Porcine Enteric Disease Caused by
Clostridium difficile and
Clostridium perfringens: Epidemiology,
Pathogenesis and Immunity

by

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SIGNED: Michael A. Anderson
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ABSTRACT

*Clostridium difficile* and *Clostridium perfringens* are among the most common agents of enteric disease in both humans and domestic animals. The former continues to increase in prevalence and diseases caused by the latter persist. Infection with a recently-emerged hypervirulent strain (NAP1/027/III) of *C. difficile* is increasingly common and serious sequelae and fatalities are much more common in these patients. In neonatal piglets, *C. difficile* infection (CDI) has become a common occurrence. Historically, isolation of *C. perfringens* type A from patients with enteric disease has been considered inconsequential due to its presence in the normal intestine and to the mild nature of disease syndromes such as porcine enteritis. However, both CDI and type A disease cause losses to the swine industry and pigs have been implicated as a possible source of *C. difficile* for infection in humans. We investigated the epidemiology and pathogenesis of porcine CDI, and immunity against porcine CDI and type A enteritis. The occurrence of CDI in integrated swine production facilities was most common in neonatal pigs. Infection in sows was rare, and finisher pigs were culture negative. All *C. difficile* strains were ribotype 078. Hypervirulent strain NAP1/027/TTIII was more virulent in neonatal pigs than both a historic human historic human strain and a porcine strain with toxin producing potential similar to ribotype 027 strains. Inoculation of anti-microbial-treated adolescent pigs with NAP1/027/III did not cause disease. Intra-gastric inoculation of pigs with purified TcdA resulted in severe small intestine damage which is uncharacteristic of natural disease; effects of TcdB were minimal. Passive immunization
of piglets against *C. difficile* TcdA or *C. perfringens* type A alpha (CPA) and beta 2 (CPB2) toxins did not prevent disease.
1. INTRODUCTION

1.1 Problem Definition

*Clostridium difficile* infection (CDI) has emerged as a leading cause of diarrhea in neonatal pigs. The vast majority of porcine strains are of ribotype 078 and > 90% produce toxins A (TcdA) and B (TcdB). In humans, there are indications that some strains are more virulent than others, but we know little of their unique virulence attributes. Multiple genetic-based methods [ribotyping, toxinotyping, pulsed-field gel electrophoresis (PFGE), microarray analysis, detection of binary toxin genes, and *tcdC* deletion analysis] have been used to create a database of human strain types; this has allowed identification and tracking of emergent hypervirulent strains. Very little is known about the transmission of *C. difficile* among different aged pigs in an integrated facility. CDI has not been described in grower or finisher pigs, but asymptomatic carrier pigs may be a reservoir for community-acquired human infection, and if CDI occurs in older pigs, it could be both a cause of economic loss and an additional source of organisms for contamination of pork products. More than 40% of retail pork products yield isolates of *C. difficile*, and the most likely source is contamination at slaughter by colonized pigs. The plausibility of immunoprophylactic control of CDI in pigs has not been reported, but immunization against TcdA and TcdB have demonstrated efficacy in humans and laboratory animals. Historically, *C. perfringens* has been the most important cause of clostridial enteric disease in domestic animals causing significant economic losses in many species. Use of commercial vaccines for diseases caused by *C.
perfringens types B, C, and D has significantly reduced mortality rates in some herds, but the currently available vaccines for protection from type A enteritis in pigs are conditionally licensed without proof of efficacy.

1.2 Objectives of the Current Research

We hypothesized that C. difficile remains associated with pigs from farrowing through finishing and can, under appropriate conditions, cause CDI in older pigs. We further hypothesize that passive immunization of piglets will protect against enteric disease caused by C. difficile and C. perfringens type A. To test these hypotheses, we proposed a plan of work based on the following objectives:

1. To determine prevalence of C. difficile strains in neonatal, nursery, grower, finishing, and breeding pigs,
2. to confirm the in vivo effects of TcdA and TcdB in piglets,
3. to assess the in vivo effects of C. difficile on neonatal and adolescent pigs,
4. to assess efficacy of anti-CDI and –C. perfringens type A passive immunity in piglets by immunization of dams.

1.3 Literature Review

1.3.1. Clostridium difficile Infection (CDI): Overview

Clostridium difficile is a Gram-positive, spore-forming, anaerobic bacillus which was first isolated from asymptomatic human infants and named Bacillus difficilis due to the difficulty involved in cultivation (Hall and O'Toole 1935). It was first associated with
human disease in 1978 (George, Sutter et al. 1978), and is currently the leading etiologic agent of nosocomial diarrhea (Moyenuddin, Williamson et al. 2002). *Clostridium difficile* infection (CDI), which has most often been associated with prior antimicrobial therapy, manifests as diarrhea and pseudomembranous colitis, and prolonged infections can result in paralytic ileus, toxic megacolon, bowel perforation and death (Harbarth, Samore et al. 2001). Detection of TcdA and TcdB in feces of clinical specimens confirms CDI diagnosis. Historically, hospital-associated CDI was the rule and community-associated disease the exception, but the latter is becoming more common. In addition to antimicrobials, drugs such as those which affect gastric acid production are now thought to predispose CDI, and may contribute to the frequency of community-associated CDI (DuPont, Garey et al. 2008). CDI has drawn considerable attention in recent years with the emergence of hypervirulent strains causing increased morbidity and mortality in the United States, Canada and Europe (McDonald, Killgore et al. 2005; Noren 2005).

CDI has also emerged as a common occurrence in pigs 1-7 days of age. Clinically, pigs present with yellow, pasty diarrhea though constipation or obstipation is common. (Waters, Orr et al. 1998; Songer, Post et al. 2000; Songer, Post et al. 2000a; Songer 2004; Songer and Anderson 2006). Small intestine is not affected, but mesocolonic edema accompanied by microscopic lesions in cecum and colon, is characteristic. The lesion consists of focal mucosal epithelial erosion or ulceration; neutrophil infiltration and exudation into the colonic lumen forms the hallmark “volcano lesion” of CDI. Accurate morbidity and mortality rates in the field are not available, but
TcdA and TcdB were detected in intestinal contents of 34% of ~1000 piglets with naturally-occurring CDI, and it was more prevalent than any other type of enteritis (Yaeger, Funk et al. 2002). Subsequent investigations revealed toxins in 50% of diseased piglets (Yaeger, Funk et al. 2002). Porcine CDI was first reported in pigs 8 weeks of age (Jones and Hunter 1983), but additional reports describing epidemiology or disease in older pigs are rare; antibiotic-associated fatal cases have been reported in periparturient sows (Kiss and Bilkei 2005).

1.3.2 Pathogenesis

As noted, the principal virulence factors of *C. difficile* are TcdA and TcdB. Toxin genes *tcdA* and *tcdB* reside in a 19.6 kb pathogenecity locus (*PaLoc*) with *tcdC* (a negative regulator of toxin production), *tcdD* (a positive regulator of toxin production), and *tcdE* (involved in toxin secretion). TcdA and TcdB belong to the family of large clostridial cytotoxins, which are characterized by a receptor binding domain at the carboxyl terminus and an enzymatic domain at the amino terminus (Schirmer and Aktories 2004). The enzymatic portions of TcdA and TcdB have similar substrate specificity, and inactivate Rho-subtype GTPases by monoglucosylation (von Eichel-Streiber, Meyer zu Heringdorf et al. 1995; Chaves-Olarte, Weidmann et al. 1997; Faust, Ye et al. 1998). The net effect is dysregulation of actin metabolism, with cell rounding and eventual death. TcdB has 1000x greater catalytic activity and is much more toxic for cultured cells than TcdA (Chaves-Olarte, Weidmann et al. 1997).
Toxins must gain entry to host cells for cytotoxic effects to manifest, and intoxication of host cells begins by receptor mediated endocytosis. Human infants often have *C. difficile* and its toxins in their stools at levels matching or exceeding those in specimens from individuals with disease, but they remain disease free because they lack receptors of mature colonocytes (Rolfe 1988). Cells of varying hosts are susceptible to TcdA, but receptors are not the same in all species. For example, TcdA binds to the carbohydrate Gal-α1-3β1-4GlcNAc-R (the so-called galactosyl epitope), but its distribution in porcine tissue is not consistent with brush border binding of TcdA (Keel and Songer 2007). Lesions in most species develop in cecum and colon; jejunal lesions occur in foals and rabbits, perhaps as a function of receptor distribution. TcdB is toxic to a variety of cultured cells, but receptor identification remains elusive. *In vivo*, TcdB does not bind to epithelial mucosa, and dogma has been that TcdB action must be preceded by mucosal epithelial damage induced by TcdA or other factors (Lyerly, Saum et al. 1985; Pothoulakis and Lamont 2001). Lack of TcdB enterotoxicity is confounded by isolation of *C. difficile* strains from clinically-ill humans which are TcdA negative but TcdB positive (Kato, Kato et al. 1998). Cellular intoxication may occur by alternative mechanisms such as pH-mediated channel formation in host cell lipid bilayers (Barth, Pfeifer et al. 2001). Perhaps TcdB emulsified in digesta could be endocytosed after the same manner as nutrients in the gut (Stange 1983).

A limited percentage of *C. difficile* strains produce a binary toxin (CDT). Among strains (n= 1,152) isolated from patients in Canada, 34% were CDT positive (Martin, Willey et al. 2008). In its intact active form, CDT is an AB-type toxin; *cdtA* encodes the
45 kDa enzymatic component and \( cdtB \) the 100 kDa binding component (Perelle, Gibert et al. 1997). Unlike other AB-type toxins, the enzymatic and binding portions of CDT are not covalently linked, but are both required for toxicity. The toxin’s ADP-ribosyltransferase activity on host cell actin results in disorganization of the cytoskeleton, with cell rounding and death. The role of CDT in pathogenesis of CDI is not known, but recently-emerged hypervirulent strains (NAP1/027/TTIII and ribotypes 078) are CDT positive, suggesting that it may contribute to increased virulence.

As noted, \( tcdA \) and \( tcdB \) are regulated negatively by \( tcdC \). Strains with partial deletions in \( tcdC \) (most 18 bp or 39 bp, but some 36 or 54 bp) have been isolated, and at least some of these have been thought to produce large amounts TcdA and TcdB (Spigaglia and Mastrantonio 2002). Strain NAP1/027/TTIII has an 18 bp deletion in \( tcdC \) and it produces 16-23 fold more toxin than other strains (Louie 2005). This may be due entirely or in part to the deletion in \( tcdC \).

CDI is initiated by colonization. Antibody titers against \( C.\) difficile surface layer proteins (SLPs), such as the flagellar cap protein FliD, flagellin FliC, and cell wall proteins Cwp66 and Cwp84, have been assessed in human patients. Diseased patients had significantly lower anti-SLP IgM antibody levels as compared to a non-CDI control group (Drudy, Calabi et al. 2004), and these reduced antibody titers can extend to TcdA and TcdB (Pechine, Janoir et al. 2005). These data suggest that immune responses to SLPs may play a role in CDI immunity and/or resistance to recurrence. Data from more direct experiments reveal that SLPs play a role in colonization by vegetative cells, but not by spores (Calabi, Calabi et al. 2002). Appendages which facilitate spore adherence have
been identified but not characterized (Panessa-Warren, Tortora et al. 1997).

Environmental populations of *C. difficile* persist as spores and ingestion of these can lead to colonization. However, not all such encounters with hosts, even by toxigenic strains, result in disease (Borriello 1998). Variability in spore colonization potential may affect the rate at which disease develops and persists.

1.3.3 Epidemiology

Traditional means of strain characterization, often based upon immunologic methods, are being replaced by genetic systems. Toxinotypes of *C. difficile* are derived from genetic polymorphisms within the *PaLoc* (Rupnik, Avesani et al. 1998), and ribotyping is based on sequence variation in the 16S/23S ribosomal intergenic sequence (Gurtler 1993). Pulsed-field gel electrophoresis (PFGE) classifies strains on the basis of polymorphisms in the locations of restriction sites of the whole genome. *tcdC* deletions in some strains contribute to the fingerprint, and binary toxin genes can be detected via PCR in a limited percentage of isolates (Spigaglia and Mastrantonio 2002; Goncalves, Decre et al. 2004). Most epidemiologic studies have relied on several methods, because, to date, no single system gives a complete reflection of strain virulence or transmission. However, ribotyping is used extensively due to its discriminatory power and relative simplicity (Brazier 2001). The recently-emerged epidemic strain is characterized as North American PFGE (NAP) type 1, ribotype 027, and toxinotype III (NAP1/027/TTIII). The strain has been isolated throughout the United States, Canada,
and Europe (Noren 2005). Thus far, these characteristics mainly occur together, suggesting recent amplification of the strain within the general population.

The emergence of epidemic strain NAP1/027/TTIII, and more recently ribotype 078, are of particular concern because of their association with increased morbidity and mortality. Both strains produce binary toxin and have deletions in \( tcdC \) (18 bp and 39 bp, respectively). Historically, hospital-associated CDI was the rule, and community-associated CDI the exception, but the latter is becoming more common (Kyne, Merry et al. 1998). Sources of infection are just being uncovered. Transmission of \( C.\ difficile \) from domestic animals to humans has not been convincingly documented; however, it has been isolated from a variety of household pets and domestic animals, and many of the strains are indistinguishable from those obtained from human disease (Borriello, Honour et al. 1983; O'Neill, Adams et al. 1993; Arroyo, Kruth et al. 2005; Keel and Songer 2006). Most swine isolates from one collection (83% of 119 isolates) were ribotype 078, and 7.5% of historical human strains were also ribotype 078 (Keel and Songer 2007; Goorhuis, Debast et al. 2008). Ribotype 078 has also been isolated from a variety of ready-to-eat beef, poultry, and pork products (Songer 2007). In the Netherlands, CDI due to infection with 078 strains was more commonly associated with community-acquired disease than were NAP1/027/TTIII infections. These data suggest the potential for transmission of \( C.\ difficile \) from pigs to humans.

1.3.4 Prophylaxis
The convalescent immune response, as well as experimental vaccination with toxoids, provides immunity against CDI or its recurrence in humans (Kotloff, Wasserman et al. 2001; Kyne, Warny et al. 2001). Circulating anti-TcdA IgG antibody titers are three times higher in asymptomatic carriers of *C. difficile* than in people with CDI (Kyne, Warny et al. 2000). Patients developing high convalescent IgG serum antibody levels were 48 times less likely to experience relapse (Kyne, Warny et al. 2001).

Human volunteers immunized with experimental toxoids developed serum IgG levels higher than those in convalescent patients (Kotloff, Wasserman et al. 2001), and disease resolved without recurrence in CDI patients vaccinated with TcdA and TcdB toxoids (Stavros, Kyne et al. 2005). In hamsters, parenteral immunization with TcdA and TcdA+TcdB toxoids protected against challenge, while TcdB monovalent toxoids did not (Kim, Iaconis et al. 1987). Animals were protected from mortality but not necessarily from morbidity. Evaluation of methods of immunization to prevent non-lethal diarrhea and mild intestinal pathology in hamsters revealed that intra-nasal and intra-peritoneal immunizations together produced full protection; both systemic and mucosal immunity may be needed (Torres, Lyerly et al. 1995). Passive protection can also be efficacious. Colostral IgG antibodies from toxoid immunized cows were efficacious against challenge when administered orally to hamsters (Lyerly, Bostwick et al. 1991; Kelly, Pothoulakis et al. 1996). Similar efficacy was observed with egg yolk-derived antibodies (IgY) (Kink and Williams 1998), although in this instance, anti-TcdA antibodies protected, and addition of anti-TcdB enhanced protection. Intravenous administration of monoclonal
antibodies against the binding domain of TcdA protected mice and hamsters from death and diarrhea (Corthier, Muller et al. 1991).

These data do not address the mechanisms or significance of mucosal colonization. As previously noted, antibody titers against \textit{C. difficile} SLP’s may contribute to disease prevention and recurrence. Anti-colonization immunity may also minimize \textit{C. difficile} concentrations in possible reservoirs of infection such as humans or pigs. Vaccine formulation strategies for both humans and domestic animals; for development of comprehensive immunoprophylactic disease management strategies, vaccine formulations should include SLP antigens.

1.4 \textit{Clostridium perfringens} type A

1.4.1 Overview

\textit{Clostridium perfringens} is perhaps the most widely-distributed pathogen in the world. It is consistently recovered from both the environment and the intestinal tract of warm-blooded vertebrates. As many as 17 exotoxins are produced by \textit{C. perfringens}, but strains are classified into five major types (A-E) based on production of one or more of the major toxins, alpha (CPA), beta (CPB), epsilon (ETX), and iota (ITX) (Brooks 1957; McDonel 1980; McDonel 1986). Type A strains may produce disease (Elder 1957; Mo’llby 1978; Smith 1979; Songer 1996), but are also common in the normal gut and the environment. Types B, C, D, and E, on the other hand are usually associated with diseased animals or proximal contaminated environments (Timoney 1988).
Due to its ubiquity, \textit{C. perfringens} type A has historically been considered normal flora and avirulent in the gut. Tissues collected ante- and post-mortem from healthy and diseased animals alike readily yield type A. Tissues collected at anything but a fresh post mortem can be overgrown with \textit{C. perfringens} type A, leading to recovery of high numbers of the organism. Caution is indicated in making a diagnosis of type A-associated disease on solely microbiological grounds.

1.4.2. Pathogenesis

\textit{Clostridium perfringens} type A produces no major toxins other than CPA, a phospholipase/sphingomyelinase C which is hemolytic, necrotizing, lethal, and is the primary virulence factor in a variety of disease syndromes (Elder 1957; Mo’llby 1978; Smith 1979; Songer 1996). Its role in pathogenesis of myonecrosis (gas gangrene) has been well characterized (Naylor, Eaton et al. 1998). Parenteral immunization of chickens with CPA toxoid significantly reduces the occurrence and severity of necrotic enteritis caused by \textit{C. perfringens} type A (Cooper, Trinh et al. 2008; Kulkarni, Parreira et al. 2008). Anemia, hemoglobinuria, and jaundice follow absorption of CPA from the gut in sheep with yellow lamb disease (McGowan 1958; Fleming 1985). Type A infection causes mild necrotizing enteritis, with villous atrophy and transient diarrhea in pigs 1-3 days of age (Collins, Bergeland et al. 1989; Johannsen 1993; Songer 1996; Songer unpublished data). Unlike disease in other species, a defined role for CPA in pathogenesis of porcine neonatal enteric disease has been elusive. Response of piglet gut loops to inoculation with CPA suggests that this toxin alone is not responsible for
enteritis (Popoff and Jestin 1985; Estrada-Correa 1988). However, its putative role in the
aforementioned disease syndromes has led to its being targeted as a protective antigen in
porcine enteritis.

Beta2 toxin (CPB2) was first identified in a type C strain from a piglet with
necrotizing enteritis (Gibert, Jolivet-Reynaud et al. 1997). The 28-kDa protein is toxic
for Chinese hamster ovary cells and causes mucosal necrosis in guinea pig ligated
intestinal loops. Epidemiologic surveys report that >90% of porcine type A strains from
diseased piglets are cpb2 positive, while prevalence in bovine and ovine strains may be
50% or less (Bueschel, Jost et al. 2003). The high prevalence of cpb2 in porcine strains
suggests a role for CPB2 in piglet enteritis, but this has not been definitively documented.

1.4.3 Prophylaxis

The potent actions of clostridal toxins are associated with rapid onset of disease
and acute death of the host; for example, the lethal dose of tetanus toxin is less than the
immunizing dose (Batty 1971); under these circumstances immunoprophylactic control
measures, rather than treatment strategies, are paramount. Clostridium perfringens types
B, C, and D cause significant economic losses in calves, pigs, and sheep (Songer 1996).
Type C necrotic enteritis can result in fatalities within 24 h postpartum, but passive
immunization by vaccination of dams can significantly reduce losses. Type A enteritis
also occurs in neonates, and immunoprophylactic control strategies are needed.
However, unlike diseases caused by C. perfringens types B, C, and D, protective
immunogens have not been identified.
1.5 Dissertation Format

The dissertation is organized into two main sections 1) an introductory chapter including problem definition, research objectives, scope of work, and literature review and 2) six appendices describing details of materials and methods, results, and conclusions, all in manuscript format in preparation for publication. Appendix F includes supplemental data, but it is not in manuscript format. The dissertation was written by the degree candidate, Michael A Anderson, and reviewed by coauthors of the manuscripts.

Appendix A describes the occurrence of Clostridium difficile infection (CDI) in an integrated swine facility. Currently, integrated swine production facilities have become cloistered, not by choice, but out of pressure from animal welfare enthusiasts and the need for biosecurity. Regular, and persistent, communication with an Arizona facility resulted in an open and productive working relationship. Collection of blood and fecal samples was organized by Michael A Anderson and the herd health veterinarian, and samples were shipped to The University of Arizona.

Appendix B describes inoculation of piglets with various strains of C. difficile. This experiment would not have been possible without preliminary disease modeling experiments which were orchestrated by Michael A Anderson. Methods developed here were applied to other infection trials.

Appendix C confirmed susceptibility of neonatal pigs to TcdA and TcdB. Toxins were purified and administered to pigs intragastrically (IG). Toxin purification and animal trials were organized and conducted by Michael A Anderson.
Appendix D addressed the plausibility of colostral passive transfer of antiCDI by vaccination of gestating dams with recombinant TcdA. The trial required acquisition of bred gilts and processing the animals through gestation, vaccinations, and farrowing. Aspects of the trial were orchestrated by Michael A Anderson.

Appendix E tested the immunizing potential of *C. perfringens* type A antigens (CPA, CPB2) against type A enteritis. Piglets were passively immunized by colostral transfer of antibodies from vaccinated dams. The trial required acquisition of bred gilts and processing the animals through gestation, vaccinations, and farrowing. Aspects of the trial were orchestrated by Michael A Anderson.

Appendix F reports preliminary results of immunohistochemistry and bacterial adherence assay data.
2. PRESENT STUDY

The methods, results and conclusions of the appended research are briefly discussed here. Details are described in each appendix.

Appendix A describes the occurrence of CDI in integrated swine facilities. We examined the distribution of \textit{C. difficile} infection in swine production facilities and assessed the potential for occurrence of CDI in adolescent pigs. Gestating sows had geometric mean TcdA and toxin B TcdB neutralizing titers of 0 and 1:16, respectively. Isolation of \textit{C. difficile} from feces sows was rare. A total of 3 strains were isolated from sows; one from gestating sow feces and 2 from postpartum fecal samples. However, 88% of litters farrowed by these sows were culture positive and TcdA and TcdB were detected in feces of 63% of litters. Only one isolate was recovered from nursery pig feces and none from grower or finisher pigs. All \textit{C. difficile} isolates were ribotype 078 and PFGE type NAP7. Adolescent pigs inoculated IG with toxigenic \textit{C. difficile} spores did not develop signs of disease.

Data presented Appendix B demonstrate the hypervirulence of \textit{C. difficile} human epidemic strain NAP1/027/TTIII in pigs. Animals were inoculated with spores of hypervirulent strain ribotype 027, porcine ribotype 078, or human historic ribotype 001. The organism was recovered from feces of all inoculates; toxins were detected in feces of all piglets inoculated with ribotypes 078 and 027 and in 25% of those inoculated with ribotype 001. Colitis was demonstrated in 80%, 14.3% and 0% of piglets challenged with ribotypes 027, 078, and 001, respectively. Thus, the human hypervirulent strain is also highly virulent in piglets. Lack of lesions and the low toxin positivity rate in piglets
inoculated with a historical strain of ribotype 001 may explain the uncommon occurrence
of non-078 strains in piglets in the US.

Data included in Appendix C demonstrate susceptibility of neonatal pigs to \emph{C. difficile} TcdA and TcdB administered IG. Piglets were inoculated intragastrically with TcdA and/or TcdB. Inoculation with TcdA alone or together with TcdB yielded catastrophic lesions in the jejunum. Synergy was observed when the two toxins were administered together. Lesions produced by TcdB alone were minor. The data demonstrates the exquisite susceptibility of pigs to purified toxins, but lesions are not consistent with clinical disease.

Appendix D includes data demonstrating lack of protection from CDI in piglets passively immunized with recombinant TcdA (rTcdA). Piglets (farrowed to vaccinated or non-vaccinated control sows) inoculated IG with spores developed diarrhea 1 day post challenge, but toxins were not detected in feces until two days post challenge. Lesions developed in both immunized and control piglets. These data suggest that the immune response elicited by vaccination with rTcdA was insufficient in nature or extent to abrogate disease. Day 1 post challenge diarrhea may have been due to alternate virulence factors such as binary toxin.

Data in Appendix E are derived from piglets farrowed to sows immunized against \emph{C. perfringens} type A antigens [alpha toxin (CPA), beta 2 toxin (CPB2)]. Piglets were not protected from challenge, but rather developed signs of disease ~12 h post IG inoculation with ~1x10^{10} CFU \emph{C. perfringens} type A. Diarrhea developed in immunized and control pigs alike. There were no differences in gross or microscopic lesions.
between immunized and control pigs. These data suggest that the immune response elicited by vaccination with CPA and/or CPB2 was insufficient in nature or extent to abrogate disease.

Appendix F; TcdB did not adhere to colonocytes of varying breeds or ages of pigs. Adherence the human hypervirulent strain vs a human historical strain were similar.
REFERENCES


APPENDIX A

*Clostridium difficile* in integrated swine production facilities

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Abstract

Historically, healthcare facility associated *Clostridium difficile* infection (HA-CDI) has been the rule and community-associated disease (CA-CDI) the exception. The latter is becoming more common, but the source of infection is not clearly defined. Porcine CDI manifests as diarrhea and typhlocolitis in piglets 1 - 7 days of age, and more than 90% of strains from multiple geographic locations in the US have been ribotype 078. Strains of this ribotype have now been isolated from retail pork products and from humans with CDI. Epidemiologic assessment of *C. difficile* colonization and occurrence of CDI within a defined population of pigs has not been reported. We examined the distribution of *C. difficile* infection in a single swine production facility and assessed the potential for occurrence of CDI in adolescent pigs. Gestating sows had geometric mean toxin A (TcdA) and toxin B (TcdB) neutralizing titers of 0 and 1:16, respectively. Isolation of *C. difficile* from feces of both gestating (1/74 positive) and postpartum (2/65) sows was rare. However, 88% of litters farrowed by these sows were culture positive and TcdA and TcdB were detected in feces of 63% of litters. One of 90 samples from different aged pigs, Nursery (n=30), grower (n=30), and finisher pigs (n=30), was culture positive. All *C. difficile* isolates were ribotype 078 and PFGE type NAP7. Adolescent pigs inoculated IG with toxigenic *C. difficile* spores did not develop signs of disease. These studies show that porcine CDI is most prevalent in the neonatal tier of pigs. Additional investigations are required to determine if there is risk for transmission to humans.
1. Introduction

*Clostridium difficile* is a Gram-positive, spore-forming, anaerobic bacillus which was first associated with human disease in 1978 (George, Sutter et al. 1978). Today, *Clostridium difficile* infection disease (CDI) is the leading cause of diarrhea in hospitalized individuals and is most often associated with prior antimicrobial therapy (Larson, Price et al. 1978). In addition to diarrhea, CDI can result in pseudomembranous colitis and, in more severe cases, toxic megacolon, bowel perforation, and death (Bartlett 1994). The primary virulence factors of *C. difficile* are toxins A (TcdA) and B (TcdB), members of the family of large clostridial toxins (Voth and Ballard 2005), and detection of toxins in stools is diagnostic. Historically, hospital-associated disease has been the rule and community-associated disease was the exception, but the latter is becoming more common (Cote 2008). Reservoirs of infection for CA-CDI remain elusive.

*Clostridium difficile* affects other species, causing enterocolitis in guinea pigs, typhlocolitis in adult horses, enterocolitis in foals, and sporadic diarrhea and gastrointestinal lesions in a variety of other animals, including dogs, cats, and, rarely, birds (Orchard, Fekety et al. 1983; Berry and Levett 1986; Weese, Weese et al. 2001; Magdesian, Hirsh et al. 2002). Porcine CDAD was first reported in 8 week-old pigs (Jones and Hunter 1983), but it has since emerged as a common occurrence in piglets 1-7 days of age (Jones and Hunter 1983; Waters, Orr et al. 1998; Songer, Post et al. 2000; Songer, Post et al. 2000a; Songer 2004).
Transmission of *C. difficile* among domestic animals and humans is not fully understood, but the organism has been isolated from a variety of household pets and food animals; many of the strains are indistinguishable from those found in humans (Borriello, Honour et al. 1983; O'Neill, Adams et al. 1993; Arroyo, Kruth et al. 2005; Keel and Songer 2006). Most swine isolates from one collection (83% of 119 isolates) were ribotype 078 (Keel, Brazier et al. 2007), which is increasingly common in humans (Goorhuis, Debast et al. 2008). These findings in company with the isolation of ribotype 078 from uncooked and ready to eat beef, poultry, and pork products (Songer 2007), suggest the potential for transmission of *C. difficile* from pigs to humans.

*Clostridium difficile* contamination of healthcare environments is often a consequence of shedding by diseased hosts (Ray, Hoyen et al. 2002; Donskey 2004). CDI epidemiology within animal populations is less well-understood. Porcine CDI manifests clinically almost exclusively in neonates, but older pigs could maintain *C. difficile* in herds via subclinical disease or asymptomatic shedding. Porcine CDI was first identified in pigs 8 weeks of age, but with the exception of peripartum disease in a sow (Kiss and Bilkei 2005), subsequent reports of disease in older pigs are rare. Recently, human disease associated with ribotype 078 strains has prompted further investigations, and *C. difficile* was found in pigs on two of three farms in the Netherlands (Rupnik, Widmer et al. 2008). We assessed the prevalence of *C. difficile* and CDI in a commercial swine production facility and investigated, on a preliminary basis, and susceptibility of older pigs to disease.
2. Materials and Methods

2.1 Epidemiology

2.1.1. Animals

Twenty-five gestating sows were selected for sample collection from each of three individual herds within an Arizona swine production facility (Herds I, II, and III). Selection was random, from a population with similar proximity of anticipated farrowing dates (~90 days gestation) and parity. Herd I animals consisted of gilts while sows from Herds II and III were multiparous. Groups, each comprising 30, of 30 nursery (6-10 wks old), grower (16-19 wks old), and finisher (19-22 wks old) pigs were sampled at random.

2.1.2. Sample Collection

Freshly-voided feces was collected from each gestating sow (~90 days gestation) via sterile tongue depressor, placed into 50 ml screw-capped tubes, shipped the laboratory on wet ice, and stored frozen at -20°C until tested. Sows were sampled again 2-3 days postpartum, at which time, fecal samples were also obtained by rectal swab (Stool Prep, TechLab, Blacksburg, VA) from five randomly-selected nursing piglets in each litter. Swabs were shipped and stored as above until testing. Nursery, grower, and finisher pigs were sampled once, as described for sows. Blood was collected from gestating sows at the time of fecal sampling, and serum stored at -20°C until testing.

2.1.3. Serology
Neutralizing antibodies (against TcdA and TcdB) were titrated via an assay in Chinese hamster ovary (CHO) cell monolayers. CHO cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Cambrex, Walkersville, MD) with 10% fetal bovine serum (Intergen, Purchase, New York) and 100 µg gentamicin per ml (Sigma-Aldrich, St. Louis, MO). For toxicity assays, cells were trypsinized and washed, and 2 x 10^4 cells placed in each well of 96-well tissue culture plates (Corning, Corning, NY). After overnight incubation at 37°C in an atmosphere of 5% CO\textsubscript{2}, 100 µl aliquots of serially diluted purified TcdA or TcdB were added to each well. Monolayers were incubated for an additional 18 h, after which the cells were fixed, stained with trypan blue, and examined microscopically. The endpoint was reported as the reciprocal of the last dilution causing 50% cytopathic effect.

For serum neutralization assays, serial, two-fold dilutions of serum samples were made in a 96-well microtitration plate. TcdA or TcdB (~20 100% tissue culture toxic doses) were added to each well and incubated at room temperature for 1 h. Aliquots of serum/toxin mixtures were transferred to CHO cell monolayers prepared as above and plates incubated overnight as described. Monolayers were fixed, stained, and examined microscopically to determine endpoint titers (the last dilution to prevent 100% cytotoxicity).

2.1.4. Bacteriologic culture

Fecal material (1 g), or rectal swabs were heat shocked (80°C, 10 min) and placed into pre-reduced TMNF broth [40 g Proteose Peptone (Becton Dickinson, Sparks, MD), 6
g fructose, 5 g Na₂HPO₄, 2 g NaCl, 1 g KH₂PO₄, 1 g taurocholic acid, 0.12 g MgSO₄, and 1 ml of 1% neutral red in 95% ethanol; moxalactam and norfloxacin were added to final concentrations of 12 µg/ml and 32 µg/ml, respectively. Broth cultures were incubated anaerobically (5% H₂, 5% CO₂, 90% N₂) at 37°C for 7 days and then ethanol-shocked by diluting 1:2 in 95% ethanol and incubating at room temperature for 30 min. After centrifugation at (2000 x g, 30 min, 25°C) the pellet was plated onto pre-reduced TMNF agar [TMNF broth with 2% technical grade agar (Becton Dickinson)] and incubated anaerobically at 37°C for 48 h. Preliminary identification of C. difficile was by colony morphology, including fluorescence under UV light, and confirmatory testing was by PCR amplification of cdd3 (Braun, Hundsberger et al. 1996)

2.1.5. Toxin Detection

Fecal samples from nursing piglet swabs were tested for TcdA and TcdB via commercial EIA test kit (Tox A/B, TechLab, Blacksburg, VA).

2.1.6. Strain Characterization

Strains were characterized by pulsed-field gel electrophoresis (PFGE) and ribotyping. PFGE was carried out according to the protocol of the Centers for Disease Control and Prevention (Klaassen, van Haren et al. 2002; McDonald, Killgore et al. 2005). Strains were grown for 48 h at 37°C under anaerobic conditions on C. difficile selective agar (CDSA) plates [37 g brain heart infusion broth, 20 g technical grade agar
(Becton Dickinson) and 1 g taurocholic acid in 1 L H$_2$O was supplemented with 5% citrated bovine blood and D-cycloserine and cefoxitin for final concentrations of 250 µg/ml and 10 µg/ml, respectively. A single colony was inoculated into 0.5 ml PYG broth [10 g tryptone, 5 g yeast extract (Becton Dickinson), 10 g dextrose, 1 L H$_2$O] and incubated at 37°C for 7 h under anaerobic conditions. Cultures were centrifuged (10,000 x g, 5 min), the pellet resuspended in 150 µl Gram positive lysis buffer (6mM Tris-HCl pH 8.0, 1M NaCl, 100mM EDTA pH 8.0, 0.5 % Brij-58, 0.2% sodium deoxycholate, 0.5% sodium laurysarcosine, 20mg RNase/ml and 10 mg lysozyme/ml) and incubated at 37°C for 5 min. Molten 2% agarose (150 µl) was added to the culture suspension, allowed to harden, and added to 3 ml lysis buffer (1.6 ml of 10 mg/ml RNAse, 4.4 ml of 100 mg/ml lysozyme, 40 ml Gram positive lysis buffer) and incubated overnight at 37°C. Lysis buffer was aspirated, replaced with ESP buffer (3 ml EDTA pH 8.0, 10 mg SDS/ml, 1 mg proteinase K/ml) and incubated overnight at 56°C. After washing the plug five times with HPLC-water, DNA was restricted overnight with SmaI. Pulsed-field gel electrophoresis parameters (gradient, 6; run time, 18 h; included angle, 120; int. Sw. time, 5 sec; fin. Sw. time, 40 sec; ramping factor, linear), (Klaassen, van Haren et al. 2002; McDonald, Killgore et al. 2005).

Ribotyping was conducted as described (Rupnik, Brazier et al. 2001). The PCR reaction mixture contained 5 U of Taq polymerase (Promega), 1.5 mM MgCl$_2$, and 100 pmol of each primer in a total volume of 100µl. Primers were RiboF (CTGGGGTGAAAGTCGTAACAAGG) and RiboR (GCGCCCTTTGTAGCTTGACC). Several colonies of *C. difficile*, cultivated on CDSA, were suspended in 100µl of 5%
Chelex (Bio-Rad), boiled for 10 min, and centrifuged at 17,000xg for 10 min. An aliquot (10µl) of supernatant was used as template. The PCR protocol was started with a 3 min denaturation step at 93°C, followed by 30 cycles at 52°C for 1 min, 72°C for 1 min, and 93°C for 1 min. The reaction ended with a 10 min incubation at 72°C. PCR products were separated electrophoretically in a 3% NuSieve agarose gel, run at 150 mV for 6 h. Every fifth lane in the gel had a 100bp ladder for accurate interpretation of band sizes.

2.2. Infection Trial

2.2.1. Animals/Groups

Thirty ~70 lbs. pigs were procured and randomly assigned to Groups I and II (n=15 per group). Group I pigs received two daily (200 mg) oral doses of moxifloxacin prior to spore inoculations and concurrently with spores for an additional three days; pigs were euthanized (by barbiturate overdose) one day after the last spore inoculation. In Group II, moxifloxacin treatment was discontinued after three days, at which time, daily spore inoculations were initiated and continued for three more days; animals were euthanized and necropsied three days after the last spore inoculation. Pigs were housed and fed antibiotic-free feed in accordance with guidelines of the Institutional Animal Care and Use Committee of The University of Arizona.

2.2.2 Inocula and Challenge
Strain JGS6125 was inoculated onto CDSA and plates were incubated under anaerobic conditions (5% H₂, 5% CO₂, 90% N₂) at 37°C for 48 h. A single colony was passed to multiple antibiotic-free BHI-CYE agar plates [37 g brain heart infusion broth, 5 g yeast extract, 20 g technical grade agar (Becton Dickinson), and 0.5 g cysteine HCl], which were incubated as before. The resulting colonies were collected on sterile cotton swabs, streaked onto BHI-CYE plates (~300) to achieve a lawn of growth and incubated anaerobically at 37°C for 7 days. Spores collected by washing each plate with 5 ml PBS were harvested by centrifugation (10,000x g, 20 min). The pellet was washed with 180 ml of 1M KCl:0.5 M NaCl and then resuspended in 100 ml 50 mM TrisHCl, pH 7.2:10 mg lysozyme per ml and incubated at 37°C for 1 h. Spores were washed three times with 100 ml of HPLC-grade water and 5 ml aliquots (~1x10⁹ CFU/ml) were stored at 4°C. Germination was induced by heat shock (80°C, 10 min), followed by addition of 5 ml CDS broth (CDSA without agar) and incubation at 37°C for 30-60 min prior to IG inoculation of pigs. Pigs received ~5x10⁹ CFU/inoculation.

2.2.3. Observations

Pigs were observed daily for signs of disease and diarrhea was rated 0-3 (0, normal; 1, yellow formed stools, perineum dry; 2, yellow, fluid feces, perineum irritated and caked with feces; 3, pig constipated or obstipated.

Rectal swabs (Stoolprep) were collected daily beginning two days before treatment with moxifloxicin, heat shocked (80°C, 10 min), streaked for isolation on CDSA, and incubated anaerobically at 37°C for 48 h. Extent of growth of C. difficile was
rated 0-4, based upon the last quadrant with colonies. *Clostridium difficile* was also enumerated (CFU/g feces) by plating dilutions of colonic contents on CDSA plates. Swabs were also tested for the presence of toxins (Tox A/B, Techlab).

Pigs in Groups I and II were euthanized and necropsied 1 and 3 days after the last spore inoculation, respectively. Gross signs of disease were recorded and intestinal tissues were collected, fixed in 10% buffered formalin, embedded in paraffin and processed for staining with haematoxylin and eosin (H&E). H&E stained sections were examined by light microscopy for lesions consistent with CDI.

### 3. Results

#### 3.1. Epidemiology

Among gestating sows and postpartum pigs, *C. difficile* was isolated most commonly from neonates. *In toto*, 140/323 (43%) piglets and 57/65 (88%) litters were culture positive. There were no differences in culture positivity between farms for individual pigs (Farm I, 42/105, 40%; Farm II, 54/110, 49%; Farm III, 44/108, 41%) or litters (Farm I, 19/21, 90%; Farm II, 20/22, 91%; Farm III, 18/22, 82%). Toxins were detected in feces of 98/323 (30%) nursing piglets and in 41/65 (63%) of litters. Farms I, II and III had 36/105 (34%), 32/110 (29%) and 30/108 (28%) toxin positive piglets; 57% (12/21), 73% (16/22) and 59% (13/22) of litters were positive, respectively (Figure 1).
No sow had a serum neutralizing titer against TcdA, but a comprehensive geometric mean anti-TcdB titer of 1:16 was within a 2-fold dilution of group means (geomeans; Farm I, 1:29; Farm II, 1:24; Farm III, 1:13.8). Sows were placed into one of three groups based on anti-TcdB titer to see if there was an association between titer and CDI, and titer did not affect CDI prevalence (Figure 2). Prevalence of CDI was also constant among sows of differing parity (Figure 3).

Isolation of *C. difficile* from sows, and feeder pigs was much less frequent. A total of 3 strains were isolated from sows; one from gestating sow feces and 2 from postpartum fecal samples. Only one isolate was recovered from nursery pig feces and none from grower or finisher pigs. All the sow and nursery pig isolates and 35/140 piglet isolates were ribotype 078 and NAP type 7.

### 3.2. Infection Trial

None of the pigs developed signs consistent with CDI. Neither diarrhea nor constipation was observed. Feces from three animals in Group I were mildly toxin positive on the first day after spore inoculation. All other samples were EIA negative. Toxin was detected in cecum and colon from one animal in Group II at post-mortem, but all others were negative. No pig exhibited mesocolonic edema or microscopic lesions characteristic of CDI.
Rectal swab samples collected from Group I pigs yielded large numbers of *C. difficile (4+)* throughout the observation period, and ~1x10⁶ CFU were detected in colonic contents. Shedding was significantly less in Group II pigs, rectal swab samples were sporadically and minimally (1+ and 2+) positive, and *C. difficile* was not detected in dilution platings.

5. Discussion

Our results define CDI occurrence throughout all stages of swine production in multiple herds within a single production system. Dogma suggests that dams serve as the reservoir of infection for nursing pigs, and this would account for disease manifestation in pigs <1 day of age. However, out of 75 gestating and 65 postpartum sow fecal samples tested, only 3 were culture positive. Never-the-less, *C. difficile* was cultured from 88% of litters tested and CDI diagnosis by way of toxin detection indicated a 63% morbidity rate. These data may be a reflection of the insensitivity and/or specificity of the culture methods. Enrichment broth containing antimicrobials and defined nutrients could select for limited phenotypes.

Vaccination/challenge trial pigs were pre-disposed to infection by treatment with moxifloxacin but neither control nor vaccinated pigs developed disease. Furthermore, pigs in Group II became culture negative upon cessation of spore inoculations. However, these results do not conclusively demonstrate that older pigs are insusceptible to CDI. Historically, antibiotic-associated CDI is preferential toward use of cephalosporin
derivatives and fluorquinolones. While moxifloxacin is a fluoroquinilone, it may not be the antibiotic of choice for disease pre-disposition in adolescent pigs. Dogma suggests that antibiotic predisposition to CDI is via elimination of normal flora competitors (Wilson and Perini 1988). Results of recent work suggest that antimicrobials may actually function in CDI by eliminating bile-salt metabolizing flora, resulting in an increased rate of germination of \textit{C. difficile} spores (Sorg and Sonenshein 2008).

Treatment of pigs with an antibiotic which targets porcine bile-salt metabolizing flora could possibly induce CDI in older pigs. Disease in pigs was first reported in 8 week old animals (Jones and Hunter 1983), but with the exception of a report of CDI in sows (Kiss and Bilkei 2005), additional reports in older pigs are rare. Antibiotics are routinely used for prophylaxis and therapy in the swine industry, so if pigs are susceptible to antimicrobial-associated CDI, spontaneous cases may occur. If so, then accompanying disease symptoms such as diarrhea would contribute to widespread dissemination of the organism and amplification of the reservoir of infection. Antimicrobial-associated disease or shedding in older pigs may go unrecognized or undiagnosed.

These data suggest that \textit{C. difficile} is confined mostly to the neonatal tier of production animals. However, samples cultured from other production facilities have yielded results differing from those reported here. In some herds, nursery pigs, grower pigs and sows were highly positive (data not shown). In other herds, sows and grower pigs were mostly negative (data not shown). With the emergence of ribotype 078 as an agent of disease in humans and its isolation from retail pork products, the possibility that
slaughter pigs are a significant source of post-harvest contamination requires further investigation.
Figure 1. Farm I, n=21; Farm II, n=22; Farm III, n=22.
Figure 2. Dams were separated into three groups based on anti-TcdB titers. Sows with titers of 1:4 or 1:8 had a geometric mean titer of 1:7. A geometric mean of 22 was calculated from sows with titers of 1:16 or 1:32 and sow with titers of 1:64 were grouped together.
Figure 3. First parity sows, n=25; parity 2-4, n=28; parity 5-8, n=12.
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References


APPENDIX B

Response of Neonatal Pigs to the Human Hypervirulent Strain of *Clostridium difficile*

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Abstract

The rate of serious disease caused by *Clostridium difficile* is low in humans infected by “historical” strains (representative PCR ribotype 001), but morbidity and mortality are abnormally high in those affected by a recently-emerged “hypervirulent” strain (PCR ribotype 027). Furthermore, strains of ribotype 078 have emerged as a leading cause of neonatal pig diarrhea in the US and elsewhere, and as important causes of serious human disease in some places. The main virulence attributes of these strains are presumed to be toxins A (TcdA) and B (TcdB), with relative production by 027 > 078 > 001. We inoculated piglets with ~4 x 10⁹ germinating spores of strains of these three ribotypes. *Clostridium difficile* was recovered from feces of all inoculates; toxins were detected in feces of all piglets inoculated with ribotypes 078 and 027 and in 25% of those inoculated with ribotype 001. Colitis was demonstrated in 80%, 14.3% and 0% of piglets challenged with ribotypes 027, 078, and 001, respectively. Without any known host susceptibility attributes, the human hypervirulent strain is also relatively highly virulent in piglets.
1. Introduction

*Clostridium difficile* is an anaerobic, Gram-positive, spore-forming bacillus which has become the leading cause of nosocomial diarrhea in humans (Gursoy, Guven et al. 2007). *Clostridium difficile* infection (CDI) manifests as diarrhea, pseudomembranous colitis, and rarely, paralytic ileus, toxic megacolon, bowel perforation, and death (Harbarth, Samore et al. 2001; Nicolasora and Kaul 2008). Disease often occurs in association with antimicrobial usage (Larson, Price et al. 1978).

CDI has also emerged as a common syndrome in pigs 1-7 days of age (Songer, Post et al. 2000). Pigs present with yellow, pasty diarrhea, though constipation or obstipation is also common (Waters, Orr et al. 1998; Songer, Post et al. 2000; Songer, Post et al. 2000a; Songer 2004; Songer and Anderson 2006). Small intestine is not affected, but mesocolonic edema, accompanied by microscopic lesions in cecum and colon, is characteristic. The lesion consists of focal mucosal epithelial erosion or ulceration; neutrophil infiltration and exudation into the colonic lumen forms the hallmark “volcano lesion” of CDI. Disease has been reproduced in piglets by intragastric (IG) inoculation with *C. difficile* (Songer, Post et al. 2000).

The principal virulence factors of *C. difficile* are toxins A (TcdA) and B (TcdB), which belong to the family of large clostridial cytotoxins. They are characterized by a receptor binding domain at the carboxyl terminus and an enzymatic domain at the amino terminus (Schirmer and Aktories 2004). The mechanisms of cytotoxicity of TcdA and
TcdB are the same; after entry via receptor-mediated endocytosis, they inactivate Rho-subtype GTPases by monoglucosylation (von Eichel-Streiber, Meyer zu Heringdorf et al. 1995; Chaves-Olarte, Weidmann et al. 1997; Faust, Ye et al. 1998). The net effect is dysregulation of actin metabolism, with cell rounding and death.

The carbohydrate Galα1-3Galβ1-4GlcNAc has been identified as a receptor for TcdA, but it is not present in all host species (Krivan, Clark et al. 1986; Tucker and Wilkins 1991). The TcdB receptor has not been identified, but evidence suggests that it occurs widely, although not ubiquitously. TcdB is toxic for a variety of cell types, and has, in fact, 1000x more catalytic activity than TcdA (Voth and Ballard 2005). However, stools from human infants often contain large amounts of toxins (Rolfe 1988), without producing lesions; this is due to a putative lack of toxin receptors. Lesions from toxins occur in colon and cecum in most species; occurrence of lesions in jejunum of foals and rabbits may be due to a unique receptor distribution (Keel and Songer 2006). In neonatal pigs, TcdA, but not TcdB, binds to intestinal brush borders of neonates (Keel and Songer 2007).

A variety of methods are used for genetic characterization of C. difficile strains (Rupnik, Avesani et al. 1998; Brazier 2001; Spigaglia and Mastrantonio 2002). Pulsed-field gel electrophoresis (PFGE) has been widely used to compare strains from outbreaks and hosts (Cote 2008). Toxinotypes are derived from genetic polymorphisms within the pathogenecity locus (PaLoc) (Rupnik, Avesani et al. 1998). Deletions in tcdC, a negative regulator of toxin production, may result in increased production of TcdA and TcdB (Spigaglia and Mastrantonio 2002). Binary toxin (CDT) is not found in historical strains,
but is produced by strains from pigs and calves (Keel, Brazier et al. 2007), and many human strains; its role in pathogenesis, if any, is unknown (Geric, Johnson et al. 2003).

PCR-ribotypes are based upon polymorphisms in the spacer between 16S and 23S ribosomal RNA genes (Brazier 2001). More than 180 ribotypes have been described, and this technique is used extensively (Stubbs, Brazier et al. 1999). To date, no single typing system provides results congruent with pathotype, and most epidemiologic studies have relied on concurrent use of several methods to produce a fingerprint. Strains historically associated with human CDI are of various North American pulsed-field types (NAP), ribotype 001, toxinotype 0, have an intact $tcdC$, and fail to produce binary toxin. The fingerprint of the recently-emerged human epidemic strain is NAP1, ribotype 027, and toxinotype III; it produces binary toxin and has an 18 bp deletion in $tcdC$ (Noren 2005). Most porcine strains are NAP7, ribotype 078, toxinotype V, CDT positive and have a 39 bp deletion in $tcdC$ (Keel, Brazier et al. 2007).

Severity of CDI may be a function of host susceptibility and/or strain virulence. CDI in humans has been known for 30 years, but it has apparently become a problem only recently in pigs (Songer, Post et al. 2000). This may be due to delayed exposure of pigs to the organism, failure to recognize the disease in pigs prior to the late 1990s, or to emergence of strains virulent for pigs. Susceptibility of the pig may also be affected by the availability of toxin receptors, in that those for TcdB apparently do not exist in neonates (Keel and Songer 2007), at least not in those of today’s predominant genetic makeup. Other breeds may have receptors for both toxins, or neither; if the latter proves true it may be that genetic manipulation of pigs for enhanced productivity has brought
with it the TcdA receptor and susceptibility to CDI. We assessed aspects of the interaction between piglets and historical, human hypervirulent, and porcine *C. difficile* strains by inoculation of neonatal pigs and characterization of their responses.

2. Materials and Methods

2.1. Pigs and Inoculation

Historic human strain JGS667 (ribotype 001), hypervirulent strain JGS6125 (ribotype 027), and porcine strain JGS725 (ribotype 078) (Table 1) were cultivated on BHI-CYE agar [37 g brain heart infusion broth, 5 g yeast extract, 20 g technical grade agar (Becton-Dickinson), and 0.5 g cysteine HCl] and incubated under anaerobic conditions (5% H$_2$, 5% CO$_2$, 90% N$_2$) at 37°C for 48 h. Growth from multiple plates was harvested onto sterile cotton swabs and used to produce lawn growth on BHI-CYE plates (n~300), which were incubated as before, but for 7 days. The lawn was harvested by washing with 5 ml PBS. After centrifugation (10,000 x g, 20 min), the pellet was washed with 180 ml 1 M KCl: 0.5 M NaCl, resuspended in 100 ml 50 mM Tris-HCl, pH 7.2 with 10 mg lysozyme per ml, and incubated at 37°C for 1 h. Spores were washed three times with 100 ml HPLC-grade water and 2 ml aliquots (~2x10$^9$ CFU/ml) were stored at 4°C.

For use, germination was induced by heat shock (80°C), followed by addition of 0.5 ml of *Clostridium difficile* selective broth [CDS broth; 37 g brain heart infusion broth (Becton Dickinson) and 1 g taurocholic acid in 1 L HPLC-grade water supplemented with 50 ml of fetal bovine serum and D-cycloserine and cefoxitin stock solutions at final
concentrations of 250 µg/ml and 10 µg/ml, respectively, after autoclaving] and incubation at 37°C for 30-60 min prior to inoculation of piglets. Colostrum deprived piglets (n=21) were inoculated IG with ~4x10^9 respective spores (ribotype 001, 4 pigs; ribotype 027, 10 pigs; ribotype 078, 7 pigs) within ~1 h of birth and again 4 h later. Pigs were housed and fed (Esbilac™ milk replacer, Pet-Ag, Hampshire, IL) in accordance with guidelines of the Institutional Animal Care and Use Committee of The University of Arizona.

Table 1. Strain summary.

<table>
<thead>
<tr>
<th></th>
<th>Human Epidemic</th>
<th>Human Historical</th>
<th>Porcine</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>JGS6125</td>
<td>JGS667</td>
<td>JGS725</td>
</tr>
<tr>
<td>Ribotype</td>
<td>027</td>
<td>001</td>
<td>078</td>
</tr>
<tr>
<td>Binary Toxin Genes</td>
<td>Pos</td>
<td>Neg</td>
<td>Pos</td>
</tr>
<tr>
<td>Motility</td>
<td>Pos</td>
<td>Pos</td>
<td>Neg</td>
</tr>
<tr>
<td>tcdC Deletions</td>
<td>18 bp</td>
<td>None</td>
<td>39 bp</td>
</tr>
<tr>
<td>Toxin Production</td>
<td>++++</td>
<td>+</td>
<td>++</td>
</tr>
</tbody>
</table>

2.2. Observations

Piglets euthanized via barbiturate overdose on the second post inoculation day were necropsied, and extent of mesocolonic edema was rated 0-3 (Table 2). Segments of small intestine and colon were fixed in 10% buffered formalin, trimmed, embedded in paraffin, sectioned (5µm), and stained with haemotoxylin and eosin (H&E). Microscopic lesions in colon were rated 0-5 (Table 2). Rectal swabs collected daily and colon
contents collected at post-mortem were examined by bacteriologic culture on *Clostridium difficile* selective agar [CDSA; 37 g brain heart infusion broth, 20 g technical grade agar (Becton Dickinson) and 1 g taurocholic acid in 1 L H₂O; 5% citrated bovine blood, D-cycloserine (250µg/ml final concentration) and cefoxitin (10µg/ml final concentration); incubation under anaerobic conditions at 37°C for 48 h] and toxin testing via commercial EIA test kit (Tox A/B, TechLab, Blacksburg, VA).

Table 2. Gross lesion scores.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No mesocolonic edema</td>
</tr>
<tr>
<td>1</td>
<td>Mild mesocolonic edema</td>
</tr>
<tr>
<td>2</td>
<td>Moderate mesocolonic edema</td>
</tr>
<tr>
<td>3</td>
<td>Severe mesocolonic edema</td>
</tr>
</tbody>
</table>
Table 3. Microscopic lesion scores.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No coagulative necrosis; small number of scattered apoptotic cells permissible</td>
</tr>
<tr>
<td>1</td>
<td>Multifocal necrosis affecting many cells or small islands of epithelial cells at luminal surface</td>
</tr>
<tr>
<td>2</td>
<td>Similar to 1, except with neutrophil infiltration (inflammation)</td>
</tr>
<tr>
<td>3</td>
<td>Multifocal coagulative necrosis of superficial epithelium with erosions/ulcers or inflammation</td>
</tr>
<tr>
<td>4</td>
<td>Extensive, coalescing ulceration/necrosis of superficial mucosa +/- extension into crypts</td>
</tr>
<tr>
<td>5</td>
<td>Necrosis of mucosal epithelium and deeper structures in lamina propria with inflammation</td>
</tr>
</tbody>
</table>

3. Results

Apparent differences in virulence between strains were revealed by results of in vivo testing. All pigs were, as expected, culture positive after inoculation. Toxins were detected in feces collected ante- and/or post-mortem from all pigs inoculated with NAP1/027/TTIII and porcine ribotype 078, but in only 25% (1/4) of pigs inoculated with the historic strain. Mesocolonic edema was present in 80%, 86% and 25% of pigs inoculated with hypervirulent, porcine and historic strains, respectively. Microscopic
lesions were present in 80% NAP1/027/TTIII inoculated pigs, but in only 14% of pigs inoculated with ribotype 078; lesions were absent from pigs inoculated with the historic strain (Figure 1).

4. Discussion

The hypervirulence of strain NAP1/027/TTIII is primarily attributable to increased toxin production, putatively resulting from a deletion in \textit{tcdC}, the negative regulator of TcdA and TcdB production (McDonald, Killgore et al. 2005; Warny, Pepin et al. 2005; Kuijper, Coignard et al. 2006). Production of binary toxin may also increase virulence, and its role in pathogenesis is of infections should be investigated (Goncalves, Decre et al. 2004). There are clear genotypic and phenotypic differences among strains JGS6125, JGS 667, and JGS725 (Table 1), and the relative hypervirulence of strain JGS6125 was demonstrated via piglet inoculation. Elevated toxin production \textit{in vivo} was evident, based upon detection of TcdA and TcdB in 100% of piglets inoculated with NAP1/027/TTIII and porcine strain JGS725, but in only 25% of pigs inoculated with the historic strain. The porcine strain, like the hypervirulent strain, has a deletion in \textit{tcdC}, which may explain the equivalent number of toxin-positives among piglets inoculated with these two strains. However, lesion production was more common in piglets inoculated with the NAP1/027/TTIII strain (80%) than those inoculated with the NAP7/078/TTV strain (14%). These results demonstrate the relative hypervirulence of NAP1/027/TTIII, but do nothing to reveal the mechanisms by which virulence has been
increased. Differences in ability to colonize may be important, and a role for CDT and other potential virulence factors should be a focus of future research.
Figure 1. A compilation of culture, toxin positivity, gross and microscopic lesion data was used to diagnose piglet CDI.
Acknowledgments

The authors would like to gratefully acknowledge Hien Trinh for technical guidance and administrative support. Assistance with animal and sample processing from Micheal Hailey was greatly appreciated.

References


APPENDIX C

Effect of Clostridium difficile toxins A and B administered intragastrically to neonatal pigs

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Abstract

*Clostridium difficile* infection (CDI) has become a common diagnosis in neonatal pigs in many swine-producing areas, in North America and elsewhere. Disease manifests clinically as diarrhea in pigs 1-7 days of age, although constipation and obstipation are also common. Gross lesions often include mesocolonic edema accompanied by focal neutrophil infiltration and mucosal epithelial erosion or ulceration. Toxins A (TcdA) and B (TcdB) are major virulence factors. We examined the effects of purified TcdA and TcdB in neonatal pigs inoculated intragastric (IG). Piglets became diarrheic within 3 h. Post mortem examination revealed effusion into the small intestine. Diffuse necrosis of superficial small intestine mucosa was unlike the findings in natural and experimental infections with *C. difficile*. Mild mesocolonic edema was observed, and cecal and colonic mucosal necrosis was focal, as in natural and experimentally-induced disease. Small intestinal lesions were most severe when TcdA was administered alone or in combination with TcdB. Synergistic effects were observed by inoculation with reduced amounts of both TcdA and TcdB, resulting in lesions similar to those caused by higher doses of TcdA alone. Lesions caused by TcdB were less severe and more focal in small intestine, and cecum and colon. These results confirm the susceptibility of pigs to the effects of *C. difficile* toxins and the potential for TcdA/TcdB synergy.

*Keywords:* *Clostridium difficile*; toxins A (TcdA) and B (TcdB); neonatal pigs; intragastric inoculation
1. Introduction

*Clostridium difficile* is a Gram-positive to Gram-variable, rod-shaped, spore-forming obligate anaerobe. It is a leading cause of antibiotic-associated diarrhea and colitis in humans and is often encountered as a nosocomial pathogen among the elderly (Harbarth, Samore et al. 2001). It is also an important cause of disease in laboratory animals and other domestic species. Clindamycin-treated hamsters are especially sensitive to infection, and develop fatal typhlitis following administration of as few as one colony-forming unit (CFU) of *C. difficile* (Larson, Price et al. 1978; Larson and Borriello 1990). *Clostridium difficile* has also been associated with fatal enterocolitis in guinea pigs, typhlocolitis in adult horses, enterocolitis in foals, and sporadic diarrhea and intestinal disease in a variety of other mammals, including New World primates, dogs, cats, bears, and prairie dogs, and, rarely, in birds (Orchard, Fekety et al. 1983; Berry and Levett 1986; Muller, Pitt et al. 1987; Jones, Adney et al. 1988; Frazier, Herron et al. 1993; Perrin, Cosmetatos et al. 1993; Gustafsson, Baverud et al. 1997; Rolland, Chalifoux et al. 1997; Weese, Weese et al. 2001; Magdesian, Hirsh et al. 2002).

*Clostridium difficile* is now well-accepted as the etiologic agent of typhlocolitis in suckling pigs (Waters, Orr et al. 1998; Songer, Post et al. 2000; Songer, Post et al. 2000a; Songer 2004; Songer and Anderson 2006). Onset is at 1-7 days of age, with diarrhea that often begins shortly after birth, though constipation and obstipation occur as well. Disease may go unrecognized due to the typical mildness of clinical signs, leading to inaccurate estimates of morbidity rates (Waters, Orr et al. 1998; Songer, Post et al. 2000).
However, diagnostic examination of ~1000 field cases of naturally-occurring piglet enteritis revealed toxins A (TcdA) and B (TcdB) in colonic contents of 34.1% of these, without identification of additional etiologic agents. CDI was more prevalent than any other cause of enteritis (Yaeger, Funk et al. 2002).

TcdA and TcdB, the principal virulence factors of *C. difficile*, belong to the family of large clostridial cytotoxins. They are characterized by a receptor binding domain at the carboxyl terminus and a functional, enzymatic domain at the amino terminus (Schirmer and Aktories 2004). The enzymatic portions of TcdA and TcdB have similar substrate specificity, and inactivate Rho-subtype GTPases by monoglucosylation (von Eichel-Streiber, Meyer zu Heringdorf et al. 1995; Chaves-Olarte, Weidmann et al. 1997; Faust, Ye et al. 1998). The net effect is dysregulation of actin metabolism, with cell rounding and eventual death. TcdB has 1000X greater catalytic activity and is much more toxic to cultured cells than TcdA (Chaves-Olarte, Weidmann et al. 1997). Dogma has been that TcdB action *in vivo* must be preceded by effects of TcdA or other damage to mucosal epithelium (Lyerly, Saum et al. 1985; Pothoulakis and Lamont 2001).

Early evidence suggested that pigs were not susceptible to *C. difficile* toxins (Sisk, Cole et al. 1998), in that lesions did not develop in porcine intestinal loops inoculated with toxins. However, disease can be reproduced experimentally by inoculation of neonates with spores (Songer and Post 2004), and toxin production in cecum and colon are common features of both natural and experimental disease.
We determined the susceptibility of piglets to IG inoculation with purified TcdA and TcdB, and report here catastrophic effects in the small intestine and milder effects in the colon, with the latter more typical of natural and experimental infections.

2. Materials and Methods

2.1. Production, purification, and assay of toxins

Colonies of strain VPI 10463 were inoculated into 10 ml BHI-CYE broth in a 20 x 150 mm Hungate tube, under an atmosphere of 5% CO₂:5% H₂:90%N₂ and incubated at 37°C overnight. This overnight culture (1-2 ml) was used to inoculate a loop of dialysis tubing (MW cutoff 10 kDa) suspended in a 4 L Erlenmeyer flask containing 4 L BHI-CYE broth. The flask was incubated at 37°C in an anaerobic incubator. After 5-7 days, the material in the dialysis tubing was collected, and the supernatant was clarified by centrifugation (15,000 x g, 20 min) and sterilized by filtration (0.22 µm pore diameter). Toxins A and B were purified by ion-affinity chromatography over DEAE sepharose (Amersham Biosciences, Uppsala, Sweden). Two ml samples of culture supernatant were applied to the matrix in a 30 x 150 mm column via an IV-7 injection valve (Amersham). Fractions (5ml) were collected via a GradiFrac™ rotary fraction collector (Amersham) and protein elution was monitored by a UV-1 ultraviolet monitor.
(Amersham). TcdA was eluted with a linear gradient of 0.05-0.25 M NaCl in 50 mM Tris-HCl at pH 7.4. TcdB remained in the column and was recovered via a linear gradient of 0.25-0.6 M NaCl in 50 mM Tris-HCl at pH 7.4. Eluted TcdA and TcdB fractions 20-40 were pooled and concentrated by centrifugal filtration (MW cutoff=100 dDa; Millipore, Bedford, MA). Protein concentration was determined by a Bradford assay per the manufacturer’s instructions (Bio-Rad, Hercules, CA). Toxin purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Toxin activity was quantitated on Chinese hamster ovary (CHO) cell monolayers. Cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM; Cambrex, Walkersville, MD) with 10% fetal bovine serum (Intergen, Purchase, NY) and 100 µg gentamicin per ml (Sigma-Aldrich, St. Louis, MO). For toxicity assays, cells were trypsinized, washed, and dispensed (2 x 10^4 cells per well) into each well of a 96-well tissue culture plate. After incubation for 4 h at 37°C in an atmosphere of 5% CO₂, 100 µl aliquots of serial dilutions of toxin preparations were added to each well. Cells were incubated, as before, for an additional 18 h. Monolayers were then fixed, stained with trypan blue, and examined microscopically. The endpoint was reported as the reciprocal of the last dilution causing 50% cytopathic effect. Protein concentration of purified toxins was measured by Bradford assay (Bio-Rad, Hercules, CA).

2.2 Pigs
Clinically-normal, 12-24 h old pigs (n =75), which were culture negative for *C. difficile* and enzyme immunoassay negative for toxins (Tox A/B II; TechLab, Blacksburg, VA) were administered 20 ml 1 M Na$_2$HCO$_3$ IG. Principals were inoculated IG 2.5 h later with purified TcdA and/or TcdB in 10 ml 1 M Na$_2$HCO$_3$ (Table 1). Controls were given 10 ml Na$_2$HCO$_3$ alone. Pigs were euthanized by CO$_2$ asphyxiation 12 h post-treatment or when moribund. Necropsies were performed immediately, and portions of stomach, duodenum, jejunum, ileum, cecum, ascending colon, descending colon, and pancreas were immersed in 10% neutral buffered formalin.
Table 1. Summary of treatment groups

<table>
<thead>
<tr>
<th>Test group</th>
<th>Treatment</th>
<th>Number of pigs</th>
</tr>
</thead>
<tbody>
<tr>
<td>TcdA low dose</td>
<td>25 µg TcdA</td>
<td>10</td>
</tr>
<tr>
<td>TcdA high dose</td>
<td>40 µg TcdA</td>
<td>12</td>
</tr>
<tr>
<td>TcdB low dose</td>
<td>25 µg TcdB</td>
<td>10</td>
</tr>
<tr>
<td>TcdB high dose</td>
<td>40 µg TcdB</td>
<td>12</td>
</tr>
<tr>
<td>TcdA + TcdB low dose</td>
<td>12.5 µg TcdA</td>
<td>6</td>
</tr>
<tr>
<td>TcdA + TcdB high dose</td>
<td>20 µg TcdA</td>
<td>13</td>
</tr>
<tr>
<td>Controls</td>
<td>Na₂HCO₃ diluent only</td>
<td>12</td>
</tr>
</tbody>
</table>

2.3 Histopathology

Fixed tissues were dehydrated through graded concentrations of alcohols and xylene, embedded in paraffin, and sections (5 µm) stained with hematoxylin and eosin.
Tissues were examined by light microscopy and lesion scores assigned in a blind manner (Table 2).

Table 2. Scoring of microscopic lesions

<table>
<thead>
<tr>
<th>Lesion Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No coagulative necrosis; small number of scattered apoptotic cells</td>
</tr>
<tr>
<td>1</td>
<td>Multifocal necrosis affecting many individual cells or small islands of epithelial cells, at the lumenal surface</td>
</tr>
<tr>
<td>2</td>
<td>Multifocal coagulative necrosis of the superficial epithelium with erosions or ulcers</td>
</tr>
<tr>
<td>3</td>
<td>Extensive, coalescing ulceration or extensive necrosis of the superficial mucosa ± extension into the crypts</td>
</tr>
<tr>
<td>4</td>
<td>Necrosis of mucosal epithelium and deeper structures in the lamina propria, or submucosa</td>
</tr>
</tbody>
</table>

3. Results

Jejunal hyperemia was the most common gross lesion in animals inoculated with TcdA, alone or in combination with TcdB (Figure 1A). Prevalence of gross lesions in low and high TcdA dose groups was 60% and 92%, respectively. Gross lesions in piglets given TcdB alone were less prevalent (low dose TcdB, 50%; high dose TcdB, 25%) and much less extensive than those caused by TcdA alone or in combination with TcdB. TcdA and TcdB together caused gross lesions similar in severity and extent to those
associated with TcdA intoxication (low dose TcdA/TcdB, 83%; high dose TcdA/TcdB, 92%). Mesocolonic edema (Figure 1B), typical of field cases of C. difficile infection, was rare in pigs in any treatment group (low TcdA 10%, high TcdA 8%, low TcdB 30%, high TcdB 17%, TcdA/B low and high 1%). None of the controls developed gross lesions (Figure 2).

Microscopic lesions in pigs inoculated with TcdA and/or TcdB differed only in prevalence and severity (Figure 3). Inoculation with TcdA, alone or with TcdB, caused the most severe lesions, with necrotic villous enterocytes throughout small intestine. There was a dose response to TcdA (average lesion scores of 1 and 3 for low TcdA and high TcdA groups, respectively). Synergistic effects of TcdA and TcdB were evident. Mean lesion scores in piglets inoculated with low or high doses of TcdA and TcdB (low TcdA/B= 2; high TcdA/B=2.3) were similar to those in piglets inoculated with TcdA alone, but with only one-half the amount of TcdA. Piglets inoculated with TcdB alone developed the mildest lesions, with mean scores ≤ 1 in both groups. Colonic necrosis was limited to the superficial epithelium. Scattered karyorhectic debris, consistent with remnant apoptotic bodies, was found in superficial lamina propria. Mean colonic lesion scores were ≤ 0.5 in all groups, except animals treated with the high dose of TcdA (mean score 0.7). Neither jejunal nor colonic lesions developed in control animals (Figure 4).
4. Discussion

Results of these studies reveal that pigs are exquisitely susceptible to the effects of purified *C. difficile* toxins administered IG. Inoculated pigs developed severe small intestinal gross and microscopic lesions, which are not encountered in natural disease. Colonic lesions more closely resembled those in field cases of porcine CDI, without inflammation. Naturally infected pigs usually present with mesocolonic edema, focal neutrophil infiltration into colonic mucosa, and mucosal epithelial erosion. Infiltrates of neutrophils are localized, with intense infiltrates associated with small ulcers and large numbers of neutrophils migrating across the mucosal surface to the intestinal lumen. The greater severity of lesions in small intestine might be due to exposure of these tissues to highest toxin concentrations following IG inoculation; there may have been less toxin to act in distal parts of the tract. Results of these experiments demonstrate severe, yet uncharacteristic, effects of *C. difficile* toxins on small intestine of neonatal piglets.

Clearly, TcdA has severe effects on small intestinal mucosa of neonatal pigs, but the effects of TcdB are not as profound. Research in other species revealed that TcdB alone has minimal or no effect when introduced by gavage, unless there is incidental epithelial trauma (Lyerly, Saum et al. 1985; Pothoulakis and Lamont 2001). In counterpoint, TcdB was as active as TcdA in intestinal loops of calves (Hammitt, Bueschel et al. 2008), but loop preparation trauma may have predisposed these tissues to TcdB-induced damage. One hypothesis contends that TcdB receptors are on the basolateral aspect of enterocytes, and that trauma provides access to receptors on damaged cells. A cascade of cell damage by TcdB and access to additional binding sites
ensues. A secondary hypothesis suggests that TcdA-induced trauma can pre-dispose cells to the effects of TcdB. However, these scenarios are called into doubt by reports of TcdA-negative, TcdB-positive strains that produce disease in humans (Komatsu, Kato et al. 2003; van den Berg, Claas et al. 2004). Receptor mediated endocytosis of TcdB may, as with TcdA, be a necessary first step in expression of its intracellular activities, but biotinylated TcdB fails to bind to piglet tissues (Keel and Songer 2007); furthermore, no TcdB receptor has been identified in any species. Thus cellular intoxication in piglets, whether in small intestine or colon, may occur by alternative mechanisms such as pH-mediated channel formation in host cell lipid bilayers (Barth, Pfeifer et al. 2001). TcdB emulsified in digesta could be endocytosed after the same manner as nutrients in the piglet gut (Stange 1983). These results show piglet sensitivity to C. difficile toxins, but do not encompass a comprehensive profile of pathogenesis.
Figure 1. Gross lesions. (A) Diffuse small intestinal hyperemia with red tinged fluid in the lumen as that caused by TcdA alone or TcdA and TcdB together and mild mesocolonic edema (B).
Figure 2. Prevalence of jejunal and colonic gross lesions, p>0.2
Figure 3. Microscopic intestinal lesions in piglets inoculated intragastrically with *C. difficile* toxins. Jejunum (A) from pig inoculated with 40 µg TcdA. Most epithelial cells are necrotic and have sloughed. Colon (B) from pig inoculated with 40 µg TcdA. Effects on epithelial cells are focal and without neutrophil infiltration.
Figure 4. Mean microscopic lesion scores, p>0.2.
Acknowledgements

The authors gratefully acknowledge Hien Trinh for expert guidance and administrative support.

References


APPENDIX D

Response to challenge of suckling pigs passively-immunized vs Clostridium difficile toxin A

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Abstract

Porcine Clostridium difficile infection (CDI) manifests as typhlocolitis in piglets 1 - 7 days of age with morbidity rates up to 90%. Virulent strains of C. difficile usually produce toxins A (TcdA) and B (TcdB) as major virulence factors, but only the former is presumed to be involved in lesion production in the porcine cecum and colon. No commercial immunoprophylactic products are available to pork producers, so we challenged the immunity of neonates farrowed by gilts immunized with recombinant TcdA. Gilts developed 1:16 geometric mean toxin-neutralizing titers following immunization with TcdA, while non-vaccinated control gilts remained seronegative. Neonates were inoculated twice with ~2x10^9 CFU of germinating spores of virulent C. difficile strain JGS6125 (ribotype 027, toxinotype III). Challenged pigs developed diarrhea by 24 h post-challenge. However, TcdA was not detected in stools until 48 h post challenge, at that time 90% of controls and 85% of principals were affected. Colitis typical of porcine CDI was demonstrated at 48 h post-challenge in 61% and 46% of piglets from non-vaccinated and vaccinated dams, respectively. These data suggest that the extent or nature of the antiTcdA immune response was insufficient to abrogate the effects of challenge. Diarrhea in vaccinated and control piglets, in the apparent absence of TcdA, may have been due to alternate virulence factors, such as binary toxin CDT.
1. Introduction

*Clostridium difficile* is an anaerobic gram-positive, spore-forming bacillus which has become the leading cause of nosocomial diarrhea in humans. *Clostridium difficile* infection (CDI) manifests as diarrhea, pseudomembranous colitis, and rarely, fatal toxic megacolon (Harbarth, Samore et al. 2001; Nicolasora and Kaul 2008). Disease most often occurs in association with antibiotic usage (Larson, Price et al. 1978).

The principal virulence factors of *C. difficile* are toxins A (TcdA) and B (TcdB). They belong to the family of large clostridial cytotoxins, which are characterized by a receptor binding domain at the carboxyl terminus and an enzymatic domain at the amino terminus (Schirmer and Aktories 2004). The enzymatic portions of TcdA and TcdB have similar substrate specificity, inactivating Rho-subtype GTPases by monoglucosylation (von Eichel-Streiber, Meyer zu Heringdorf et al. 1995; Chaves-Olarte, Weidmann et al. 1997; Faust, Ye et al. 1998). The net effect is dysregulation of actin metabolism, with cell rounding and death. TcdB has 1000-fold greater catalytic activity and is much more toxic for cultured cells than TcdA (Chaves-Olarte, Weidmann et al. 1997). Dogma has been that TcdB action in vivo must be preceded by mucosal epithelial damage induced by TcdA or other factors (Lyerly, Saum et al. 1985; Pothoulakis and Lamont 2001).

CDI is also a cause of enterocolitis in guinea pigs, typhlocolitis in adult horses, enterocolitis in foals, and sporadic diarrhea and intestinal disease in a variety of other animals, including dogs, cats, and, rarely, in birds (Orchard, Fekety et al. 1983; Berry and Levett 1986; Weese, Weese et al. 2001; Magdesian, Hirsh et al. 2002). Disease modeling in laboratory rodents such as hamsters has yielded invaluable information on
pathogenesis; hamsters pre-treated with clindamycin develop disease when inoculated with even a single *C. difficile* spore (Larson, Price et al. 1978; Lyerly, Saum et al. 1985; Larson and Borriello 1990).

CDI is now recognized to be common in pigs 1-7 days of age. Pigs present with yellow, pasty diarrhea, although constipation or obstipation is common. (Waters, Orr et al. 1998; Songer, Post et al. 2000; Songer, Post et al. 2000a; Songer 2004; Songer and Anderson 2006). Small intestine is not affected, but mesocolonic edema, accompanied by focal mucosal epithelial erosion or ulceration in cecum and colon, is characteristic. Neutrophil infiltration and exudation into the colonic lumen forms the hallmark “volcano lesion” of CDI. Accurate morbidity and mortality rates in the field are not available, but (TcdA) and (TcdB) were detected in intestinal contents of 34% of ~1000 piglets with naturally-occurring enteritis, and CDI was more prevalent than any other source of enteritis (Yaeger, Funk et al. 2002).

Immunization with TcdA and TcdB has protected against CDI in laboratory rodents (Kim, Iaconis et al. 1987; Lyerly, Bostwick et al. 1991; Kelly, Pothoulakis et al. 1996). Hamsters were protected by passive immunization with bovine hyperimmune colostrum against TcdA and TcdB (Lyerly, Bostwick et al. 1991). Immunization with anti-TcdA monoclonal antibodies protected mice against CDI (Corthier, Muller et al. 1991). Human asymptomatic carriers of toxigenic *C. difficile* are more likely to lack significant titers of anti-TcdA antibodies, and convalescent patients are much less likely to experience recurrent CDI if they develop anti-TcdA humoral immunity (Kyne, Warny et al. 2000).
In pigs, immunohistochemical studies revealed that TcdA, but not TcdB, binds to neonatal intestinal epithelium (Keel and Songer 2007). Furthermore, TcdA, but not TcdB, produced lesions in porcine neonatal colonic explants (Keel and Songer 2007). The apparent primacy of TcdA in pathogenesis of porcine CDI led us to focus on TcdA as a potential immunogen. Our aim in the work described here was to assess the efficacy of passive protection of piglets by immunization of dams against recombinant TcdA (rTcdA).

2. Materials and Methods

2.1. Animal Treatments

Six bred gilts were randomly assigned to one of two groups (Group I, non-vaccinated controls; Group II, vaccinated principals). During gestation, gilts received two, 4 ml intramuscular (IM) doses of experimental C. difficile vaccine at 5 and 2 weeks before farrowing. Commercial Escherichia coli vaccine (Novartis Animal Health, Porcine Pilishield™) was administered to gilts in both test groups according to label recommendations (two, 2 ml IM doses, 5 and 2 weeks pre-farrowing). Gilts were housed and fed (antibiotic-free feed) in accordance with The University of Arizona Institutional Animal Care and Use Committee and University Animal Care guidelines. Pregnant gilts were transported from gestation pens to farrowing crates 3 days prior to the expected parturition date. Twenty-two piglets from Group I and 21 from Group II were inoculated with spores of C. difficile strain JGS6125. Eight principals and 4 controls were
euthanized and necropsied 1 day post inoculation; 13 principals and 18 controls were euthanized and necropsied 2 days post inoculation.

2.2. Vaccine

A 1221 bp fragment of tcdA was amplified by PCR from genomic DNA of strain VPI10463 (Primers: HISTOXAF, CAGAACAAATTGGATCCTTTGAATGGC, and HISTOXAR, TTAGCCAAAGCTTCAAGGGGCTTTTACTCC). The PCR product was digested with BamHI and HindIII and cloned into BamHI/HindIII- digested pTrcHis B. The plasmid was transformed into E. coli DH5α, creating strain JGS2534. The plasmid encoded the C-terminus of TcdA (rTcdA), a 440 amino acid protein comprising 407 amino acids of the C-terminus portion of tcdA with an N-terminal extension of 33 amino acids including a histidine tag. DNA inserts were sequenced for confirmation.

JGS2534 cultivated in 2 L Luria Bertani (LB; Becton Dickenson) broth to OD$_{600}$ 0.6 was induced by addition of IPTG to a final concentration of 2.5 mM. Incubation was continued for an additional 3 h to allow protein expression. Cells were harvested by centrifugation (10,000 x g, 20 min) and resuspended in of 20 ml binding buffer (20mM Tris, 100mM NaCl, pH 8.0). Following freeze thaw at -20°C, cells were lysed in a French pressure cell (1000 p.s.i.) and HIS tagged proteins were purified on a metal affinity column (Talon Metal Affinity Resin, Clonetech Laboratories, Mountain View, CA). Resin (20 ml) was washed twice in binding buffer via centrifugation (500 x g, 20 min) and binding buffer was mixed with cell lysate (~20 ml) and washed resin (~10 ml) for a final volume of ~50 ml. The resin and cell lysate mixture were incubated at room
temperature with agitation for 20 min, washed twice with binding buffer and dispensed into a column. Bound protein was eluted from the column by gravity flow with 50 mM imidazole/20 mM tris/100 mM NaCl solution, pH 8.0. Fractions (1.5 ml) with highest purity (determined by SDS-PAGE) were pooled. Protein concentrations were determined by Bradford assay as per manufacturer’s instructions (BioRad, Hercules, CA).

Adjuvant in completed products consisted of 25% v/v of 10% w/v aluminum potassium sulfate (alum) (E.M. Sergeant Pulp and Chemical, Clifton, NJ). Alum solution was titrated into the soluble recombinant antigen solutions until pH 6.0-6.3. The remaining volume of alum was added while maintaining pH 6.0-6.3 via addition of 5 N NaOH. Vaccine was formulated to contain 200 µl of rTcdA per 4 ml dose.

2.2. Serology

Blood was collected from gilts prior to immunizations and ~1 week post second farrowing. Piglet sera were collected at the time of necropsy. Serum was separated and stored at -20°C until tested.

Toxin-neutralizing antibodies (against TcdA and TcdB) were titrated in Chinese hamster ovary (CHO) cell monolayers. CHO cells were maintained in Iscove's modified Dulbecco's medium (IMDM; Cambrex, Walkersville, MD) with 10% fetal bovine serum (Intergen, Purchase, New York) and 100 µg gentamicin per ml (Sigma-Aldrich, St. Louis, MO). For toxicity assays, cells were trypsinized and washed, and 2 x 10^4 cells were placed in each well of 96-well tissue culture plates (Corning, Corning, NY). After overnight incubation at 37°C in an atmosphere of 5% CO₂, 100 µl aliquots of serially
diluted purified native TcdA or TcdB were added to each well. Cells were incubated for an additional 18 h. Monolayers were then fixed, stained with trypan blue, and examined microscopically. The endpoint was reported as the reciprocal of the last dilution causing 50% cytopathic effect.

For serum neutralization assays, serial, two-fold dilutions of serum samples were made in a 96-well microtitration plate. TcdA or TcdB (~20 100% tissue culture toxic doses) was added to each well and incubated at room temperature for 1 h. Aliquots of serum/toxin mixtures were transferred to CHO cell monolayers and plates incubated overnight as above. Monolayers were fixed as previously described and examined microscopically to determine endpoint titers (last dilution preventing 100% cytotoxicity).

2.4. Inoculations

Strain JGS6125 was inoculated onto Clostridium difficile selective agar (CDSA); brain heart infusion broth (37 g), 20 g technical grade agar (Becton Dickinson) and 1 g taurocholic acid in 1 L HPLC-grade water was supplemented with 5% citrated bovine blood, D-cycloserine/cefoxitin (250 µg/ml final concentration) and cefoxitin (10 µg/ml final concentration), after autoclaving. Plates were incubated under anaerobic conditions (5% H₂, 5% CO₂, 90% N₂) at 37°C for 48 h. Single colonies were passed to multiple antibiotic free BHI-CYE agar plates [37 g brain heart infusion broth, 5 g yeast extract, 20 g technical grade agar (Becton Dickenson), and 0.5 g cysteine HCl]. Plates were incubated as before. Culture harvested onto sterile cotton swabs was used to prepare lawns on BHI-CYE plates (~300) incubated anaerobically at 37°C for 7 days. Spores
collected by washing each plate with 5 ml PBS were harvested by centrifugation
(10,000x g, 20 min), washed with 180 ml of 1M KCl:0.5 M NaCl, resuspended in 100
ml 50 mM Tris-HCl, pH 7.2:10 mg lysozyme per ml and incubated at 37°C for 1 h.
Spores were washed three times with 100 ml amounts of HPLC-grade water and 5 ml
aliquots (~1x10^9 CFU/ml) were stored at 4°C.

Germination was induced by heat shock (80°C), followed by addition of 0.5 ml of
Clostridium difficile selective broth (CDSA without agar) and incubation at 37°C for 30-
60 min prior to inoculation of piglets. Before ingestion of colostrum, piglets were
inoculated IG with ~2x10^9 spores. Animals not euthanized on the first post challenge day
received an additional ~2x10^9 spores after 24 h.

2.5. Observations

Diarrhea was scored twice daily on a scale 0-3 (Table 1). Piglets euthanized via
barbiturate overdose were necropsied (one or two days post inoculation), and
mesocolonic edema was rated 0-3 (Figure 1, Table 2). Samples of small intestine and
colon were fixed in 10% buffered formalin, trimmed, embedded in paraffin, sectioned
(5µm), and stained with haemotoxylin and eosin (H&E). Microscopic lesions in the colon
were rated 0-5 (Table 3).
### Table 1. Clinical scores

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal, brown solid feces, perineum dry</td>
</tr>
<tr>
<td>1</td>
<td>Yellow, semi-solid/fluid feces, perineum dry</td>
</tr>
<tr>
<td>2</td>
<td>Yellow, semi-solid/fluid feces, irritated perineum caked with feces or wet with liquid feces</td>
</tr>
<tr>
<td>3</td>
<td>Pig constipated or obstipated</td>
</tr>
</tbody>
</table>

### Table 2. Gross lesion scores.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No mesocolonic edema</td>
</tr>
<tr>
<td>1</td>
<td>Mild mesocolonic edema</td>
</tr>
<tr>
<td>2</td>
<td>Moderate mesocolonic edema</td>
</tr>
<tr>
<td>3</td>
<td>Severe mesocolonic edema</td>
</tr>
</tbody>
</table>
Table 3. Microscopic lesion scores.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>No coagulative necrosis; small number of scattered apoptotic cells permissible</td>
</tr>
<tr>
<td>1</td>
<td>Multifocal necrosis affecting many cells or small islands of epithelial cells at luminal surface</td>
</tr>
<tr>
<td>2</td>
<td>Similar to 1, except with neutrophil infiltration (inflammation)</td>
</tr>
<tr>
<td>3</td>
<td>Multifocal coagulative necrosis of superficial epithelium with erosions/ulcers or inflammation</td>
</tr>
<tr>
<td>4</td>
<td>Extensive, coalescing ulceration/necrosis of superficial mucosa +/- extention into crypts</td>
</tr>
<tr>
<td>5</td>
<td>Necrosis of mucosal epithelium and deeper structures in lamina propria with inflammation</td>
</tr>
</tbody>
</table>

Fecal specimens collected daily via rectal swab were examined by bacteriologic culture on CDSA plates, and numbers of colony-forming units (CFU) per gram of colonic contents (collected at necropsy) were determined by dilution plating. Fecal samples were also tested for TcdA and TcdB via commercial EIA test kit (Tox A/B, TechLab, Blacksburg, VA).

2.6. Other Diagnostics
Piglets from each litter were tested for pathogenic *E. coli*. Small intestinal contents collected at post-mortem were examined by bacteriologic culture on Tergitol-7 (Sigma-Aldrich, St. Louis, MO) agar plates, incubated overnight aerobically at 37°C. Isolates from plates with high numbers of *E. coli* were examined via multiplex PCR for the genes for Stx2e, StB, LT, StaP, F18, F41, K88, K99, and 987P.

3. Results

3.1. Serology

Geometric mean post vaccination serum titers against TcdA in immunized sows and their piglets were 15 and 16, respectively. Non-immunized control sows and piglets remained sero-negative (Figure 2). Sows in both groups had pre-vaccination anti-TcdB titers which persisted throughout the trial and carried over to the piglets (sow/piglet geometric means; Group I, 20/11; Group II, 32/10).

3.2. Post Inoculation Observations

On day 1 post-challenge, both control and principal pigs had semi-solid to liquid feces and mean diarrhea scores of 1.1 and 1.3, respectively. On day 2, the clinical presentation changed to pasty diarrhea, as reflected in mean scores of 0.7 in both test groups (Figure 3).

Rectal swab samples from all pigs in both test groups were culture positive on both day 1 and day 2 post-challenge. Immunized pigs had a mean of $3.8 \times 10^4$ CFU per g and controls had $1.1 \times 10^5$ CFU per g, in the form of spores, in contents. Spore prevalence
was apparently unrelated to toxin positivity in either day 1 or day 2 post challenge. On day 1 post challenge, only 1 of 22 (4.8%) fecal samples from immunized pigs and 1/22 (4.5%) samples from control pigs were mildly toxin positive. Post-mortem testing of gut contents confirmed absence of toxin, with one mildly-positive immunized pig and no positive control pigs. A marked increase in positivity was noted on the second day post-inoculation; 67% and 69% of fecal samples from controls and principals, respectively, were positive. Prevalence of toxin in colonic contents of controls (89%) and immunized pigs (85%) paralleled that in feces collected antemortem via rectal swab (Figure 4).

Gross lesions, unlike microscopic lesions, were noted in both day 1 and day 2 animals. Controls (100%) and principals (88%) necropsied had mesocolonic edema. The edema persisted through 48 h in 78% of control pigs and 69% of immunized animals. On the other hand, no pigs in either test group had microscopic lesions 24 h after infection, but after 48 h, 61% of controls and 46% of principals had lesions (Figure 5).

4. Discussion

Piglets from dams vaccinated with rTcdA were not protected from CDI; immunization with rTcdA elicited a 16-fold post vaccination increase in serum anti-toxin titers against TcdA, yet piglets from immunized dams developed clinical disease and lesions similar to those of control animals. Pre-existing anti-TcdB titers were higher than those vs TcdA, without active immunization against TcdB. However, lateral comparisons of anti-TcdA and anti-TcdB antibodies are inappropriate. TcdB is 1000x more cytotoxic than TcdA, but in both assays, toxins are standardized to contain ca. 20
100% tissue culture toxic doses of TcdA or TcdB, respectively. The molar concentration of TcdB is much lower than TcdA, resulting in an anti-TcdB assay that is much more sensitive than the anti-TcdA serologic assay. Therefore, pre-existing anti-TcdB titers may be negligible. In any case, anti-toxin titers, regardless of toxin, were not protective.

Cellular intoxication by either TcdA or TcdB occurs by receptor mediated endocytosis. Endosomal acidification facilitates translocation of enzymatic N-termini into the cytoplasm where inactivation of GTPases causes cell death. The cytotoxic mechanisms of TcdA and TcdB are the same, but their C-terminal binding domains differ. TcdA binds to carbohydrates such as host specific Galalpha1-3beta1-4GlcNAc-R (Tucker and Wilkins 1991). Immunohistochemical studies showed that TcdA, but not TcdB, binds to colonocyte brush borders of neonatal pigs (Keel and Songer 2007). Identification of TcdB receptors remains elusive; however, cellular intoxication may occur by alternative mechanisms, such as pH-mediated channel formation (Barth, Pfeifer et al. 2001). The commercial EIA test kit used for toxin detection does not discriminate between TcdA and TcdB. Immunization against rTcdA may have been adequate to prevent the effects of TcdA, but alternate TcdB intoxication mechanisms may have played a role in pathogenesis.

Without any other known etiology, human CDI is diagnosed in clinically-ill patients (diarrhea) upon detection of TcdA, TcdB and/or toxigenic C. difficile in feces, identification of pseudomembranous colitis via surgery or endoscopy, presence of microscopic lesions consistent with CDI (McDonald, Coignard et al. 2007). Diagnosis of porcine CDI is based upon similar criteria (diarrhea, culture and toxin positivity,
mesocolonic edema and erosions or ulcerations of colonic mucosa with evidence of inflammation). We demonstrated that any combination of the preceding criteria may present in pigs. For disease-modeling purposes, the presence of microscopic lesions is desirable for making definitive diagnoses. On day 1 post challenge, nearly all pigs were toxin negative and without microscopic lesions, but diarrhea and mesocolonic edema were common. Exposure to potential virulence factors such as binary toxin may produce diarrhea and mesocolonic edema before TcdA or TcdB appear. This requires further study, but detection of binary toxin might serve as an early diagnostic finding in porcine CDI. Additional experiments are necessary to ensure proper vaccine formulation strategies. In conclusion, these data showed that passive immunization of piglets against TcdA did not yield protective immunity from CDI.
Figure 1. Diarrhea progressed from yellow, liquid feces on day 1 post challenge (A) to yellow, pasty feces or constipation by day 2 post challenge. Mesocolonic edema (B) was present 1 and 2 days post inoculation.
Figure 2. For the purpose of calculating geometric means, negative samples were assigned a titer of 1.
Figure 3. Vaccinates vs Controls, p>0.8.
Figure 4. Cumulative rectal swab and colonic fecal content toxin positive samples; vaccinates vs controls, p=1.0.
Figure 5. Microscopic lesion prevalence is shown. Mean lesion scores of 0.8 in both test groups on day 2 post inoculation are not shown. There were no lesions in day 1 post inoculation pigs. Vaccinates vs controls, p>0.9.
Acknowledgements

The authors gratefully acknowledge Hien Trinh for expert guidance and administrative support. The University of Arizona diagnosticians R. Glock, DVM, Ph.D. and S. Dial, DVM, Ph.D. were invaluable resources for interpretation of gross and microscopic lesions. Extension veterinarian P. Cuneo, DVM provided animal husbandry support and guidance. Thanks goes out to Emily Richie and Micheal Hailey for animal and sample processing.

References


APPENDIX E

Response to Challenge of Nursing Piglets Passively Immunized Against Clostridium perfringens Alpha and Beta2 Toxins

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Abstract

Porcine Clostridium perfringens type A infection in neonatal piglets usually manifests as transient enteritis, with diarrhea and mild villous atrophy. All strains isolated from affected piglets produce alpha toxin (CPA) and > 90% produce beta2 toxin (CPB2). Roles for both in pathogenesis have been hypothesized but not proven. We challenged the immunity of nursing piglets farrowed by nonimmunized control gilts (Group I) or gilts immunized with native CPA toxoid (Group II) or recombinant CPA (rCPA) and recombinant CPB2 (rCPB2) toxin (Group III). Gilts in Groups II and III developed geometric mean CPA-neutralizing titers of 1:4.6 and 1:7.0, respectively. Those Groups I and II had no EIA titers against rCPB2, while those in Group III had post-vaccination geometric mean titers against rCPB2 of 1:1213. Neonates were inoculated IG with \( \sim 2 \times 10^{10} \) CFU of C. perfringens type A strain JGS4024, which was cpb2-positive by PCR. Mean diarrhea and enteritis scores among groups (Group I: 1.9/1.2; Group II: 1.4/0.9; Group III: 1.6/0.9) were not significantly different. CPA was detected in feces of 69%, 6%, and 54% of piglets in Groups I, II, and III, respectively. Immunization with native toxoid reduced the rate of CPA detection in feces of challenged piglets, but did not eliminate disease symptoms. These data suggest (a) that immune responses to native or recombinant CPA and recombinant CPB2 were inadequate in nature or extent to control disease and/or (b) that other virulence factors are sufficient to enable C. perfringens type A to produce piglet enteritis.
1. Introduction

_Clostridium perfringens_ is perhaps the most widely-distributed pathogen in the world. It is consistently recovered from both the environment and the intestinal tract of warm-blooded vertebrates. As many as 17 exotoxins are produced by _C. perfringens_, but strains are classified into five major types (A-E) based on production of one or more of the major toxins, alpha (CPA), beta (CPB), epsilon (ETX) and iota (ITX) (Brooks 1957; McDonel 1980; McDonel 1986). Type A strains may produce disease (Elder 1957; Mo¨llby 1978; Smith 1979; Songer 1996), but are also common in the normal gut and the environment. Types B, C, D and E, on the other hand are usually associated with diseased animals or proximal contaminated environments (Timoney 1988).

Due to its ubiquity, _C. perfringens_ type A has historically been considered normal flora and avirulent in the gut. Ante- and post-mortem tissues from healthy and diseased animals alike readily yield type A. Tissues collected post mortem can become overgrown with _C. perfringens_ type A, leading to recovery of high numbers of the organism. Caution is indicated in making a diagnosis of type A-associated disease solely on microbiological grounds.

_Clostridium perfringens_ type A produces no major toxins other than CPA, a phospholipase/sphingomyelinase C which is hemolytic, necrotizing, lethal, and is the primary virulence factor in a variety of disease syndromes (Elder 1957; Mo´llby 1978; Smith 1979; Songer 1996). Its role in pathogenesis of myonecrosis (gas gangrene) has been well characterized (Naylor, Eaton et al. 1998). Parenteral immunization of chickens with CPA toxoid significantly reduces the occurrence and severity of necrotic enteritis...
caused by *C. perfringens* type A (Cooper, Trinh et al. 2008; Kulkarni, Parreira et al. 2008). Anemia, hemoglobinuria, and jaundice follow absorption of CPA from the gut in sheep with yellow lamb disease (McGowan 1958; Fleming 1985). Type A infection causes mild necrotizing enteritis, with villous atrophy and transient diarrhea in pigs 1-3 days of age (Collins, Bergeland et al. 1989; Johannsen 1993; Songer 1996; Songer unpublished data). Unlike disease in other species, a defined role for CPA in pathogenesis of porcine neonatal enteric disease has been elusive. Response of piglet gut loops to inoculation with CPA suggests that this toxin alone is not responsible for enteritis (Popoff and Jestin 1985; Estrada-Correa 1988). However, its putative role in the aforementioned disease syndromes has led to its being targeted as a protective antigen in porcine enteritis.

Production of beta2 toxin (CPB2) was first identified in a type C strain from a piglet with necrotizing enteritis (Gibert, Jolivet-Reynaud et al. 1997). The 28-kDa protein is toxic for Chinese hamster ovary cells and causes mucosal necrosis in guinea pig ligated intestinal loops. Epidemiologic surveys report that >90% of porcine type A strains from diseased piglets are *cpb2* positive, while prevalence in bovine and ovine strains may be 50% or less (Bueschel, Jost et al. 2003). The high prevalence of *cpb2* in porcine strains suggests a role for CPB2 in piglet enteritis, but this has not been documented.

We report here that immunization of gilts with native or recombinant CPA and CPB2 fails to protect piglets against necrotizing enteritis in the face of challenge with *C. perfringens* type A.
2. Materials and Methods

2.1. Animal Test Groups

Fifteen bred gilts obtained from an Arizona pig production facility were randomly assigned to three groups (Table 1). During gestation, commingled gilts in test groups II and III received 2ml intramuscular doses of the respective experimental vaccines at 5 and 2 weeks prior to farrowing. Commercial *E. coli* vaccine (Porcine Pilishield™, Novartis Animal Health, Larchwood, IA) was administered to gilts in all test groups according to label recommendations. Gilts were housed and fed (antibiotic-free feed) in accordance with guidelines of the Institutional Animal Care and Use Committee of The University of Arizona. Pregnant gilts were transported from gestation pens to farrowing crates three days prior to the expected parturition date. Piglets were challenged (n=106) or remained as non-challenged contact controls (n=40).
Table 1. Animal Test Group Treatments.

<table>
<thead>
<tr>
<th>Test Group</th>
<th>Vaccine Treatment</th>
<th>Gilts</th>
<th>Inoculated Piglets</th>
<th>Contact Control Piglets</th>
</tr>
</thead>
<tbody>
<tr>
<td>I</td>
<td>Non-Vaccinated</td>
<td>5</td>
<td>40</td>
<td>16</td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>II</td>
<td>Native Alpha Toxoid</td>
<td>5</td>
<td>35</td>
<td>14</td>
</tr>
<tr>
<td>III</td>
<td>rCpa+rCpb2</td>
<td>5</td>
<td>31</td>
<td>10</td>
</tr>
<tr>
<td>Total</td>
<td></td>
<td>15</td>
<td>106</td>
<td>40</td>
</tr>
</tbody>
</table>
2.2 Vaccines

Late log-phase fermenter culture of *C. perfringens* type A strain GL785 (CPA⁺, CPB2⁺) was inactivated with formalin (0.6% v/v, 96 h, 33°C, pH 7.0) and cells removed via hollow fiber ultra-filtration (300 kDa pore size). The resulting toxoid was concentrated via hollow fiber ultra-filtration (10 kDa pore size). CPA antigen concentration was determined by its ability to combine with a standardized CPA anti-toxin, and endpoint toxoid values were reported in combining power units (CPU) as previously described (Batty 1971). Native CPA toxoid vaccine was formulated to contain 30 CPU/2ml dose.

For production of rCPA and rCPB2, 1292 (CPA) and 723 (CPB2) bp fragments, were amplified from *C. perfringens* genomic DNA by PCR (Primers: CPAF, CTAAGTCTCGAGTTGGGATGG and CPAR, CCTCTGATAACATCGTGTAAG; CPB2F, AATGAAAGCCTCATGC and CPB2R, GTCACTTCCATATTCTTCTATGC). The CPA PCR product was digested with XhoI and HindIII and cloned into XhoI/HindIII-digested pTrcHis B. The CPB2 PCR product was digested with BamHI and EcoRI and cloned into BamHI/EcoRI-digested pTrcHis B. The sequence of the resulting plasmids was confirmed, and they were then transformed into *E. coli* DH5α, yielding JGS2445 and JGS2431. pJGS2445 encoded 6xHIS-tagged CPA (rCPA), a 466 amino acid protein comprising 430 amino acids of the C-terminus portion of *cpa* with an N-terminal extension of 33 amino acids including a 6xHIS sequence. pJGS2535 encoded 6xHIS-tagged CPB2 (rCPB2), a 274 amino acid
protein consisting of 241 amino acids of the C-terminus of \textit{cpb2} with the same N-terminal sequence as rCPA.

JGS2445 and JGS2431 cultivated in 2 L Luria Bertani (LB) broth (Becton Dickinson) to $\text{OD}_{600}$ 0.6 were induced by addition of IPTG and then incubated for a further 3 h to allow protein expression. Cells were harvested by centrifugation (10,000 x g, 20 min) and the pellet was resuspended 20 ml binding buffer (20mM Tris, 100mM NaCl, pH 8.0). Following freeze (-20°C), and thawing, cells were lysed in a French pressure cell (1000 p.s.i.) and HIS-tagged proteins were affinity purified on a metal affinity column (Talon Metal Affinity Resin; Clontech Laboratories, Mountain View, CA). Resin (20 ml) was washed twice with binding buffer. Binding buffer (~20 ml) was mixed with cell lysate (~20 ml) and washed resin (~10 ml) and incubated at room temperature (RT) with agitation for 20 min. After washing twice with binding buffer, this mixture was poured into a column. Bound protein was eluted gravity flow, with 50 mM imidazole/20mM Tris/100mM NaCl pH 8.0. Fractions (1.5 ml) were examined by SDS-PAGE, and those containing the highest purity proteins were collected. Protein concentrations were determined by Bradford reagents as per manufacturer’s instructions (BioRad, Hercules, CA).

Adjuvant in completed products consisted of 25% v/v of 10% w/v aluminum potassium sulfate (alum) (E.M. Sergeant Pulp and Chemical, Clifton, NJ). Alum solution was titrated into the soluble recombinant antigen solutions until the pH 6.0-6.3. The remaining volume of alum was added while maintaining pH 6.0-6.3 via addition of 5 N NaOH. Vaccine was formulated and pigs were immunized as outlined in Table 2.
Table 2. Experimental Vaccine Formulations.

<table>
<thead>
<tr>
<th>Experimental Vaccines</th>
<th>Antigen Input</th>
</tr>
</thead>
<tbody>
<tr>
<td>Vaccine</td>
<td>Antigen</td>
</tr>
<tr>
<td>Native Toxoid</td>
<td><em>C. perfringens</em> Strain GL785 Cpa Toxoid</td>
</tr>
<tr>
<td>rCpa+rCpb2</td>
<td><em>C. perfringens</em> rCpa</td>
</tr>
<tr>
<td></td>
<td><em>C. perfringens</em> rCpb2</td>
</tr>
</tbody>
</table>

2.3. Serology

Blood was obtained from gilts prior to vaccination and 1 week post-farrowing. Piglets were sampled at the time of necropsy. Serum was separated and stored at -20°C until tested.

Anti-CPA antibodies in gilt and piglet serum were titrated via neutralization assay. A mixture of NaN₃ (0.4 g) and agar (8 g) in HPLC-grade water (342 ml) and 10x PBS (0.1 M, pH 7.4, 38 ml) was sterilized by autoclaving. One fresh egg yolk, mixed with 5 ml sterile 5M NaCl and 15 ml sterile HPLC-grade water, was added, and 25 ml aliquots were poured into 100 mm square Petri dishes. After solidifying, 3 mm wells were punched.
Gilt and piglet sera were carried through two-fold serial dilutions through 1:128 in PBS in a 96-well microtitration plate. An aliquot (40 µl) of each dilution was mixed with 40 µl of a standardized CPA solution. After incubation at 37ºC for 1 h, 25 µl of this mixture was added to each of two wells and the plate incubated overnight at 37ºC. Endpoint neutralizing titers were reported as the reciprocal of the highest dilution which prevented hydrolysis of egg yolk lecithin. To calculate geometric means, serum neutralizing titers <1 (undiluted) were reported as 0.5.

Gilt and piglet sera were tested for anti-CPB2 antibodies via an enzyme immunoassay (EIA). Wells of 96-well Immulon IV microtitration (Dynatech Laboratories, Chantilly, VA) were coated with 20 µg of rCPB2 in 100 µl of carbonate buffer (pH 9.6) and incubated overnight at 4ºC. Between each incubation step, wells were washed 5x with PBS-Tween wash buffer (8 g NaCl, 0.2 g KCl, 1.15 g Na₂HPO₄, 0.2 g KH₂PO₄, 1 L H₂O, 3 ml Tween-20). BSA (3%) dissolved in wash buffer was added to each well (200 µl/well) and plates were incubated 1 h at 37ºC. Sera were diluted 1:100 in PBS Tween wash buffer two-fold serial dilutions prepared in the same solution. These dilutions were transferred to the coated and washed microtitration plates and incubated at 37ºC for 1 h. After washing, 100 µl peroxidase-labeled, goat anti-swine IgG (H+L; KPL, Gaithersburg, MD) diluted 1:2000 in wash buffer was added to each well and plates were incubated at 37ºC for 1 h. (KPL). OPD chromagen (100 µl/well; SIGMAFAST™ OPD, Sigma-Aldrich, St.Louis, MO) was added to washed plates and incubated at RT for 10 min. The reaction was stopped by addition of 3% H₂SO₄ (50 µl/well). Endpoint serum titers were
reported as the reciprocal of the highest dilution with an OD$_{490}$ \textgreater 0.4. For calculation of geometric means, serum samples with titers <100 were arbitrarily assigned a value of 50.

2.4. Piglet Challenge

*Clostridium perfringens* type A (strain JGS-4024) was cultivated on 5% sheep blood agar plates, with overnight incubation in an atmosphere of 5% CO$_2$: 5% H$_2$: 90% N$_2$ at 37°C. A single colony was then inoculated into 50 ml of pre-reduced Tryptic Soy Broth (TSB) and incubated as before. This culture was diluted 1:20 in 400 ml TSB and incubated as before, until the culture reached OD$_{600nm}$ of 1.3. Cultures were centrifuged (10,000xg, 20 min, 4°C) and the pellet resuspended in 40 ml of culture supernatant fluid. The resulting 10x concentrate was dispensed into 4x10 ml aliquots each containing \( \sim2\times10^{10} \) cfu. Aliquots were stored at 4°C for no longer than 12 h. Piglets were inoculated intragastrically with \( \sim2\times10^{10} \) CFU within 4 h of birth. Contact controls were not inoculated.

2.5. Observations

Diarrhea was rated on a scale from 0-3 and scores were assigned to each piglet twice daily (Table 3). Approximately half of all challenged piglets were euthanized [by barbiturate overdose] and necropsied 1 day post inoculation, while the remainder were euthanized and necropsied 2 days post inoculation. Gross lesions were rated and scored 0-3 (Table 4).
<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal, brown solid feces, perineum dry</td>
</tr>
<tr>
<td>1</td>
<td>Yellow/brown, semi-solid feces, perineum dry</td>
</tr>
<tr>
<td>2</td>
<td>Yellow/brown, fluid feces, irritated perineum with caked feces or wet with liquid feces</td>
</tr>
<tr>
<td>3</td>
<td>Moribund or dead</td>
</tr>
</tbody>
</table>
Table 4. Gross lesions. Gross lesions were scored at the time of necropsy.

<table>
<thead>
<tr>
<th>Score</th>
<th>Description</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>Normal, no gas in colon or small intestine, no hemorrhage, firm colonic fecal contents, normal semi-fluid small intestine fecal contents.</td>
</tr>
<tr>
<td>1</td>
<td>Gaseous, distended colon with pasty fecal contents, normal small intestine</td>
</tr>
<tr>
<td>2</td>
<td>Gaseous, distended colon and ileum, pasty to watery fecal contents in colon and small intestine.</td>
</tr>
<tr>
<td>3</td>
<td>Gaseous, distended colon, ileum and jejunum, colon and small intestine with pasty to watery fecal contents. Ventral abdominal bruising.</td>
</tr>
</tbody>
</table>

Fecal samples were examined by bacteriologic culture on *Clostridium perfringens* selective agar (CPSA) [37 g brain heart infusion agar (BHI; Beckton Dickinson, Sparks, MD), 5 g yeast extract (Beckton Dickinson), 1 g sodium metabisulfite, and 10 g technical grade agar (Becton Dickenson) in 1 L H₂O]. Autoclaved CPSA was supplemented with D-cycloserine (500 µg/ml final concentration) and 5% ml defibrinated bovine blood. Small intestinal contents were also tested for the presence of CPA via a commercial EIA test kit (CPA Toxin Antigen Detection Kit, BioX Diagnostics, Jemelle, Belgium).

Tissues fixed for ≤ 48 h in 10% buffered formalin were trimmed, embedded in paraffin, sectioned (5µm thickness) and stained with haematoxylin and eosin (H&E).
Quantitative analysis of jejunal villous atrophy was based upon villous length in micrometers (Figure 4).

2.6. Exclusion of Other Pathogens

Piglets from each litter were examined by bacteriologic culture for pathogenic *E. coli*. Intestinal content collected post-mortem was cultured on Tergitol-7 (Sigma-Aldrich, St. Louis, MO) agar and incubated overnight at 37°C. Isolates of *E. coli* were tested via multi-plex PCR for virulence genes, including those for Stx2e, StB, LT, StAP, F18, F41, K88, K99 and 987P.

3. Results

3.1. Serology

Gilts in Groups II (native CPA toxoids) and III (rCPA and rCPB2) developed geometric mean CPA-neutralizing titers of 1:4.6 and 1:7.0, respectively. Group III gilts had post-vaccination geometric mean EIA titers against rCPB2 of 1:1213, while non-vaccinated animals (Group I) remained sero-negative to both antigens. Piglet serum titers were similar to those of their respective dams (Figures 1 and 2).

3.2. Post Challenge Clinical Observations

 Clinically-diseased piglets presented with transient diarrhea typical of type A infections, as demonstrated by diminishing scores in Group I piglets of 1.7 and 1.3 on days 1 and 2 post-challenge, respectively. There were no statistically significant
differences in cumulative or daily diarrhea scores between vaccinated and control pigs. However, differences between each test group and its respective population of non-challenged contact controls were statistically significant; p<.001 (Figure 5).

Gross findings consistent with *C. perfringens* type A infection included gas- and fluid-filled colon, ileum, and/or jejunum (Figure 3). Distention due to liquid, gaseous content was common in ileum and colon, but rare in jejunum. Cumulative day 1 and day 2 post-challenge necropsy scores in Groups I, II and III were 1.16, 0.94, and 0.9, respectively. However, contact pigs in Group I had a mean score of 0.81 and those from vaccinated dams (Groups II and III) did not have gross lesions (Figure 6).

Specimens with villous atrophy (Figure 4B) were rare. Therefore, mean jejunal villous lengths are only subtly different between non-immunized and passively immunized piglets (Group I, 1099 µm; Group II, 1226 µm; Group III, 1233 µm; p>0.2). Differences in villous atrophy/degeneration between test groups was more apparent, but upon quantitative assessment of microscopic lesions, were not significant. Small intestine degeneration was observed in 37% of control pigs, 25% of Group II pigs and 20% of Group III piglets. Prevalence and severity of villous atrophy in non-challenged pigs was reduced to less than half of that of inoculated litter-mates (Figure 7).

Bacteriologic culture of gut contents obtained at necropsy revealed large numbers of *C. perfringens* and elevated levels of CPA. All pigs were culture positive following challenge and pigs in each test group had ~2x10⁷ CFU per gram of feces (Figure 8). CPA was detected in feces of 69%, 6% and 54% of pigs in Groups I, II and II, respectively (Figure 9). Large numbers of *E. coli* were isolated from 5 pigs (Group I, 3 pigs; Group
II, 1 pig; Group III 1, pig). One isolate was Stx positive (Group III pig), but the virulence genes for which we tested were absent from all other isolates.

4. Discussion

These experiments demonstrate the complexity behind porcine *C. perfringens* type A enteritis pathogenesis and identification of protective immunizing antigens. *Clostridium perfringens* produces more than 17 toxins, adherence proteins, acid resistance proteins, and bacteriocins all of which could play a role in the development of disease. Genomic comparisons between strains of different origins (i.e., poultry vs porcine) reveal differences of ≥300 genes (Songer unpublished data). Due to CPA’s potency and CPB2’s prevalence in porcine type A strains, these two toxin have been hypothesized to have a role in piglet diarrhea. Findings from evaluation by vaccination:challenge approach suggest that typical disease occurs in the face of immunization with CPA and/or CPB2, even with CPA neutralized in feces (Group II) suggest that other factors are involved.

Piglets in all treatment groups inoculated with challenge strain JG4024 developed signs of type A enteritis and these studies contribute to disease characterization. Transient diarrhea was the most prominent indicator of disease. Not withstanding, gross lesions were most apparent in colon which serves as the end-stream receptacle for diarrhea created in the small intestine, but microscopic lesions in jejunum confirm the source of the diarrhea. Intestinal distention resulting from gaseous, liquid fecal contents progressed proximally with an increase in disease severity. Moribund field specimens
appear bloated due to extreme jejunal distention. Evidence of *C. perfringens* Type A disease was additionally confirmed by reduced disease severity in contact control pigs and results of exclusive diagnostics.

Immune responses elicited by native CPA or rCPA and rCPB2 vaccine formulations were inadequate in nature or extent to control type A enteritis. Testing of fecal contents for the presence of CPA best demonstrates this. Unlike immunization with rCPA or rCPB2, passive immunization with native toxoid was great enough to affect GI tract CPA concentrations, yet these animals had diarrhea and gross and microscopic lesions similar to that experienced by challenged control pigs. Interestingly, mean gross lesion scores in contact control pigs from non-immunized dams were similar to those of challenged litter mates while non-challenged/immunized pigs had few or no gross lesions.

This information demonstrates the complexity of *C. perfringens* Type A disease. Late log phase cultures such as those used here contain a plethora of proteins unaccounted for. Immunization against CPA and/or CPB2 may stifle, but not eliminate, pathogenesis in low level, passively infected, pigs such as experienced by contact controls. However, challenged piglets did not have immunity enough to prevent development of clinical disease, gross or microscopic lesions. Most likely, different virulence factors manifest in a variety of disease symptoms. A multivalent vaccine formulation will be required for complete protection for type A enteritis. In-depth genomics studies are needed for identification of additional virulence genes which could result in at better understanding and control of pathogenesis.
Figure 1. Cpb2 Serology. Serum samples were pre-diluted 1:100. For the purpose of calculating geometric mean titers, samples with a titer of <100 were assigned a titer of 50.
Figure 2. Cpa Serology. Serum dilutions started at 1:1 (undiluted). For the purpose of calculating geometric mean titers, samples with a titer of <1 were assigned a titer of 0.5.
Figure 3. Gross lesions. *C. perfringens* Type A enterocolitis manifests with a distended fluid filled colon (a). More advanced clinical specimens reveal distended colon and small intestine (b).
Figure 4. Microscopic lesions. Villi were measured in micrometers from crypt base to villous tip. Mean jejunal villous length of healthy (a) small intestine was 1200-1300 μm. Severe (b) jejunal tissues demonstrating atrophy present with mean villous lengths of <700 μm.
Figure 5. Diarrhea Scores. Vaccinates vs controls, p>0.2. Challenged vs respective contacts, p<0.001.
Figure 6. Gross Pathology Summary. Neither daily nor cumulative (day 1 and day 2 post inoculation) mean lesion scores of challenged pigs between controls and vaccine test groups were significant (ANOVA; p>0.2). Cumulative scores are presented.
Figure 7A. Length was measured in µm from crypt base to villous tip. Vac vs control, p>0.2.

Figure 7B. Positive tissues were identified by sparsely populated, blunted, and fused villi with apical epithelial cell irregularities. Fisher’s exact two-tailed p=0.3.
Figure 8. Differences *C. perfringens* colonization between test groups was not significant (ANOVA).
Figure 9. Detection of CPA in feces. Challenged piglets: Native toxoid vs controls; Fisher’s exact two-tailed p<0.0001. Controls vs rCpa+rCpb2; Fisher’s exact two-tailed p=0.3.
Acknowledgments

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References


APPENDIX F

Supplemental data.

Immunohistochemistry in varying ages and breeds of pigs.

*In vitro* colonization of hypervirulent vs. historical strains of *Clostridium difficile*.

1. Introduction

*Clostridium difficile* is an anaerobic, gram-positive, spore-forming bacillus which has become the leading cause of nosocomial diarrhea in humans (Gursoy, Guven et al. 2007). *Clostridium difficile* infection (CDI) manifests as diarrhea, pseudomembranous colitis, and rarely, paralytic ileus, toxic megacolon, bowel perforation, and death (Harbarth, Samore et al. 2001; Nicolasora and Kaul 2008). Disease often occurs in association with antimicrobial usage (Larson, Price et al. 1978). The emergence of a hypervirulent strain (NAP1/027/III) causes increased morbidity and mortality rates as a result of increased toxin production (McDonald, Killgore et al. 2005).

CDI has also emerged as a common syndrome in pigs 1-7 days of age (Songer, Post et al. 2000). Pigs present with yellow, pasty diarrhea, though constipation or obstipation is common (Waters, Orr et al. 1998; Songer, Post et al. 2000; Songer, Post et al. 2000a; Songer 2004; Songer and Anderson 2006). The small intestine is not affected, but mesocolonic edema, accompanied by microscopic lesions in cecum and colon, is characteristic. The lesion consists of focal mucosal epithelial erosion or ulceration; neutrophil infiltration and exudation into the colonic lumen forms the hallmark "volcano
lesion” of CDI. Disease has been reproduced in piglets by intragastric (IG) inoculation with *C. difficile* (Songer, Post et al. 2000).

The principal virulence factors of *C. difficile* are toxins A (TcdA) and B (TcdB), which belong to the family of large clostridial cytotoxins. They are characterized by a receptor binding domain at the carboxyl terminus and an enzymatic domain at the amino terminus (Schirmer and Aktories 2004). The mechanisms of cytotoxicity of TcdA and TcdB are the same; after entry via receptor-mediated endocytosis, they inactivate Rho-subtype GTPases by monoglucosylation (von Eichel-Streiber, Meyer zu Heringdorf et al. 1995; Chaves-Olarte, Weidmann et al. 1997; Faust, Ye et al. 1998). The net effect is dysregulation of actin metabolism, with cell rounding and death.

The carbohydrate Galα1-3Galβ1-4GlcNAc has been identified as a receptor for TcdA, but it is not present in all species (Krivan, Clark et al. 1986; Tucker and Wilkins 1991). The TcdB receptor has not been identified. However, evidence suggests that it occurs widely, but not ubiquitously. TcdB is toxic for a variety of cell types, and has, in fact, 1000x more catalytic activity than TcdA (Voth and Ballard 2005). However, stools from human infants often contain large amounts of toxins (Rolfe 1988), but without producing lesions; this is due to a putative lack of toxin receptors. Lesions from toxins occur in the colon and cecum in most species, but lesions develop in jejunum of foals and rabbits; probably because of unique receptor distribution (Keel and Songer 2006). In neonatal pigs, TcdA, but not TcdB, binds to intestinal brush borders of neonates (Keel and Songer 2007).
CDI is preceded by colonization. Antibody titers against *C. difficile* surface layer proteins (SLPs), such as the flagellar cap protein FliD, flagellin FliC, and cell wall proteins Cwp66 and Cwp84, have been assessed in human patients. Diseased patients had significantly lower SLP IgM antibody levels as compared to a non-CDI control group (Drudy, Calabi et al. 2004; Pechine, Janoir et al. 2005), and these reduced antibody titers can extend to TcdA and TcdB (Pechine, Janoir et al. 2005). These data suggest that immune responses to SLPs may play a role in CDI immunity and/or resistance to recurrence, but they tell us nothing about strain-to-strain differences in colonization potential.

Data from more direct experiments reveal that SLPs play a role in colonization by vegetative cells, but not by spores (Calabi, Calabi et al. 2002). Appendages which facilitate spore adherence have been identified but not characterized (Panessa-Warren, Tortora et al. 1997). Environmental populations of *C. difficile* persist as spores and subsequent CDI occurs via ingestion, but not all such encounters with hosts, even by toxigenic strains, result in disease (Borriello 1998). Variability in spore colonization potential may affect the rate at which disease develops.

Severity of CDI may be a function of host susceptibility and/or strain virulence. Human CDI has been known for 30 years, but it has apparently become a problem only recently in pigs (Songer, Post et al. 2000). This may be due to delayed exposure of pigs to the organism, failure to recognize the disease in pigs prior to the late 1990s, or to emergence of strains virulent for pigs. Susceptibility of the pig may also be affected by the availability of toxin receptors, in that those for TcdB apparently do not exist in
neonates (Keel and Songer 2007), at least not in those of today’s predominant genetic makeup. Other breeds may have receptors for both toxins, or neither, if the latter, it might be that genetic manipulation of pigs for enhanced productivity has brought with it the TcdA receptor and susceptibility to CDI. We examined the binding of biotinylated TcdA and TcdB to colonocytes of pigs from different breeds and ages, and compared rates of adherence of historic and hypervirulent strains to Caco-2 cells.

2. Materials and Methods

2.1. Immunohistochemistry

2.1.1. Toxins

Colonies of strain VPI 10463 were inoculated into 10 ml of brain heart infusion agar with yeast extract (0.5%), cysteine hydrochloride (0.05%) (BHI-CYE) broth in a Hungate tube, under anaerobic conditions (5% CO₂: 5% H₂: 90% N₂) and incubated at 37°C overnight. Toxins were produced in a 4 L Erlenmeyer flask containing 4 L BHI-CYE, in which a loop of dialysis tubing (MW cutoff 10 kDa) filled with HPLC-grade water was suspended. The tubing was inoculated with 1 - 2 ml of the overnight culture and the flask incubated at 37°C in an aerobic incubator for 5 - 7 days. Supernatant was clarified by centrifugation (15,000 x g, 20 min) and filtration (0.22 µm pore diameter). Toxins were purified by ion-affinity chromatography over DEAE sepharose (Amersham Biosciences, Uppsala, Sweden). Two ml samples of culture supernatant were applied to the column automatically via an IV-7 injection valve (Amersham). Fractions of 5 ml were collected on a GradiFrac™ rotary fraction collector (Amersham) and protein elution
was monitored by a UV-1 ultraviolet monitor (Amersham). TcdA was eluted with a linear gradient of 0.05-0.25 M NaCl in 50 mM Tris-HCl at a pH of 7.4. TcdB remained in the column and was recovered via a linear gradient of 0.25-0.6 M NaCl in 50 mM Tris-HCl at pH 7.4. Fractions of interest were pooled and concentrated by centrifugal filtration (MW cutoff= 100 kDa; Millipore, Bedford, MA). Toxin concentration was determined by a Bradford assay per the manufacturer's instructions (BioRad, Hercules, CA). Toxin purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

Purified TcdA and TcdB solutions were prepared for biotinylation by exchanging Tris solution substrate with PBS via flow through desalting column (Zeba™, Pierce Chemical Co., Rockford, IL). First, the column was prepared by adding PBS directly to the column and centrifuging (1000 x g, 2 min). Purified toxins were applied directly to the spin column, centrifuged (1000 x g, 2 min) and collected. Protein concentrations of desalted toxins were determined by the methods of Bradford (Bio-Rad, Hercules, CA). Biotin (Pierce), in 20 mM excess of protein concentration, was added to each desalted toxin preparation and incubated at room temperature for 30 min. Unbound biotin was removed by centrifugation (1000 x g, 2 min) through a desalt spin column (Zeba™).

2.1.2. Immunohistochemistry

Colon tissues were collected from pigs of varying genetic backgrounds (Yorkshire, Hampshire, PIC genetics), and ages (~8 weeks; slaughter, ~22 weeks; multiparous sow), fixed in 10% buffered formalin for <48 h, trimmed and embedded in paraffin. Sectioned (5μm) tissues were deparaffinized and blocked by avidin (DAKO
Corp., Carpenteria, CA) followed by biotin for 15 min each at room temperature. Slides were washed between each step with 50 mM Tris-HCL pH 7.2. Biotinylated TcdA or TcdB diluted 1:8 in antibody diluents (DAKO) were added and slides were incubated as before. Antibody diluent was added to negative control slides. Streptavidin-horseradish peroxidase and diaminobenzidine chromagen (DAKO) was added for 7 min, and slides were washed and counterstained with haemotoxylin (2 min). Slides were examined by light microscopy for bound toxins.

2.2. Aherence Assay

Strains JGS6125 and JGS667 were cultivated and harvested as described above and purified by density gradient centrifugation (Tamir and Gilvarg 1966). Caco-2 BBE (C2BBE) human intestinal epithelial cells were maintained in high-glucose (25 mM) Dulbecco’s Minimal Eagle Medium (DMEM) with 10% fetal bovine serum, 20 mM HEPES, 100 IU/ml penicillin, 100 µg/ml streptomycin and incubated at 37°C in an atmosphere of 5% CO₂. Cells were grown to confluent monolayers (1x10⁶ cells/well) in 6 well tissue culture plates (Corning, Corning, NY). Twenty four hours prior to inoculation with C. difficile spores, media was exchanged with antibiotic-free and serum-free DMEM. Centrifuged spores were resuspended in DMEM and applied to C2BBE monolayers at 20 or 60 multiplicities of infection (spores:C2BBE) in a volume ≥ monolayer submersion. Infected monolayers were incubated at 37°C for 40 min in an atmosphere of 5% CO₂. Monolayers were washed three times with PBS, for removal of non-adherent spores, and harvested via cell scraper. Adherent spores were enumerated
by dilution plating and results were reported as percent adherence (calculations; recovered spores/input spores x 100).

3. Results

Biotinylated TcdA bound to colonocytes of neonates, regardless of breed. The age of the pig was more discriminatory. Evidence of receptor mediated binding was apparent in nursery and slaughter aged pigs, but labeled toxin did not bind to tissue from sows (Figure 1). Biotinylated TcdB did not bind to any of the colon tissue tested.

Historic strain JGS 667 and hypervirulent strain JGS6125 had adherence percentages of 5 and 3.5, respectively with a multiplicity of infection (moi) ratio of 20. Adherence between the two strains was closer (JGS667, 2.8%; JGS6125, 3%) with a moi ratio of 60.

4. Discussion

Results from these IHC experiments confirmed lack of TcdB binding to porcine colonic brush borders in alternate ages and breeds. However, these data do not preclude a role for TcdB in pathogenesis. Recent experiments suggest that TcdB from ribotype 027 strains may possess a unique binding domain which could lend to receptor mediated endocytosis (Stabler, Dawson et al. 2008). Purified toxin used in these experiments were not from the hypervirulent strain. Or, cellular intoxication may occur by alternative mechanisms such as pH-mediated channel formation in host cell lipid bilayers (Barth,
Pfeifer et al. 2001). TcdB emulsified in digesta could be endocytosed mechanisms of nutrient uptake instead of by receptor mediation (Stange 1983).

*In vitro* assessment of the two strains showed similar percent adherence between the two strains suggesting that hyper-virulence is not attributable to increased colonization potential of spores. Future experiments with vegetative cells may not agree.
Figure 1. Biotinylated TcdA binds to colonocyte brush borders of neonates (A), nursery aged (B), and slaughter pigs (C), but not sows (D). Labeled TcdB did not bind to any of the tissues tested.
References


