype V CDAD may occur from an environmental source of *C. difficile* common to humans and animals, direct or indirect contact with infected animals, or meat consumption from infected animals (Jhung et al 2008). It is important to be mindful that these are all hypothetical as they have yet to be proven.

Hypervirulent epidemic human strains have been isolated from animals as well. Ribotype 027 has been isolated from a hospital visitation dog as well as dairy, beef, and veal calves, and adult cattle (Lefebvre et al 2006, Rodriguez-Palacios et al 2007). Isolation of an epidemic human isolate from a hospital visitation dog suggests that the animal acquired the strain during visits to the hospital (Lefebvre et al 2006).
V. Research

V.1. Materials and methods

Animals and housing.

Thirty-two conventional neonatal pigs were enrolled in the study. All pigs had antibodies to porcine reproductive and respiratory syndrome virus (PRRSV), and all were negative for PRRSV by PCR (Hermann et al 2006). At the farm of origin, three sows were monitored for signs of parturition, and a sterile surgical drape was placed in the farrowing crate behind the sow. This was done for each experimental replicate and two different time points, one week apart. Sows were allowed to farrow naturally and immediately post delivery individual piglets were removed from the sow, dried, and placed in a clean plastic tote underneath a heat lamp. Umbilical cords were clamped, cut, and then sprayed with gentle iodine. Colostrum was collected from the three farrowing sows in sterile 50 ml centrifuge tubes. Equal amounts of colostrum from the three sows were then mixed and each individual neonatal pig received 10 ml of colostrum mixed with 20 ml of commercial puppy milk replacer (Esbilac liquid puppy formula; Pet-Ag, Hampshire, IL) in the same 60 ml syringe. The 30 ml mixture of colostrum and milk replacer was delivered via an oral-gastric tube consisting of an eight gauge rubber French catheter (Sovereign™, Tyco/Healthcare, Mansfield, MA). Pigs were then transported to a BSL-2 animal facility at Iowa State University. At the research facility, pigs were housed in one of two identical raised plastic pens partitioned into eight individual pens (approximately 0.7 x 0.7 m) with solid dividing walls (Figure 1) and individual feeding bowls. All pigs were housed in the same airspace. Room temperature was maintained at 29°C and heat lamps were suspended over the pigs to keep the immediate environment at 35°C. Pigs were bowl-fed 60 ml commercial milk replacer three times daily. Feedings were spaced approximately seven to nine hours apart.
**Experimental Design.**

The study was performed in two replicates of 16 pigs each. In each replicate, pigs were randomly allocated using several random number iterations in Microsoft Excel (Microsoft Excel 2007, Microsoft, Redmond, WA) into either one negative control group ($n=3$) or two different treatment groups ($n= 6$ and $7$). Four different *C. difficile* isolates were used in this study, with two different isolates per replicate. Approximately four hours after birth, pigs were inoculated gastrically with the sham (negative controls) or treatment using an eight gauge rubber French catheter as an oral-gastric tube. At 24, 48, and 72 hours post inoculation (hpi), one negative control pig and two or three pigs per treatment group were randomly selected based on previously determined random number iterations and euthanized by an intravenous overdose of pentobarbital (Fatal-Plus®, Vortech Pharmaceutical, LTD, Dearborn, MI) . The experimental design is summarized in Table 1. The experimental protocol was approved by the Iowa State University Institutional Animal Care and Use Committee. (IACUC protocol # 5-09-6743-S)

**Inoculum.**

Four different isolates of *C. difficile* were selected for experimental infection (Table 2). JGS6125 is a strain of human origin. Isolates 13912-1, 02792-1, and 15454-1 were isolated at the ISU VDL from the feces or intestinal contents of neonatal pigs presented with the complaint of diarrhea. In all pigs, high levels of toxin (4+) were detected by ELISA (*Clostridium difficile* tox A/B II kit, TechLab, Blacksburg, VA). All isolates were stored at -80°C until culture preparation.

Lawns of isolates were cultivated on anaerobic brain heart infusion agar with 0.5% yeast extract and 0.05% L-cysteine (BHI-CYE) and incubated in an anaerobic glovebox (Bugbox™, Ruskinn Life Sciences LTD, Pencoed, UK) with an atmosphere of 10% CO$_2$: 10% H$_2$: 80% N$_2$ at 37°C for 7 days. Lawns were harvested with 5 ml phosphate-buffered saline (PBS; 0.01M, pH 7.2) and centrifuged at 10,000 x g for 20 min at 4°C. The pellet was washed with 1M KCl: 0.5M NaCl, centrifuged as before and resuspended in 25 ml 50mM
Tris-HCl, (pH7.2) with 10 mg lysozyme per ml. After 1 hr incubation at 37°C, the material was washed 3 times with 25 ml sterile nanopure water, using the same centrifugation conditions as before. Before centrifugation of the third wash the spores were counted on a hemacytometer. The pellet was adjusted to a final concentration of ~ 2x10⁹ spores/ml in sterile nanopure water and stored at 4°C. Immediately prior to challenge, spores were heat shocked in a waterbath at 80°C for 10 min. BHI broth with 0.1% taurocholic acid and 5% fetal bovine serum was added to the heated spore suspension at a concentration of 25% v/v and incubated 1 hr at 37°C. BHI broth was used in place of spores in the control inoculum. Final cell counts were determined by plating 10⁻⁵ to 10⁻⁹ dilutions of the final challenge inoculum in duplicate on anaerobic BHI-CYE plates and incubating as before. Toxinotype, identity, and presence of tcdA and tcdB toxin genes were determined or confirmed by PCR as previously described (Braun et al 1996, Rupnik et al 1998, Kato et al 1998).

**Inoculation.**

Oral-gastric inoculation was done for all groups. The control group in each replicate was given 1.25 ml of media and then flushed with 15 ml of milk replacer. For all treatment groups, 1.25 ml of challenge preparation containing heat shocked C. difficile spores was given followed by 15 ml of liquid milk replacer. Administration of challenge material or media only was done at approximately four hours after birth. Pigs were allowed access to milk replacer (via bowls) immediately after administration of challenge.

**Sample collection.**

Rectal swabs for aerobic and anaerobic culture (Culturette, BD, Franklin Lakes, NJ) and C. difficile toxin detection (Stool-Prep, TechLab, Blacksburg, VA) were collected from each pig prior to inoculation. Rectal swabs for toxin assay were collected daily from each pig post inoculation until necropsy. Swabs were tested within one hour post sampling.
Necropsy.

Gross observations at necropsy were scored as previously described (Yaeger et al 2007) and are listed in Table 3. Body condition, hydration status, perineal fecal staining, consistency of colonic contents, mesocolonic edema, and visible luminal necrosis were assessed. Fresh and formalin fixed tissues were collected with sterilized instruments and included: ileum, jejunum, descending colon, cecum, and a cross section through the spiral colon containing 4-5 loops. Colonic and cecal contents were collected in a sterilized plastic cup.

Culture and toxin detection.

The contents and mucosa swabbed from two sections of the spiral colon, approximately three to four cm long were combined and cultured. A sterile swab was inserted into the intestinal loop with the assistance of sterile forceps. The loop was swabbed vigorously and the swab was then dipped into leaked intestinal content fluid in the specimen bag. The material on the swab was plated to Clostridium difficile selective agar (CDSA; Remel, Lenexa, KS) both direct and following 30 minute room temperature incubation in 0.5 ml absolute ethanol, vortexing at the beginning, after 15 minutes, and at the end of incubation. Twenty microliters of the swab contents and alcohol suspension was transferred to the plate using sterile pipette tips. All plates were incubated at 37°C for 48 hr. in an anaerobic chamber (Songer, Kinyon personal communication).

Plates were positioned under a woods lamp and two grey, slightly alpha hemolytic, umbonate, fluorescing colonies with a ground glass texture typical of C. difficile were subcultured to CDSA and incubated under the same conditions. Two fluorescing colonies with typical morphology from these plates were again subcultured to anaerobic blood agar and incubated as before for 48 hr. Blood agar plates were examined for pure cultures with typical colony morphology, a characteristic ‘horse stable-like’ odor, and fluorescence under a woods lamp. Identity was confirmed by gram stain revealing long, thin, straight gram positive rods, a positive L-proline aminopeptidase disk test (PRO-disc, Remel, Lenexa, KS;
Fedorko et al (1997), and PCR as previously described (Braun et al 1996). PCR was used for toxinotyping as well as detection of toxin genes in isolates and challenge material using methods described previously (Kato et al 1998, Rupnik et al 1998). Pigs received a score of 0 or 1 for negative or positive C. difficile culture, respectively.

Pooled colon and cecum contents were assayed for C. difficile toxins A and B with the same toxin ELISA as before according to manufacturer instructions. Toxin levels were graded on a scale from 0 (no toxin detection) to 4+ (marked toxin detection) based on optical density values. This value was used as the toxin score. The toxin ELISA detects both TcdA and TcdB but does not differentiate between them (Techlab product insert).

Swabs of small intestine contents were tested by routine aerobic and anaerobic culture methods to test for Salmonella spp, hemolytic or mucoid Escherichia coli, and Clostridium perfringens. To test for Salmonella spp swabs were streaked directly onto MacConkey (MC) and Brilliant green agar (BG), incubated 18-24 hr at 37°C and were also enriched in 10 ml tetrathionate broth, incubated at 42°C and subcultured to XLT4 and BG after 24 and 48 hr of incubation. Subcultured plates were incubated 24 hr at 37°C. All plates were observed for Salmonella suspect colonies to be confirmed by biochemical testing (Kligler’s, Sulfur-Indol-Motility, and Urease). For the isolation of E. coli, swabs were streaked directly onto 5% blood agar plates (BA) and MC, incubated 24 hr at 35°C. Plates were observed for suspect E. coli colonies which were confirmed by biochemical testing (Kligler’s, Sulfur Indol Motility, and Urease). Isolation of C. perfringens was achieved by streaking swabs directly onto aerobic and anaerobic BA and Clostridium perfringens selective agar, incubated anaerobically at 35°C for 48 hr, and observed for suspect C. perfringens colonies with double zone hemolysis. Two samples of cultured hemolytic or mucoid E. coli and three samples of C. perfringens were randomly selected for PCR genotyping (Meer and Songer 1997, Casey and Bosworth 2009).

**Histopathology.**

Fixed tissue sections were collected in 10% neutral buffered formalin, routinely processed, and stained with hematoxylin and eosin. Tissues were examined by a veterinary
pathologist blinded to group designation and were assessed for the percentage of epithelial cells lining glands in the cecum and colon composed of goblet cells, quantity of neutrophils in the lamina propria, mucosal alterations, and mesenteritis. Microscopic lesions were assessed and scored as described previously (Yaeger et al 2007) with slight modifications. Table 4 summarizes the scoring system used.

**Scoring.**

Three categories of scores were compared: 1) clinical signs, 2) gross lesions, and 3) microscopic lesions. Clinical signs scores were created by summing scores for body condition, hydration status, and perineum staining. A pig was considered to be diarrheic if it received a perineum staining score of 1 or greater. Gross lesion scores were created by summing scores for necrotizing lesions, mesocolonic edema, culture, and toxin, and the microscopic lesion score was the sum of scores for all histopathology categories.

**Statistical analysis.**

Scores for clinical signs and gross and microscopic lesions were analyzed by ANOVA to determine if differences existed between comparisons of control groups to treatment groups, and treatment groups to each other. Significant differences between group means were determined using Tukey’s HSD test. Fisher’s Exact Test was used to determine if differences in presence of clinical signs and lesions between controls and treatment pigs were significant. Significance of correlation occurring between events were calculated by Pearson’s product-moment correlation. JMP 8 (SAS, Cary, NC) statistical software was used to perform analyses.
V.2. Results

*Bacterial Culture.*

*Clostridium difficile* was isolated from the colon of all pigs at necropsy including controls. Day 0 rectal culture for *C. difficile* was negative for all but one pig, which had a single colony. Isolates recovered from pigs were the same toxinotype as the material they were challenged with, except for challenge isolate JGS6125 (Table 5). Pigs in this group were of either toxinotype IIIb \((n=2)\) or 0 \((n=3)\). At the time of writing this manuscript, results are still pending for one pig in this group. Results are also pending on a retest of the toxinotype of JGS6125. Controls were one of three toxinotypes: 0 \((n=2)\), V \((n=3)\), and IIIb \((n=1)\). No *Salmonella* spp was isolated from any pig. Hemolytic *E. coli* was isolated from the intestinal contents of 5/32 pigs (15.6%) and mucoid *E. coli* was isolated from 1/32 pigs (3.1%). Of mucoid or hemolytic isolates, three were randomly selected for genotyping and all were nontoxigenic. *Clostridium perfringens* was isolated from the intestinal contents of 27/32 (84.3%) pigs. Two *C. perfringens* isolates were randomly selected for genotyping and were alpha and beta-2 toxin positive.

*Clinical signs.*

Clinical signs were independently scored for all pigs and are listed below by necropsy time point.

24 hpi. Control pigs had normal to thin body condition, and all pigs were normally hydrated with no diarrhea. The same was observed in all challenged pigs. Clinical signs data are listed in Tables 6 and 7.

48 hpi. One control pig was thin, mildly dehydrated, and had moderate diarrhea. The other necropsied control pig was normal. Challenged pigs had normal to thin body conditions. Four of 8 (50%) were mildly to moderately dehydrated and 7/8 (87.5%) had mild to mostly moderate and severe diarrhea.

72 hpi. One control pig was moderately dehydrated, thin, and had diarrhea. The other control was thin but had normal stool consistency and hydration. Most of the challenged pigs
were thin or emaciated (5/10 and 2/10, respectively), were mildly to moderately dehydrated (8/10), and had severe diarrhea (8/10).

Differences in clinical signs between control and challenged pigs were not statistically significant. Mean clinical sign scores at 24 hpi were significantly lower from those at 48 (p=0.0096) and 72 (p<0.0001) hpi.

**Gross lesions.**

**24 hpi.** Control pigs had no to mild mesocolonic edema and normal colon contents. Mild edema and pudding-like colon contents were observed in 5/8 (62.5%) challenged pigs. Necrotizing lesions were not observed in any pigs. Gross lesion data is summarized in Tables 6 and 7.

**48 hpi.** No to moderate mesocolonic edema and pudding-like to watery colon contents were observed in the controls. Mild to severe mesocolonic edema (Figure 2) was observed in 6/8 (75%) challenged pigs, but all had pudding-like to mostly watery colon contents. Visible necrotizing lesions in the cecum or colon were not observed in any pigs.

**72 hpi.** Mesocolonic edema was not observed in controls. However, one control pig had watery colon contents. Mild to severe mesocolonic edema was observed in 8/10 (80%) challenged pigs. The same number of pigs also had pudding-like to mostly watery colon contents. Moderate necrotizing lesions were observed in the colon of one challenged pig.

There were no significant differences in gross lesions between control and challenged pigs. Mean gross lesion scores of all groups at 24 hpi were significantly lower than mean scores at both 48 (p=0.0007) and 72 (p=0.0061) hpi.

**Microscopic lesions.**

**24 hpi.** Control pigs had normal tissues except for a single observation of mild neutrophilic infiltration in the small intestine lamina propria. Mild neutrophilic infiltration of the lamina propria in the colon, cecum, or small intestine was observed in 4/8 (50%) challenged pigs. A summary of microscopic lesions is listed in Tables 6 and 7.
48 hpi. One control pig had moderate to severe neutrophilic inflammation in the cecum and colonic lamina propria, and had mild goblet cell loss. Characteristic fibronecrotic ‘volcano lesions’ were observed in multiple challenged pigs with severe neutrophilic infiltration at 48 hpi (Figures 3 & 4) along with multifocal mucosal erosions in the colon. In the treatment groups, neutrophilic infiltration of the colon, cecum, or small intestine ranged from mild to severe in 6/8 (75%) pigs. Severe infiltration was limited to the colon or cecum. Mild to moderate goblet cell loss was observed in 6/8 (75%) pigs. Mild to severe mucosal alterations in either the colon or cecum were observed in half of the pigs, whereas mild mesenteritis only occurred in 2/8 (25%) pigs.

72 hpi. Control pigs had normal tissues except for mild colonic and small intestinal neutrophilic inflammation in one pig. Neutrophilic infiltration of the colon, cecum, or small intestine ranged from mild to severe in 8/10 challenged pigs. Mild to moderate goblet cell loss occurred in 7/10 pigs. Mild to severe mucosal alterations in the colon and cecum were observed in 4/10 pigs. Similar to challenged pigs necropsied at 48 hpi, fibrinonecrotic colitis with “volcano lesions” was observed in 2/10 challenged pigs. Mild mesenteritis was observed in 3/10 pigs.

Differences in the presence of microscopic lesions between control and challenged pigs were not statistically significant. Significant differences in mean lesion scores existed between isolates 15454-1 and 02792-1 (p=0.0081) as well as 15454-1 and controls (p=0.0198). Mean lesion scores of all groups at 24 hpi were significantly lower than mean scores at 48 (p=0.0046) and 72 (p=0.0121) hpi.

Toxin.

Prior to sham inoculation or challenge with C. difficile, rectal swabs from all pigs were negative for both toxins A and/or B.

24 hpi. One of the two controls had low levels of toxin detected in the colon (score=1). Rectal swabs were negative for toxin. Low levels of colon toxin (score=1) were detected in 2/8 (25%) challenged pigs. All rectal swabs were negative. Toxin data from day of necropsy is listed in Tables 7 and 8.
48 hpi. Rectal swabs of both controls were negative, but one control had a high colon toxin score of 3+. This is the same control that had moderate to severe intestinal and cecal neutrophilic infiltration, severe colonic mucosal alterations, and moderate mesocolonic edema, but had no diarrhea and clinical appearances were otherwise normal. Fecal toxin was detected in a total of 6/8 (75%) challenged pigs; high levels of toxin (3+ to 4+) were detected in 3 (37.5%) of the pigs, while the other 3 had lower levels (1 to 1+). Colon toxin was detected in 5/8 (62.5%) of challenged pigs. High colonic toxin levels (3+ to 4+) were detected in three of these pigs, while low to moderate (1 to 2+) toxin levels were detected in the other two pigs.

72 hpi. Low (1) colonic toxin was detected in one control. Only 3/10 challenged pigs were positive for colon toxin, with scores ranging from 1 to 4+. Fecal toxin was detected in 5/10 challenged pigs, with scores ranging from mostly 1 to 4+.

All challenge and recovered isolates possessed genes for TcdA and TcdB as determined by PCR. Toxin was observed in a total of 14/26 (53.8%) treatment and 3/6 (50%) control pigs at necropsy. This difference was not statistically significant. Eight of twenty-six (30.7%) challenged pigs had fecal toxin at least once during the study but no colon toxin on the day of necropsy. Fecal toxin scores ranged from 1 to 4+ in these pigs. A smaller number (3/26, 11.5%) of challenged pigs had toxin detected in the colon and never in the feces. Scores of these three were 1, 3+ and 4+. Colon and fecal toxin scores were significantly correlated on day of necropsy (p=0.0329; r=0.3781;). Correlations between toxin and microscopic lesion scores for both fecal (p=0.0016; r=0.5351) and colon (p=0.0007; r=0.5691) toxin were also significant.

V.3. Discussion

To date, a number of studies have identified C. difficile or its toxins occurring at frequencies of 25% to 50% in pig populations (Yaeger et al 2002, Songer 2004, Songer et al 2006, Yaeger et al 2007, Alvarez-Perez et al 2009, Norman et al 2009). Moreover, C. difficile cases in humans have been consistently increasing in recent years (McDonald et al 2006, Sunenshine and McDonald 2006, Redelings et al 2007, Jhung et al 2008).
appropriate responses to these findings call for research in treatments, therapies, and mechanisms of disease, but an efficient means to do so is required. Experimentally-induced disease has been described in hamsters, mice, rats, and rabbits (Lyerly et al 1985, Keel and Songer 2006), but the importance of this disease in pigs necessitates a species specific model. The purpose of this study was to reproduce *C. difficile*-induced disease using conventional pigs as an animal model and to note whether differences exist between human and porcine challenge isolates.

In this study, disease was reproduced by all four isolates initially recovered from field cases. Commonly observed lesions and indications of CDAD, including mesocolonic edema (19/26), toxin (18/26), diarrhea (15/26), neutrophilic infiltration of colonic (12/26) and cecal (13/26) lamina propria, and mucosal ulceration or erosion of the colon (8/26) and cecum (3/26) were observed in challenged pigs. The hallmark ‘volcano lesions’ resulting from excessive neutrophilic infiltration into the intestinal lamina propria were observed in 5/26 (19.2%) pigs. These observations are similar to those described by others (Post et al 2000, Songer et al 2000, Yaeger et al 2002, Yaeger et al 2007).

A recent study published during the preparation of this manuscript describes a challenge model using gnotobiotic pigs (Steele et al 2010). While one benefit to the use of gnotobiotic pigs is the ability to maintain a controlled flora and prevent colonization of potentially pathogenic and confounding ubiquitous organisms, gnotobiotic pigs are more costly, have lower availability, are more labor intensive to breed and rear than standard cross-bred conventional pigs, and are less representative of a typical pig from a production setting. Though Steele and others (2010) stated that colostrum deprivation, starvation, and treatment with antibiotics may be required to enhance susceptibility to infection, the current study was able to reproduce signs of disease after administration of colostrum and without starvation or antibiotic pretreatment. Depending on the application, one source of pig may be more desirable over the other.

A previous study not published in the peer-reviewed literature was able to experimentally induce disease in crossbred pigs without starvation or antibiotic pretreatment, although colostrum was withheld (Post et al 2000, Songer and Anderson 2006). Attempts to withhold colostrum from pigs prior to challenge in work leading up to the current study were
unsuccessful as pigs were unhealthy and necropsy revealed lesions indicative of secondary confounding pathogens (unpublished data). This was not observed in the current study when pigs were fed colostrum before inoculation.

Numerous studies have indicated the protective and therapeutic effects of systemic and local antibody in controlling CDAD (Songer 2004). Despite the efficacy of antibody, the presence of *C. difficile* or its toxins is becoming increasingly widespread in pig populations. This suggests that the passive transfer of antibodies to *C. difficile* in sow’s colostrum may be insufficient or nonexistent, or that *C. difficile* is poorly immunogenic in pigs as sows have almost certain exposure through the environment or as neonates. Even though rates of *C. difficile* isolation in pigs decrease as the pig matures, this could be due to a reduction of toxin receptors or the establishment of a mature exclusionary gut flora rather than anti-*C. difficile* or anti-TcdA/B antibodies (Alvarez-Perez et al 2009, Norman et al 2009). Thus, the role of porcine colostrum in the intervention of CDAD may be less than it is presumed to be.

The data from this study consistently demonstrated that clinical signs and lesions, both gross and microscopic, were significantly more severe at 48 and 72 hpi than at 24 hpi. This is of use to future studies as some time is required for disease to fully develop. Though it could not be statistically demonstrated, there is a slight observable trend in diminishing disease severity from 48 to 72 hpi that suggests lesions could decrease if periods between inoculation and necropsy were longer.

Few differences associated with disease were attributable to challenge isolate in this study. All of the isolates were toxinotype V, which has genes for TcdA, TcdB, and binary toxin. Toxinotype V isolates also have a 39 bp deletion in the toxin regulatory gene *tcdC* (Goorhuis et al 2008). A retest of the toxinotype of isolate JGS6125 is pending. Ribotype 078, which is toxinotype V, is the predominant ribotype circulating in swine and accounts for 83% of swine isolates (Keel et al 2007). Regardless of their genotypic similarities, isolate 02792-1 produced relatively mild disease and lesions throughout the entire study, whereas isolate 13912-1 and 15454-1 produced some of the most severe disease and microscopic lesions. Isolate JGS6125 is a strain of human origin and while lesion scores of the other three isolates generally peaked at 48 hpi, JGS6125 appeared to take longer to produce maximum disease and lesion scores peaked at 72 hpi. The only statistically significant
differences between isolates were between mean microscopic lesion scores of 15454-1 and 02792-1 (p=0.0081) and 15454-1 and the controls (p=0.0198).

Though the controls have generally lower scores in most categories than challenged pigs, these differences were not statistically significant with the exception of the previously stated differences in microscopic lesion scores of isolate 15454-1. Much of this can be attributed to statistical limitations due to the small sample size as well as the study design, which only allowed for two or three challenged pigs to be compared to a single control pig at any given necropsy. Because of these small comparisons, care must be taken in the interpretation of data. For instance, the data listed in Table 5 for each necropsy period can be misleading as the means among challenged pigs are only based on sample sizes of two or three pigs, whereas the data for the controls are not a mean but the actual scores of a single pig because only one control was observed at each necropsy per replicate. Thus, an outlier such as the control pig in replicate one at 48 hpi can have considerable influence on the overall outcome and perception of differences in disease.

The controls may have been inadvertently contaminated as they shared the same room and grate as the challenged pigs (Figure 1), even though considerable care was taken in the measures to prevent such cross contamination. Possible sources for this contamination could have been from the animal care staff or the investigative staff. Washing the floors could have aerosolized bacteria or mechanically transferred it to the bins housing the control pigs. Contaminating bacteria could have also moved under the plexiglass divider into the adjacent pen. Finally, it is possible that the signs and isolation of *C. difficile* could have been from exposure to the organism at the farm. In an attempt to reduce this possibility, pigs were snatched in clean (but not sterile) conditions, exposure to the immediate production environment was brief and limited, and the farm had no history of diarrhea or *C. difficile* of which its management was aware. Initial tests for fecal *C. difficile* organism or its toxin were negative. One pig did have a single colony of *C. difficile* on its day 0 rectal swab, but a single colony is of questionable significance (Kinyon, personal communication).

It is unclear why some of the pigs in group JGS6125 were toxinotype IIIb while the others in the same group were type 0, none of which match the toxinotype of the challenge isolate, which was toxinotype V. As a result of this confusion, a toxinotype retest of
JGS6125 is pending. Cultured isolates from all other groups were the same toxinotype as that of the respective challenge strain.

Future studies should consider the use of separate rooms for housing of control and challenged pigs. In the current study, space and time was limited so this option was not a possibility at the time the study was conducted. In addition to housing in separate rooms, the use of a nontoxigenic isolate as used by Steele and others (2010) as a control rather than media could fill gut niches and exclude potential toxigenic *C. difficile* or other confounding organisms that could result from farm exposure. However, this could have a treatment effect of its own as it could conceal the naturally existing state of the controls.

The fact that the controls were not completely free of *C. difficile* or other potentially pathogenic organisms does reveal one of the disadvantages to using standard conventional pigs and only underscores the fact that an understanding of *C. difficile* transmission in swine is lacking (Keel et al 2007). Even though numerous precautions were taken by investigators to prevent accidental cross contamination, transmission of disease could occur much easier than it is currently thought to.

*Clostridium perfringens* A was isolated from most (27/32, 84.3%) of the pigs. This does potentially confound some of the data as it is difficult to say how the overall outcome may have been affected by its presence, if at all. As this organism is very ubiquitous it could have persisted in the study room environment, on fomites, or possibly have been acquired at the farm (Garcia and Heredia 2009). Even though piglets were removed from the immediate production environment as soon as possible following birth, the organism replicates very quickly and had an uninhibited environment in the pig’s sterile gut (Yaeger et al 2002, Garcia and Heredia 2009). Mucoid or hemolytic *E. coli* was isolated from 6/32 (18.7%) pigs. This is likely to be less problematic as three of the isolates were randomly selected for genotyping and were found to be nontoxigenic.

This study has raised additional points of interest that may require further investigation and could employ the use of this model. If disease is dose dependent, the determination of the minimum infectious dose could aid in the study of disease transmission. Studying the roles of different toxinotypes in disease can benefit the understanding of existing epidemic strains and allow for the identification of other strains with epidemic
potential. The model can also be used to determine if the presence of potentially pathogenic secondary bacteria such as *E. coli* or *C. perfringens* have an influence on the development or severity of disease. Such information could add to existing knowledge of disease pathogenesis as well as facilitate disease control strategies in swine production centers. Perhaps the most promising use for the model and pressing need for the control of CDAD in swine and humans is the evaluation of intervention strategies.

In this study, conventional pigs obtained from a production setting were experimentally inoculated with four different isolates of *C. difficile*. Clinical signs and lesions characteristic of CDAD were reproduced by all four isolates although some potentially confounding variables were present, such as the presence of *C. perfringens*. Because conventional pigs were used, this model can be productive yet economical. The data generated in this study provide evidence that this can be an effective challenge model.
VI. Conclusions

*C. difficile* is emerging as a global epidemic among porcine populations and hospitalized humans. Continuing research and new methods to allow for continuing research are a global priority. The current research describes an animal disease model to facilitate these needs. All typical signs and lesions of disease were observed. General trends or differences between challenged and control groups were noticeable even though these differences were not statistically significant. Because of this, the study cannot conclusively demonstrate that any observable effects of disease were entirely from the experimental challenge. While exposure to the organism at the farm is a possibility, the discrepancies observed in some of the controls are likely the result of contamination. In future studies, farrowing the sow in a controlled research facility rather than at the farm, better separation of treatment and control groups, sampling the sow for *C. difficile*, and larger sample sizes can minimize confounding variables and reduce variance within groups.

This research lays the groundwork for a new method in which disease transmission, pathogenesis, and intervention studies can take place. In summary, this study generated data supporting a promising challenge model for *C. difficile* in conventional pigs.
Research Acknowledgements

Funding for this project was supported by the National Pork Board. I would like to thank co-authors of the manuscript submitted for publication for which the majority of this thesis was used: Drs. Darin Madson, Kent Schwartz, Hank Harris, Brad Bosworth, and Alex Ramirez and Ms. Joann Kinyon. I would also like to thank Dr. Paulo Arruda, Efrain Figueroa and Andrea Holcombe for their assistance with animal care and collection of samples during the study and the Iowa State University Laboratory Animal Resources staff for daily animal care. I also thank Dr. Glenn Songer for kindly providing the JGS6125 Clostridium difficile isolate for use in this model development, Hien Trinh in the Songer lab and Dr. Maja Rupnik for toxinotyping the Clostridium difficile isolates, Stephen Gaul in the Harris Lab for assistance with spore preparation and PCR, and Robert Foster for statistical consultation.
Appendix A. Figures

Figure 1. Piglets were housed in raised plastic pens partitioned into eight individual pens with solid dividing walls on a common grate.

Figure 2. Severe edema of the mesocolon.
Figure 3. Fibrinonecrotic lesions composed of an exudate of neutrophils, fibrin, and cellular debris form a pseudomembrane (arrow) over intestinal villi.

Figure 4. Severe neutrophilic infiltration (greater than 50 neutrophils) of the lamina propria results in a neutrophil breach into the gut lumen, forming a characteristic 'volcano lesion.' (arrow)
Appendix B. Tables

**Table 1.** Experimental design for conventional pigs inoculated with sham (group A) or challenged with four different *C. difficile* isolates. The design was run in two different replicates with necropsies performed at 24, 48, and 72 hour post inoculation.

<table>
<thead>
<tr>
<th>Group</th>
<th>Isolate</th>
<th>Dose (CFU/ml)*</th>
<th>Replicate</th>
<th>n</th>
<th>Necropsy†</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td>24 hpi‡</td>
</tr>
<tr>
<td>A</td>
<td>Negative</td>
<td>0</td>
<td>1&amp;2</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>B</td>
<td>JGS6125</td>
<td>$1.8 \times 10^9$</td>
<td>1</td>
<td>6</td>
<td>2</td>
</tr>
<tr>
<td>C</td>
<td>13912-1</td>
<td>$7.5 \times 10^7$</td>
<td>1</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>D</td>
<td>02792-1</td>
<td>$4.7 \times 10^7$</td>
<td>2</td>
<td>7</td>
<td>2</td>
</tr>
<tr>
<td>E</td>
<td>15454-1</td>
<td>$2.6 \times 10^6$</td>
<td>2</td>
<td>6</td>
<td>2</td>
</tr>
</tbody>
</table>

* Colony forming units (CFU) of inoculation after spores were shocked. After production spores were initially adjusted to a concentration of $2 \times 10^9$ spores/ml and aliquoted. Inoculation was given by oral gavage.
† Number of pigs necropsied per group over time
‡ Hours post inoculation

**Table 2.** Data summary for toxinotype and origin of *C. difficile* challenge isolates.

<table>
<thead>
<tr>
<th>Group</th>
<th>Isolate</th>
<th>Toxinotype</th>
<th>Origin</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>JGS6125</td>
<td>V*</td>
<td>Human</td>
</tr>
<tr>
<td>C</td>
<td>13912-1</td>
<td>V</td>
<td>Pig in Missouri</td>
</tr>
<tr>
<td>D</td>
<td>02792-1</td>
<td>V</td>
<td>Pig in Illinois</td>
</tr>
<tr>
<td>E</td>
<td>15454-1</td>
<td>V</td>
<td>Pig in Missouri</td>
</tr>
</tbody>
</table>

*retest pending
### Appendix B. Tables (continued)

**Table 3.** Clinical and gross lesion scoring system at necropsy.

<table>
<thead>
<tr>
<th></th>
<th>Normal</th>
<th>Thin</th>
<th>Emaciated</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Condition</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Hydration Status</td>
<td>Normal</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>Perineum Staining</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>Distal LI* Contents</td>
<td>Normal</td>
<td>Pudding-like</td>
<td>Watery</td>
</tr>
<tr>
<td>Necrotizing Lesions in SI †</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
<tr>
<td>Mesocolonic Edema ‡</td>
<td>None</td>
<td>Mild</td>
<td>Moderate</td>
</tr>
</tbody>
</table>

* Large intestine
† Small intestine
‡ mild = 1 mm separation between loops, moderate = 2-3 mm separation between loops, severe = >3 mm separation between loop
## Appendix B. Tables (continued)

**Table 4.** Scoring system used for intestinal histopathology.

<table>
<thead>
<tr>
<th>Score</th>
<th>Lesion</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td><strong>Goblet cell loss</strong></td>
</tr>
<tr>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td>1</td>
<td>less than 25% goblet cells in 2 or more adjacent glands</td>
</tr>
<tr>
<td>2</td>
<td>30-50% glands with less than 25% goblet cells</td>
</tr>
<tr>
<td>3</td>
<td>greater than 50% of glands with less than 25% goblet cells</td>
</tr>
<tr>
<td></td>
<td><strong>Neutrophils (PMNs)</strong></td>
</tr>
<tr>
<td>0</td>
<td>no PMNs in lamina propria</td>
</tr>
<tr>
<td>1</td>
<td>less than 20 PMNs in the superficial lamina propria</td>
</tr>
<tr>
<td>2</td>
<td>20-50 PMNs in the lamina propria</td>
</tr>
<tr>
<td>3</td>
<td>greater than 50 PMNs</td>
</tr>
<tr>
<td></td>
<td><strong>Mucosal alterations</strong></td>
</tr>
<tr>
<td>0</td>
<td>normal</td>
</tr>
<tr>
<td>1</td>
<td>rare mucosal erosions (less than or equal to 4)</td>
</tr>
<tr>
<td>2</td>
<td>greater than or equal to 5 erosions</td>
</tr>
<tr>
<td>3</td>
<td>1 or more ulcerations</td>
</tr>
<tr>
<td></td>
<td><strong>Mesenteritis</strong></td>
</tr>
<tr>
<td>0</td>
<td>none/normal</td>
</tr>
<tr>
<td>1</td>
<td>mild infiltrate</td>
</tr>
<tr>
<td>2</td>
<td>moderate</td>
</tr>
<tr>
<td>3</td>
<td>severe</td>
</tr>
</tbody>
</table>
### Table 5. Challenge isolate toxinotypes and corresponding toxinotypes cultured from pigs.

<table>
<thead>
<tr>
<th>Challenge Isolate</th>
<th>Challenge Isolate Toxinotype</th>
<th>Toxinotype(s) cultured from pigs</th>
<th>n</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS6125</td>
<td>V</td>
<td>IIIb</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>0</td>
</tr>
<tr>
<td>13912-1</td>
<td>V</td>
<td>V</td>
<td>7</td>
</tr>
<tr>
<td>15454-1</td>
<td>V</td>
<td>V</td>
<td>6</td>
</tr>
<tr>
<td>02792-1</td>
<td>V</td>
<td>V</td>
<td>7</td>
</tr>
<tr>
<td>Controls</td>
<td></td>
<td>IIIb</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>V</td>
<td>3</td>
</tr>
</tbody>
</table>
Table 6. Mean score at necropsy for clinical signs, gross lesions, and microscopic lesions at three time points for each isolate.

<table>
<thead>
<tr>
<th>Isolate</th>
<th>Clinical signs</th>
<th>Gross lesions</th>
<th>Microscopic lesions</th>
<th>Clinical signs</th>
<th>Gross lesions</th>
<th>Microscopic lesions</th>
<th>Clinical signs</th>
<th>Gross lesions</th>
<th>Microscopic lesions</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS6125</td>
<td>0</td>
<td>2.5</td>
<td>0.5</td>
<td>2.5</td>
<td>5</td>
<td>4</td>
<td>2.5</td>
<td>8.5</td>
<td>10</td>
</tr>
<tr>
<td>13912-1</td>
<td>0</td>
<td>2.5</td>
<td>0</td>
<td>1</td>
<td>8.5</td>
<td>12</td>
<td>3</td>
<td>5</td>
<td>9</td>
</tr>
<tr>
<td>02792-1†</td>
<td>0.5</td>
<td>2.5</td>
<td>0.5</td>
<td>4</td>
<td>4</td>
<td>0</td>
<td>6</td>
<td>3.66</td>
<td>0.66</td>
</tr>
<tr>
<td>15454-1†</td>
<td>0.5</td>
<td>2.5</td>
<td>2</td>
<td>4</td>
<td>6</td>
<td>11.5</td>
<td>6.5</td>
<td>5.5</td>
<td>6.5</td>
</tr>
<tr>
<td>Control b1‡</td>
<td>1</td>
<td>2</td>
<td>1</td>
<td>0</td>
<td>7</td>
<td>13</td>
<td>1</td>
<td>2</td>
<td>2</td>
</tr>
<tr>
<td>Control b2‡</td>
<td>0</td>
<td>2</td>
<td>0</td>
<td>4</td>
<td>3</td>
<td>0</td>
<td>6</td>
<td>3</td>
<td>0</td>
</tr>
</tbody>
</table>

*Means of all categories significantly differed from those at 48 and 72 hpi.
† Differed significantly in microscopic lesion score means.
‡ Differed significantly from 15454-1 in microscopic lesion score means.

Table 7. Pooled data summary values of clinical sign or lesion presence for all pigs and isolate groups*.

<table>
<thead>
<tr>
<th>Pig Group</th>
<th>Mesocolonic edema</th>
<th>Toxin</th>
<th>Diarrhea</th>
<th>Neutrophilic Infiltration</th>
<th></th>
<th>Mucosal alterations</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Colon</td>
<td>Cecum</td>
<td></td>
<td>Colon</td>
<td>Cecum</td>
<td>Colon</td>
</tr>
<tr>
<td>Challenge (n=26)</td>
<td>19 (73%)</td>
<td>18 (69.2%)</td>
<td>15 (57.6%)</td>
<td>12 (46.1%)</td>
<td>13 (50%)</td>
<td>8 (30.7%)</td>
</tr>
<tr>
<td>Control (n=6)</td>
<td>2 (33.3%)</td>
<td>3 (50%)</td>
<td>2 (33.3%)</td>
<td>2 (33.3%)</td>
<td>1 (16.6%)</td>
<td>1 (16.6%)</td>
</tr>
</tbody>
</table>

*Differences between challenge and controls not significant
Appendix B. Tables (continued)

Table 8. Average colon and fecal toxin scores at each necropsy.*

<table>
<thead>
<tr>
<th>Isolate</th>
<th>24 hpi Colon</th>
<th>24 hpi Fecal</th>
<th>48 hpi Colon</th>
<th>48 hpi Fecal</th>
<th>72 hpi Colon</th>
<th>72 hpi Fecal</th>
</tr>
</thead>
<tbody>
<tr>
<td>JGS6125</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>2.5</td>
<td>3.5</td>
<td>2.5</td>
</tr>
<tr>
<td>13912-1</td>
<td>0</td>
<td>0</td>
<td>4</td>
<td>2</td>
<td>1</td>
<td>1.6</td>
</tr>
<tr>
<td>02792-1</td>
<td>1</td>
<td>0</td>
<td>1</td>
<td>1</td>
<td>0</td>
<td>1</td>
</tr>
<tr>
<td>15454-1</td>
<td>1</td>
<td>0</td>
<td>2</td>
<td>2</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Control b1</td>
<td>1</td>
<td>0</td>
<td>3</td>
<td>0</td>
<td>1</td>
<td>0</td>
</tr>
<tr>
<td>Control b2</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
<td>0</td>
</tr>
</tbody>
</table>

*Scores significantly correlated.
Works Cited


86. Weese JS, HE Weese, TL Bourdeau, HR Staempfli. (2001a) Suspected *Clostridium difficile*-associated diarrhea in two cats. JAVMA. 218(9):1436-1439


