THE PATHOGENESIS OF *CLOSTRIDIUM DIFFICILE*-ASSOCIATED DISEASE IN NEONATAL PIGS

by

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As members of the Final Examination Committee, we certify that we have read the dissertation prepared by Michael Kevin Keel entitled THE PATHOGENESIS OF CLOSTRIDIUM DIFFICILE-ASSOCIATED DISEASE IN NEONATAL PIGS and recommend that it be accepted as fulfilling the dissertation requirement for the Doctor of Philosophy.

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ABSTRACT

Clostridium difficile-associated disease (CDAD) in neonatal pigs has emerged as a serious economic concern for swine producers throughout North America. The disease has been diagnosed clinically and reproduced in experimental inoculation trials in pigs, but little is known of the epidemiology or pathogenesis of the disease in pigs.

Strain characteristics and distribution of C. difficile isolates from pigs, calves, dogs, horses, and humans were assessed by PCR-ribotyping. Porcine and bovine isolates were dominated by a single ribotype. This ribotype was uncommon among isolates from other host species; it was particularly uncommon from humans, suggesting there is little transfer of isolates between humans and calves or pigs. The reason for a single common ribotype circulating among distinct bovine and porcine populations is unknown.

The intragastric inoculation of newborn pigs demonstrated their sensitivity to C. difficile toxins. Toxin B (TcdB) surprisingly resulted in more severe lesions than Toxin A (TcdA). The two toxins together acted synergistically. Colon explants were sensitive to TcdA in a dose-dependent manner. However, TcdB did not cause significant lesions in the explants, nor was there any synergism with TcdA. Electron microscopy of colon explants treated with TcdA revealed severe, ultrastructural lesions that accrued in a dose-dependent manner by two h post infection.

Direct immunohistochemistry assays demonstrated specific binding of biotinylated TcdA throughout the gastrointestinal tract of neonatal pigs. The density of bound toxin in different segments correlated with the severity of lesions in those segments from pigs gavaged with TcdA. TcdB did not bind any tissues, though it was
fully active in cell-culture assays. A monoclonal antibody to Galα1-3β1-4GlcNAc-R (α-Gal epitope), a putative receptor for TcdA in pigs, specifically bound the brush border of enterocytes, but the distribution of binding did not correlate with the distribution of TcdA binding.

Specific TcdA binding to the plasmallema of microvilli was also confirmed by immunoelectron microscopy. By five min post inoculation some toxin was already visible in endosomes or free in the cytoplasm. TcdA localized to the mitochondria of epithelial cells and, less frequently, to the nuclei. Endothelial cells and leucocytes in the superficial lamina propria were similarly labeled by toxin.
1. INTRODUCTION

1.1 Problem Definition

*Clostridium difficile* is the single most common bacterial cause of antibiotic-associated diarrhea in humans. In spite of the fact that it has been intensively studied for over 30 years, there are still huge gaps in our knowledge of the fundamental mechanisms of pathogenesis of the disease. A receptor has never been identified for TcdB, one of the principal virulence factors of *C. difficile*, but receptor-mediated endocytosis is widely regarded as an essential first step in toxin action. A variety of receptors have been proposed for TcdA, but their prevalence and distribution on host tissues are poorly characterized. The well-documented resistance of neonates of most species to CDAD is hypothesized to be related to the age-dependent acquisition of epithelial receptors. However, receptor densities have not been properly assessed in the neonates of most species.

1.1.1 Significance of *Clostridium difficile*

Study of CDAD in neonatal pigs would be warranted, just as an additional model of the human disease. However, *C. difficile* has become a significant economic concern for the pig industry in North America and elsewhere. In an industry where profit margins are notoriously thin, any reduction in profits is of considerable consequence to the economic viability of individual producers. Though it is reportedly a disease of relatively low mortality in pigs, CDAD is characterized by high morbidity and can cause significantly reduced weights of hogs at slaughter.
There is currently no information on the source of infection in farrowing units. The pigs develop disease at or near birth, indicating they acquire it from the sow or the environment. Environmental burden is a well-documented problem in hospitals, but patients with CDAD are the obvious source. CDAD has not been diagnosed in sows. A method of strain typing that is discriminatory and is easily performed is needed to better investigate the epidemiology of this disease in pigs.

Information on the susceptibility of pigs and the characteristics of the disease are based on limited case reports and the experimental inoculation of colostrum deprived pigs. These reports are confounded by the failure of experimental inoculation of porcine intestinal loops, with *C. difficile* toxins, to produce lesions. It remains to fully define *C. difficile* as a pathogen of pigs.

1.1.2 Objectives of the Current Research

The principal aims of this research were to 1) establish the distribution of strain types in neonatal pigs, 2) confirm the assumption that neonatal pigs are fully susceptible to the effects of *C. difficile* toxins *in vivo*, 3) establish the dose-dependent response of porcine colon explants to toxins *in vitro* 4) to characterize the gross, light-microscopic and ultrastructural lesions induced by *C. difficile* toxins, 5) to demonstrate that pigs have significant concentrations of toxin receptors at the mucosal surface of intestinal epithelial cells, and 6) to assess the importance of the α-Gal epitope, known to occur on pig cell membranes, in the pathogenesis of CDAD in pigs.
1.2 Literature Review

1.2.1 Overview of CDAD

*Clostridium difficile* is a Gram-positive, anaerobic, spore-forming bacillus commonly associated with diarrhea and colitis in humans and other mammals. It was first isolated from feces and meconium of asymptomatic newborn infants, and was originally named *Bacillus difficilis* due to its morphology and the difficulties encountered in cultivating it (Hall and O'Toole 1935). Though the initial report described the production of a potent toxin, the organism was not considered a significant pathogen due to the high carrier rate among asymptomatic human infants. In the past 25 years, however, *C. difficile* has been implicated as the principal infectious cause of antibiotic-associated diarrhea in adult humans, and similar clinical conditions in a variety of other mammals. It is now recognized as one of the most important nosocomial pathogens of humans. Clinically-affected humans often experience diarrhea without significant lesions, but the disease can progress, resulting in the severe tissue changes associated with pseudomembranous colitis. More serious sequelae, including prolonged ileus (toxic megacolon), intestinal perforation, and peritonitis, are less common.

The relevance of *C. difficile* to disease in animals has also become more obvious. Lesions in non-human mammals are similar to those in humans but vary widely in severity and distribution within the gastrointestinal tract. This variation is evident for both different species and different age groups within a species. Among the animals commonly used for laboratory research, Syrian hamsters (*Mesocricetus auratus*) are the most sensitive to naturally-acquired disease. Lesions attributed to *C. difficile* infection
have also been documented in guinea pigs (*Cavia porcellus*), mice (*Mus musculus*), rats (*Rattus norvegicus* and *Rattus rattus*) and rabbits (*Oryctolagus cuniculus*), and all these species are used as animal models of the human disease. *Clostridium difficile* is also an etiologic agent of intestinal disease in a variety of other species including horses (*Equus caballus*), hares (*Lepus* spp.), pigs (*Sus scrofa*), nonhuman primates, domestic dogs (*Canis familiaris*), domestic cats (*Felis catus*), ostriches (*Struthio camelus*) and black-tailed prairie dogs (*Cynomus ludovicianus*). This review addresses the pathogenesis of *C. difficile*-associated disease (CDAD) and compares the lesions and clinical conditions among known susceptible species.

### 1.2.2 Pathogenesis of CDAD

The essential virulence factors of *C. difficile* are toxins A (TcdA) and B (TcdB). The corresponding genes, *tcdA* and *tcdB*, respectively, are located on a large pathogenicity island in the bacterial chromosome (Hundsberger, Braun et al. 1997). TcdA is a potent enterotoxin. TcdB is a potent cytotoxin, *in vitro*, but apparently has little activity *in vivo* unless there is prior damage to mucosal epithelium (Lyerly, Saum et al. 1985). The toxins act synergistically; TcdA creates widespread damage to the mucosa, permitting TcdB to affect epithelial cells. Though production of TcdA is more consistently associated with disease, there are reports of CDAD in patients infected solely by TcdA-negative, TcdB-positive strains of *C. difficile* (van den Berg, Claas et al. 2004).

TcdA and TcdB consist of single, large polypeptide chains with folds stabilized by disulfide bonds. Amino-termini are highly-conserved and are catalytic; both function
in the target cell cytoplasm, by the same mechanism. The carboxy-terminus includes the receptor-binding domain and differs significantly between the two toxins. Carbohydrate receptors for TcdA include Galα1-3β1-4GlcNAc-R (α-Gal epitope), Galβ1-4{Fuα1-3}GlcNAc (Lewis X), Galβ1-4GlcNAcβ1 (Lewis Y), Galβ1-14GlcNAcβ1-3Galβ1-4(Glc) (Lewis I), and sucrose-isomaltase (Krivan, Clark et al. 1986; Tucker and Wilkins 1991; Pothoulakis, Gilbert et al. 1996). These receptors are on the brush border of susceptible intestinal epithelial cells and may occur at other sites as well. A receptor for TcdB has never been identified, but may be present on the basolateral aspect of epithelial cells, hidden when the mucosa is intact. Physical trauma, or the effects of another toxin such as TcdA, may compromise cell-cell contacts, providing TcdB access to receptors that are normally unavailable to molecules on the luminal aspect of the mucosa.

Receptor binding by TcdA initiates endocytosis by coated pits and the vesicles formed subsequently fuse with lysosomes. The mechanism of cell attachment and endocytosis have not been described for TcdB. Acidification of the endolysosome produces a conformational change in the toxin, which is followed by its activation and escape into the cytoplasm (Qa'Dan, Spyres et al. 2000). In the cytoplasm, toxin specifically inactivates Rho, and other Rho-subtype GTPases, by glycosylation of threonine at site 37/35 (Just, Selzer et al. 1995; Just, Wilm et al. 1995; Genth, Aktories et al. 1999). This class of GTPases comprises signal transduction molecules associated with apoptosis and maintenance and regulation of cytosolic actin filaments. Actin filaments are a necessary part of certain cell adhesion molecules (e.g. zonula adherens, tight junctions and focal adhesions) and their disruption results in loss of cell-cell contacts,
increased paracellular permeability of mucosal surfaces, cell rounding, and, eventually, cell death (Pothoulakis and Lamont 2001).

TcdA and TcdB have many effects and amplify the disease process beyond disruption of function and death of mucosal epithelial cells. Particularly important is initiation of an inflammatory cascade that can result in increased damage to host tissues and exudation of fluid. TcdA causes mitochondrial dysfunction, with depletion of ATP and the intracellular generation of reactive oxygen intermediates (He, Hagen et al. 2000). The oxidative burst is rapidly followed by degradation of IκB and nuclear translocation of NF-κB, leading to elaboration of IL-8 and possibly other proinflammatory mediators (He, Sougioultzis et al. 2002). Degradation of IκB occurs before glycosylation of Rho and is inhibited by antioxidant pretreatment of TcdA-exposed cells. IL-8 is a chemokine that is associated with mobilization, activation, and degranulation of neutrophils, as well as chemotaxis of basophils and T-cells. Another potent chemotaxin of neutrophils, macrophage inhibitory protein-2 (MIP-2), is elaborated by rat ileal epithelial cells within 30 min of exposure to TcdA (Castagliuolo, Keates et al. 1998). TcdA can also directly stimulate in vitro migration of neutrophils (Kelly, Becker et al. 1994). Neutrophils play a prominent role in the pathophysiology of CDAD and directly contribute to necrosis of host tissues (Triadafilopoulos, Pothoulakis et al. 1989). Inhibition of neutrophil infiltration by blocking the activity of CD18 or MIP-2 reduces the severity of lesions and decreases exudation of fluid into the intestinal lumen (Kelly, Becker et al. 1994; Castagliuolo, Keates et al. 1998).
Macrophages and monocytes are also involved in the inflammatory cascade associated with CDAD. TcdA causes macrophages in the lamina propria to express cyclooxygenase-2 (Cox-2), with subsequent production of prostaglandin E2 (PGE2) (Alcantara, Stenson et al. 2001). The effect is probably due to direct action of TcdA on intestinal macrophages, as opposed to the influence of intermediary cytokines released by epithelial cells or some other cell type. Circulating monocytes produce IL-1, IL-6, IL-8 and tumor necrosis factor-α (TNFα) in response to application of TcdA (Flegel, Muller et al. 1991; Linevsky, Pothoulakis et al. 1997). PGE2 inhibits sodium chloride and water absorption in the intestine and induces chloride secretion by enterocytes. In addition, it induces vasodilation that may contribute to effusion across the intestinal mucosa. The secretory effects of TcdA were inhibited by administration of a Cox-2 inhibitor (Alcantara, Stenson et al. 2001).

Mucosal mast cells may also be involved in development of CDAD. Administration of TcdA causes degranulation of mast cells within 15-30 min of toxin exposure (Castagliuolo, LaMont et al. 1994). Mast-cell deficient mice have reduced infiltration of neutrophils and intestinal secretion in response to TcdA challenge, but reconstitution of the mice fully restores their susceptibility (Wershil, Castagliuolo et al. 1998).

The disease and lesions of CDAD also have a neural component. Release of substance P apparently results from direct stimulation of primary sensory neurons by TcdA; this, in turn, stimulates mast-cell degranulation (Pothoulakis, Castagliuolo et al. 1994; McVey and Vigna 2001). Substance P is also associated with mast-cell
independent pathways of inflammation, through its potent vascular effects and its ability to increase vascular permeability by directing formation of endothelial gaps in venules. TcdA inhibits release of norepinephrine from sympathetic postganglionic nerve fibers in the small intestine and directly causes excitation of enteric secretomotor neurons in the submucosal plexus (Xia, Hu et al. 2000). The net result is increased secretion of water and electrolytes into the intestinal lumen. Denervation of ileal loops greatly reduces both secretion and inflammation due to TcdA, but the protective effect is overcome by higher dosages of toxin (Mantyh, McVey et al. 2000; Sorensson, Jodal et al. 2001).

A small percentage of C. difficile strains produce an actin-specific ADP-ribosyltransferase, CDT, which is unrelated to TcdA or TcdB (Perelle, Gibert et al. 1997). CDT is a binary toxin formed by separate, unlinked polypeptides, CDTa and CDTb. CDTb binds a cell-surface receptor and mediates translocation of the enzymatic component, CDTa. Once in the cytosol, CDTa catalyzes the ADP-ribosylation of monomeric actin leading to depolymerization of actin filaments and disruption of the cytoskeleton. The binary toxin is directly toxic to CHO cells and may be an additional virulence factor. However, the vast majority (approximately 95%) of clinical isolates have TcdA and/or TcdB but not CDT (Goncalves, Decre et al. 2004). The binary toxin has not yet been identified in isolates lacking tcdA and tcdB.

The pathogenesis of CDAD is a complex process involving direct effects of C. difficile toxins (TcdA and TcdB) on the mucosal epithelium, initiation of an inflammatory cascade, and direct involvement of the peripheral nervous system. However, the most important step in this process is receptor-mediated uptake of toxins.
If toxin endocytosis is blocked by antibodies against the carboxy terminus of the proteins, or by truncation of the toxins, lesions do not develop (Kink and Williams 1998). TcdA receptors have been identified on the brush border of enterocytes from multiple species, but a receptor for toxin B remains to be discovered. CDT is not an essential virulence factor, and most isolates associated with clinical cases do not carry the genes for this binary toxin.

1.2.3 Epidemiology and Prevalence

The carrier rate of *C. difficile* varies among asymptomatic individuals by species and, within a species, by age and other population characteristics. Up to 80% of human infants are culture positive, though disease is almost unheard of in this group. Infants may be refractory to disease because they lack toxin receptors. The next highest prevalence of asymptomatic infections is among hospital patients, of whom approximately 20% are culture positive (McFarland, Mulligan et al. 1989; Johnson, Clabots et al. 1990). The bacteria are usually acquired from the hospital environment, and culture positivity and incidence of CDAD increase with length of stay (Johnson, Clabots et al. 1990; Clabots, Johnson et al. 1992; Bignardi 1998).

The carrier rate of *C. difficile* is very low in both asymptomatic foals and adult horses. The rate of isolation from clinically-normal adults is 0-4.3%, and most studies found that normal foals are culture negative (Jones, Adney et al. 1987; Madewell, Tang et al. 1995; Baverud, Gustafsson et al. 1997; Weese, Staempfli et al. 2001). In contrast, one investigation found a 29% culture-positive rate for asymptomatic foals less than 14-days
old and a 0.6% carrier rate for older foals (Baverud, Gustafsson et al. 2003). The rate of
*Clostridium difficile* isolation from adult horses with diarrhea varies from 12.7-90%, and 16.7-63%
of diarrheic foals are infected (Jones, Adney et al. 1987; Madewell, Tang et al. 1995;
Weese, Staempfli et al. 2001). However, the highest culture rates were found in CDAD
outbreaks and might not accurately reflect the rate in all cases of equine diarrhea. Of all
equine diarrhea cases at one veterinary teaching hospital, 20-25% are attributed to *C.
difficile* (Weese, Staempfli et al. 2000).

Prevalence of *C. difficile* in normal cats may range from less than 10% to nearly
40%, although many isolates may be nontoxigenic (Borriello, Honour et al. 1983; Riley,
Adams et al. 1991). The situation is much the same in normal dogs, with culture
positivity ranging from approximately 20% to 40% and perhaps 50% of isolates found to
be nontoxigenic (Borriello, Honour et al. 1983; Riley, Adams et al. 1991; Struble, Tang
et al. 1994; Madewell, Bea et al. 1999). These results are in stark contrast to a study in
which 35 laboratory beagles, used in a bone marrow graft study, were culture-negative
for *C. difficile* though a varied flora was present in all dogs (Haralambie and Schmidt-
Weinmar 1988).

Data concerning carrier rates or prevalence of CDAD in other species are lacking.
Nonetheless, numerous case reports document CDAD in other mammal species and
rarely among other vertebrates.

1.2.4 Predisposing Factors
The major predisposing factors for most species are antibiotic therapy and, for humans and horses, hospitalization (Johnson, Gerding et al. 1990; Madewell, Tang et al. 1995; Baverud, Gustafsson et al. 1997). Hospitalization increases the risk of CDAD due to persistent environmental contamination with *C. difficile*. Rates of isolation from surfaces in human hospitals vary from 11.7% to 29% (Kim, Fekety et al. 1981; McFarland, Mulligan et al. 1989). The contamination rate in one veterinary teaching hospital, where CDAD was commonly diagnosed in horses, was 6.3%, but another hospital had contamination rates less than half that figure (Weese, Staempfli et al. 2000; Baverud, Gustafsson et al. 2003). Hospitalization has not been documented as a predisposing factor for CDAD in species other than humans and horses but environmental burden of *C. difficile* spores is likely related to the incidence of disease.

CDAD can potentially develop after administration of any antibiotic, even those that are efficacious against *C. difficile* (Kelly and Lamont 1993). The LD$_{50}$ of subcutaneous clindamycin for hamsters is 50 to 100 $\mu$g / Kg and typhlitis occurs as soon as 48 hours after treatment (Lusk, Fekety et al. 1978). Applied topically, 1 mg of clindamycin was uniformly fatal to hamsters and 0.1 mg caused 50% mortality due to CDAD (Feingold, Chen et al. 1979). Hamsters are much more sensitive to *C. difficile* than other species and, as a consequence, are much more likely to develop CDAD as a sequelae of antibiotic administration.

For many species, clindamycin and vancomycin therapy are a greater risk factor than other antibiotics. However, human cases of CDAD are more often associated with ampicillin and cephalosporins due to their more extensive use (Thomas, Stevenson et al.
Similarly, CDAD in horses has been closely-associated with administration of β-lactam antibiotics, presumably due to their prevalence of use (Baverud, Gustafsson et al. 1997). Erythromycin has also been implicated as a risk factor for acute colitis due to *C. difficile* in horses (Gustafsson, Baverud et al. 1997; Baverud, Franklin et al. 1998).

For both hamsters and guinea pigs, fatal enteric disease has long been observed in association with antibiotic administration (Knoop 1979; Lowe, Fox et al. 1980). CDAD is most commonly associated with antibiotic therapy but there is a report of *C. difficile*-associated typhlitis in hamsters without any treatment (Rehg and Lu 1982). In that case the hamsters were housed in a room where multiple hamsters died from *C. difficile* after receiving tetracycline, suggesting that an increased environmental burden of *C. difficile* increased the likelihood of CDAD in untreated hamsters. Anecdotal reports of CDAD in hamsters without antibiotic treatment suggests it is a common occurrence. This is analogous to the increased prevalence of CDAD among hospital patients, who are often housed in an environment rich in *C. difficile* spores.

Sporadic case reports of disease in captive exotic species have also been associated with antibiotic administration. A captive Kodiak bear developed pseudomembranous colitis after initiation of antibiotic therapy for a draining lumbosacral abscess (Orchard, Fekety et al. 1983). Kanamycin was administered for 1 week, then the treatment was changed to tetracycline. Eight days after initiation of the tetracycline, the bear developed bloody diarrhea. CDAD followed cefoxitin therapy in prairie dogs and this species was proposed as a potential model for human pseudomembranous colitis (Muller, Pitt et al. 1987). *Clostridium difficile* was cultured from all antibiotic treated
animals but from none of the controls. All treated animals developed diarrhea and 4 of the 5 had pseudomembranous typhlitis.

Maturity is a well documented, but species-dependent, prerequisite for CDAD; neonates of many species (e.g. humans, hamsters, and rabbits) are unaffected by *C. difficile* infection. In one study, more than half of the clinically normal human infants tested were carriers of *C. difficile*, though normal adults were all culture negative (Larson, Price et al. 1978). Human cases of CDAD nearly always occur among adults, predominantly among elderly patients (Bignardi 1998). TcdA does not affect ileal explants from 5-day-old rabbits, even at dosages that cause severe lesions in ileal explants from adults (Eglow, Pothoulakis et al. 1992). A prominent hypothesis to explain the resistance of such neonates is that they lack the proper toxin receptors until later in life. TcdA binding to ileal brush borders is decreased in neonatal rabbits, but maximal binding is observed in rabbits 90 days old (Eglow, Pothoulakis et al. 1992). However, the binding kinetics of TcdA to intestinal brush borders of hamsters were similar for adults and infants and could not account for the age-related susceptibility to CDAD (Rolfe 1991).

In contrast to neonatal rabbits, newborn hares are sensitive to the enteric effects of *C. difficile*. Inoculation of young hares resulted in inflammation and necrosis, primarily, of the duodenum, cecum, and colon (Dabard, Dubos et al. 1979). However, the severity of disease was greater in co-infections with *Clostridium perfringens* and/or *Clostridium tertium*, even though these species failed to cause disease in monoassociated hares. The susceptibility of adult hares to CDAD has not been described.
Horses are susceptible as adults or foals, and may develop CDAD in the first few days of life (Jones, Adney et al. 1988; Madewell, Tang et al. 1995; Weese, Parsons et al. 1999). However, spontaneous disease in pigs is apparently an exclusive disease of neonates, with a mean age of 5 days (range 1 to 14 days) (Waters, Orr et al. 1998). There is a single case report of pseudomembranous typhlocolitis in 8-week-old pigs, but *Salmonella typhimurium* was cultured in addition to *C. difficile* (Jones and Hunter 1983). Our unpublished data and anecdotal evidence confirm this as a neonatal disease of pigs.

Dietary factors reportedly can contribute to development of the disease in laboratory animals. An atherogenic diet, high in saturated fat and cholesterol, purportedly led to mortality due to *C. difficile* when fed to Syrian hamsters (Blankenship-Paris, Chang et al. 1995). High levels of dietary casein and corn starch greatly increased the likelihood that *C. difficile*-associated typhlitis would develop in hamsters, after treatment with vancomycin (Michelich, Nunez-Montiel et al. 1981). More extensive epidemiologic investigations are needed to better delineate the risk factors of CDAD in various animal species, particularly with regard to the role of environment, normal husbandry practices, and length of stay at a veterinary clinic.

1.2.5 Sensitivity and Host Susceptibility

Hamsters are exquisitely sensitive to *C. difficile* infection and, as with humans, disease manifested itself as antibiotic-associated enterocolitis long before a definitive pathogen was identified (Small 1968). Hamsters pretreated with clindamycin can develop fatal disease when inoculated with as few as one colony forming unit (CFU) of
C. difficile (Larson, Price et al. 1978). TcdA administered intragastrically (IG) in doses as low as 0.08 mg/kg causes diarrhea and lesions in hamsters, whereas 0.16 mg/Kg is uniformly fatal (Lyerly, Saum et al. 1985). Spontaneous disease is uncommon in mice, but they are susceptible to the toxins and experimental infection, though much less so than hamsters. Intragastric inoculation of mice with 2 mg TcdA/Kg caused diarrhea and lesions but no deaths (Lyerly, Saum et al. 1985). Rats seem to be the most resistant laboratory animals to the effects of C. difficile toxins; the same dosage (2 mg/kg administered IG) that caused disease in mice, and was fatal for hamsters, had no effect on rats (Lyerly, Saum et al. 1985). In spite of their decreased sensitivity to toxin, they are often used as experimental models, especially in situations where a surgical model (e.g. inoculation of intestinal loops) is needed and mice or hamsters are simply too small. Though naturally-occurring CDAD has apparently never been reported in rats, gnotobiotic rats develop clinical disease and lesions characteristic of C. difficile infection when inoculated with toxigenic strains (Czuprynski, Johnson et al. 1983).

CDAD occurs in laboratory rabbits, although Clostridium spiriforme is probably a much more significant cause of spontaneous disease. Peracute death, without clinical signs, is a common feature of CDAD in rabbits (Carman and Evans 1984; Perkins, Fox et al. 1995). Rabbits are an adequate experimental model, but, like rats, are most often used in experiments requiring surgery, particularly the ligation of intestinal loops.

Chronic diarrhea in dogs has been attributed to C. difficile infection. Treatment with metranidazole may be followed by persistent relapse of the disease after cessation of therapy (Berry and Levett 1986). Clostridium difficile toxins were detected in 21% of
dogs with diarrhea, but also in 7% of asymptomatic dogs (Weese, Staempfli et al. 2001). In the same study, 2% of diarrheic dogs, but no asymptomatic dogs, were culture positive for *C. difficile*. Two cats from the same household were diagnosed with CDAD, based upon detection of toxins, responsiveness of clinical signs to metranidazole therapy and, for one of the cats, the exclusion of other potential etiologic agents (Weese, Weese et al. 2001).

CDAD is rarely reported in non-human primates, but Old-World and New-World monkeys are known to be susceptible to naturally-occurring disease and to the effects of purified toxins. Post-antibiotic diarrhea was reported in *C. difficile*-infected cotton-top tamarins (*Saguinus oedipus*) (Rolland, Chalifoux et al. 1997). Affected tamarins were 9 months to 4-years old and died 9-38 days after the last dose of antibiotics. The 2 individuals with the most severe clinical signs had relatively mild lesions, consisting of only mucosal congestion and crypt dilatation. Individuals with severe lesions had mucosal to transmural necrosis extending from the cecum to the anus. Juvenile rhesus monkeys were susceptible to intravenous or intraperitoneal inoculation of TcdA or TcdB but spontaneous disease has never been reported in this species (Arnon, Mills et al. 1984). *Clostridium difficile* toxins were identified in both long-tailed and pig-tailed macaques (*Macaca fascicularis* and *M. nemestrina*, respectively) with diarrhea (Tsai 1986). The rate of *C. difficile* isolation was lower from macaques without diarrhea, leading the author to conclude that CDAD may have been responsible for at least some of the cases.
There are apparently no published reports of *C. difficile* causing disease in apes, but it has been diagnosed in a single orangutan (*Pongo pygmaeus*) (J.S. Brazier, personal communication). Case reports of CDAD in primates describe a clinical syndrome and lesions similar to that described in other species. It may be that the relative infrequency of cases is due to under-reporting or a lack of predisposing factors, rather than a resistance to infection or the effects of *C. difficile* toxins.

*Clostridium difficile* has been cultured from asymptomatic birds and some poikilotherms, but it is generally a disease of mammals. There is one case report of CDAD in a group of 9-day-old ostriches with severe hemorrhagic typhlitis and colitis (Frazier, Herron et al. 1993). Fecal samples yielded cultures of toxigenic *C. difficile*. The flock responded favorably to dietary changes, inoculation with adult gastrointestinal flora and a change in antibiotic therapy from sulfamerazine to penicillin. *Clostridium difficile* is also an infrequent cause of hepatitis in ostrich chicks (Shivaprasad 2003).

**1.2.6 Clinical Syndromes & Lesions**

The distribution and severity of lesions varies among susceptible species infected with *C. difficile*. Generally, however, the lesions associated with naturally-occurring CDAD have an aboral distribution, primarily affecting the colon and/or cecum and variably the ileum. The disease among individuals of any species can vary greatly and this has been most consistently documented in humans. The majority of people infected with *C. difficile* remain asymptomatic, but a portion will develop diarrhea with or without evidence of colitis. Usually diarrhea occurs in the absence of grossly-visible colitis or...
systemic signs (McFarland, Mulligan et al. 1989). In most cases the colitis is not associated with pseudomembrane formation, but may be characterized by diffuse or patchy erythema. Clinically, such patients describe watery diarrhea, abdominal pain, nausea, anorexia and malaise. Some also have low-grade fever and polymorphonuclear leukocytosis. A more-severe colitis is characterized by pseudomembrane formation, usually confined to the rectosigmoid region. It occasionally extends to more proximal parts of the colon rarely involving the entire colon. The clinical syndrome associated with pseudomembranous colitis is similar to that already described, but symptoms are often more severe. A minority of patients, approximately 3%, will develop severe colitis that may progress to more serious, life-threatening complications such as chronic ileus, megacolon, and intestinal perforation (Rubin, Bodenstein et al. 1995).

Lesions in species other than humans generally maintain the aboral distribution but are usually more extensive. Even the few case reports in nonhuman primates describe lesions that extend much further proximally in the gastrointestinal tract than are normally seen in human cases. For example, cotton-top tamarins with CDAD had pseudomembranous lesions extending from cecum to anus (Rolland, Chalifoux et al. 1997). The orangutan described with CDAD had severe colitis but a complete description of the lesions was not available. Other reports of *C. difficile*, in Old-World primates (macaques), also described diarrhea but not associated lesions (Tsai 1986).

The distribution of lesions in hamsters and guinea pigs is predominantly cecal, with occasional extension into the ileum or colon (Bartlett, Chang et al. 1978; Lusk, Fekety et al. 1978; Lowe, Fox et al. 1980; Rehg and Lu 1982). The cecum is usually
distended by fluid and is often hemorrhagic. Affected hamsters die after a very brief bout of diarrhea or without any clinical signs (Bartlett, Chang et al. 1978; Blankenship-Paris, Walton et al. 1995; Sambol, Tang et al. 2001). There are also a few reports of chronic CDAD in hamsters (Chang and Rohwer 1991; Ryden, Lipman et al. 1991; Godec 1992). Mucosal hyperplasia is described with chronic typhlitis, cholangiohepatitis, and amyloidosis of liver, kidneys, and intestinal wall. In one case, however, some individuals had lesions typical of acute disease, principally hemorrhagic and necrotizing typhlitis (Chang and Rohwer 1991).

Inoculation into axenic mice produces ulcerative typhlitis (Vernet, Corthier et al. 1989). Infection of gnotobiotic mice with C. difficile also reduced their typical cecal hypertrophy (Mahe and Corthier 1988). Reduction in cecal size was directly proportional to toxin production, and occurred even in the presence of clinical disease.

Mucosal lesions of pigs are limited to the cecum and colon. They are typically mild, but vary from grossly inapparent, multifocal necrosis of surface epithelial cells to transmural necrosis (Waters, Orr et al. 1998). Pigs with spontaneous disease typically have small microscopic ulcers with effusion of fibrin and neutrophils into the lumen, so-called “volcano ulcers”. Edema is a common feature of internal organs and a lesion that is unique to porcine CDAD. It manifests as hydrothorax, ascites, and/or mesocolonic edema. Diarrhea in pigs is variably present and some pigs are apparently obstipated. Obstipation in humans with CDAD is seen only with severe fulminant colitis with chronic ileus. In pigs, however, obstipation seems to occur with relatively mild disease.
Other clinical signs include dyspnea, mild abdominal distension, and scrotal edema (Waters, Orr et al. 1998; Songer, Post et al. 2000).

Colonoscopy revealed multiple ulcers covered with necrotic debris in a Kodiak bear (Orchard, Fekety et al. 1983). A diagnosis of CDAD was based on the presence of \textit{C. difficile} toxins in the feces, culture of \textit{C. difficile}, and lack of evidence for the presence of \textit{Salmonella} spp. or other potential enteric pathogens.

Naturally-occurring CDAD has been described in both neonatal and adult horses, although the lesions differ significantly between the two groups. As with many other species, lesions in the adults are isolated to the cecum and ascending colon (Perrin, Cosmetatos et al. 1993; Gustafsson, Baverud et al. 1997). Microscopic lesions do not differ remarkably from those associated with CDAD in other species. Affected segments of intestine may have edema, multifocal mucosal erosions and some blood vessels with fibrinoid change. Hemorrhagic necrotizing typhlocolitis has been reported in more severe cases (Gustafsson, Baverud et al. 1997). Lesions are confined to the cecum and ascending colon and are characterized by edema and generally superficial ulceration or erosions.

In contrast to adult horses, and to most other susceptible species, the small intestine of foals is consistently and severely affected. The small intestine and colon of diseased foals may be hemorrhagic and have patchy mucosal erosions or ulcers, with exudation of fibrin and adherent ingesta (Jones, Adney et al. 1988; Magdesian, Hirsh et al. 2002). Severe necrosis of villus epithelium, with adherent Gram-positive, rod-shaped bacteria, are observed microscopically. Crypt epithelium may be intact (Jones, Adney et
al. 1988). Foals with colitis have been reported, but such cases are apparently rare (Magdesian, Hirsh et al. 2002). Foal disease can begin immediately after birth and is associated with a high mortality rate, even with intensive medical therapy (Traub-Dargatz and Jones 1993).

Like foals, rabbits are most likely to develop lesions in the small intestine. Severe lesions are seen in the ileum, while those in the cecum are milder and more variable (Perkins, Fox et al. 1995).

A different distribution and/or density of toxin receptors in the gastrointestinal tract may explain the species-specific distribution of *C. difficile*-associated lesions, but it may also relate to the regions of the gastrointestinal tract in which *C. difficile* is able to proliferate to significant numbers. Higher numbers of *C. difficile* probably produce greater quantities of toxin, which would be predicted to lead to greater risk of lesion development. For instance, though *C. difficile* infection culminates in predominantly cecal lesions in hamsters and mice, direct application of culture filtrate, to either species, reveals that stomach and small intestine are also susceptible to toxin effects (Lyerly, Saum et al. 1985). The more limited distribution of lesions in naturally-occurring disease may reflect the distribution of *C. difficile* in the gastrointestinal tract. Experimental inoculation of clindamycin-treated hamsters is associated with proliferation of *C. difficile* in cecum but not in small intestine (Wilson, Sheagren et al. 1985).

1.3 Dissertation Format
This dissertation is organized with the introductory material, research objectives and summary of the work presented in the 2 initial chapters. These are followed by appendices representing manuscripts that are ready for publication. Chapter 1 includes the objectives of the current research and relates the significance of this work to resolving the problems presented by Clostridium difficile in pigs and other species. The literature review is also included in this chapter. The second chapter summarizes the methods, results and conclusions of the research, described in detail, in the individual appendices.

The research and findings in support of this dissertation are included as 4 appendices. The appendices are manuscripts that are ready for submission to journals or that have already been submitted. All were written by the degree candidate, Kevin Keel, with criticism provided by the coauthors.

Appendix A describes the ribotypes of C. difficile occurring in pigs and the prevalence of these ribotypes. These data are compared to the prevalence of ribotypes occurring in calves, dogs, horses, and man. Kevin Keel performed all polymerase chain reaction (PCR) assays to determine the ribotypes and presence of toxin genes in isolates examined. Isolates were provided by Karen Post, Beth Marlowe, Ferric Fang and Scott Weese, or were previously collected and entered in the strain collection housed in the Songer lab at the University of Arizona.

Appendix B illustrates the susceptibility of neonatal pigs to partially purified or purified toxins of C. difficile. All aspects of the described experiments were planned and performed by Kevin Keel. Toxins were isolated and purified by Kevin Keel with some assistance by Mike Anderson.
Appendix C demonstrates the distribution and density of receptors for TcdA on the gastrointestinal epithelium of newborn pigs. The distribution of TcdA binding is compared to the distribution of the α-Gal epitope on pigs, demonstrating that other receptors play the dominant role in TcdA pathophysiology. The immunohistochemistry (IHC) procedure was adapted from published references (Eglow, Pothoulakis et al. 1992). IHC procedures were perfected and all were performed by Kevin Keel with the exception of the staining of neonatal hamster tissues by Matt McDaniels. All tissues were processed and sectioned by Kevin Keel and all stained sections were microscopically evaluated by Kevin Keel.

Appendix D describes the ultrastructural lesions created by TcdA in porcine colon explants. The results of immunoelectron microscopy experiments proving the specific interaction of TcdA with microvilli and the intracellular distribution of toxin are also discussed. All organ culture experiments performed as part of this work were conducted by Kevin Keel. Toxins used in the studies were prepared by Kevin Keel and Mike Anderson. Dave Bentley sectioned the tissue blocks for the immunoelectron microscopy experiments. All other tissue preparation, sectioning, microscopy, and photography were performed by Kevin Keel.
2. PRESENT STUDY

The methods, results, and conclusions of this research are described in the papers appended to the thesis. The following discussion summarizes the most important findings in each of these papers.

Appendix A describes the ribotypes of *C. difficile* isolates that occur in pigs and the prevalence of each ribotype. Isolates from calves, dogs, horses, and humans were also typed by this method and the findings were contrasted with the data from pigs. Ribotypes which occurred in humans were much different from those present in other species, indicating little possibility of zoonotic or anthropozoonotic disease. Dogs and horses also had a different suite of ribotypes and the overall diversity of ribotypes was greater than that in pigs and calves or humans. There was very little diversity of ribotypes occurring in pigs and calves. Furthermore, one ribotype, 078, was overwhelmingly dominant among both of these host species. This ribotype was rarely identified from humans and was not common in dogs or foals. Calves and pigs almost certainly did not come in contact with each other. Many of the pig isolates were also geographically distinct with little possibility of a common source of infection. The reasons for a common porcine and bovine ribotype, among distinct populations, remain to be identified.

Appendix B describes the susceptibility of newborn pigs to intragastrically administered toxins of *C. difficile*. Initially, pigs were gavaged with 750 mg or 250 mg of partially purified culture supernatant. The animals developed extreme clinical disease and severe intestinal and extraintestinal lesions. The mucosa of the entire small intestine
became necrotic and was associated with severe effusion into the lumen. Necrosis was also present in the cecum, colon and stomach, but it was limited to the most superficial epithelial cells and was not associated with sloughing, as in the small intestine. Purified TcdA caused the same types of lesions. TcdB was associated with more severe lesions than TcdA, when inoculated alone, and acted synergistically when introduced with TcdA. Subcutaneous edema, mesocolonic edema, hydrothorax and ascites also occurred. Colon explants developed lesions two h after inoculation with TcdA. In the organ culture study, however, TcdB alone did not cause significant lesions. When TcdB was inoculated with TcdA the lesions that developed were not significantly different than those due to TcdA alone.

Appendix C is a report on the distribution of receptors for *C. difficile* toxins in pig mucosa. Specific interaction of TcdA was seen by IHC throughout the GI tract and in the gall-bladder epithelium. Binding to enterocytes was limited to the brush borders and was most intense and widely distributed in the jejunum. In the cecum and colon, where lesions occur in spontaneous cases, TcdA binding was limited to epithelial cells on the very surface of the mucosa. TcdB did not bind to any of the tissues. A monoclonal antibody was used, in an IHC assay, to demonstrate the distribution of the α-Gal epitope. This trisaccharide is reported to be a receptor for TcdA and is known to be especially abundant in pig tissues. There was limited staining at the brush border of villus enterocytes in the jejunum and ileum. Most of the intestinal epithelial cells stained by the TcdA method were not labeled for the α-Gal epitope. In addition, the α-Gal IHC technique did not stain gall bladder epithelium but it did specifically stain endothelial
cells and epithelial cells of the pancreatic duct. This indicates that pigs have abundant receptors for TcdA but they are apparently something other than the α-Gal epitope.

Appendix D describes the ultrastructural lesions that occurred due to TcdA as well as the attachment of TcdA and its distribution within host tissues. Colon explants were inoculated for two h with varied concentrations of TcdA. Tissues were immediately fixed and prepared for electron microscopy. Negative control tissues did not develop lesions after being maintained for two h in organ-culture media. Tissues inoculated with TcdA did develop lesions in a dose dependent manner. The cellular lesions did not appear to be specific to TcdA and included cell swelling, swelling of organelles, necrosis, karyorhexis, and apoptosis. Severely-affected cells were often adjacent to less affected cells. In the appendix, the observation that individual cells stained by the IHC for TcdA sometimes had much more dense staining than surrounding cells is discussed.

Immunoelectron microscopy demonstrated that TcdA was bound to microvilli at the plasmalemma 5 min after inoculation with colon explants. By this time it was also present within endosomes and free in the cytoplasm. At later time points it occurred with greater frequency in the cytoplasm, attached to mitochondria and in cell nuclei. Epithelial cells were labeled for TcdA as well as endothelial cells of blood vessels in the lamina propria and leucocytes in the lamina propria. The localization of TcdA to mitochondria has previously been reported using other techniques but its presence within nuclei is apparently a new observation (He, Hagen et al. 2000).
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countries by amplified fragment length polymorphism and PCR ribotyping."


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APPENDIX A.

Prevalence of *Clostridium difficile* Ribotypes Among Neonatal Pigs and Other Species, Including Humans

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ABSTRACT

Clostridium difficile-associated disease (CDAD) has emerged as an important cause of economic loss for swine producers in North America and elsewhere in the world. Little is known of the epidemiology of CDAD, including the cycling of the organism among sows, piglets, and the swine-production environment. Differences in the size and copy number of the intergenic sequences separating the 16S and 23S ribosomal-RNA (rRNA) genes can be determined by examination of products of PCR amplification (PCR ribotyping). This has been used extensively as a typing method on which to base epidemiologic studies of human CDAD, and the method is acknowledged to be an effective and precise means of differentiating among a large number of strains. We applied PCR ribotyping to isolates obtained from neonatal pigs with CDAD. Template prepared from fresh cultures of C. difficile and primers specific for the 3' end of the 16S rRNA gene and the 5' end of the 23S rRNA gene were used in a standard PCR protocol. Products were examined by agarose gel electrophoresis. Isolates examined from pigs (n=146) consisted of four distinct ribotypes, 078 (prevalence = 82%), 126 (prevalence = 11%), 002 (prevalence = 5%) and 033 (prevalence = 2%). Isolates were also tested from calves, dogs, horses, and humans. Thirty-one of the 32 isolates (97%) from calves were ribotype 078. The only other isolate examined from a calf was ribotype 033. Ribotype 078 was rare among human isolates, which were also dominated by a single ribotype not found in pigs or calves. Ribotype 078 was also found at a lower frequency in horses and did not occur in dogs. Ribotypes among canine isolates differed considerably from those
of calves and pigs. These results suggest that there is little interspecies transfer of \textit{C. difficile}. 
INTRODUCTION

*Clostridium difficile* is the predominant, infectious cause of human antibiotic-associated diarrhea and is an important etiology of gastrointestinal disease among many other mammal species. It is a Gram-positive, spore-forming rod which only proliferates under strictly anaerobic conditions. The spores are very resistant to harsh environmental conditions and concentration of spores in the environment may play a role in the acquisition of *C. difficile* (Nakamura, Yamakawa et al. 1985; Berild, Smaabrekke et al. 2003; Khan and Cheesbrough 2003). This is most notable among human hospital patients who are much more likely to develop *C. difficile*-associated diarrhea (CDAD) than are outpatients treated with antibiotics (Jobe, Grasley et al. 1995; Levy, Stergachis et al. 2000; Riley 2004).

Little is known about the transmission of strains among animals. The risk factors for CDAD in many species are apparently similar to those of human cases, principally antibiotic use and hospitalization, or at least housing in an environment with a high burden of *C. difficile* spores. CDAD among horses has been associated with antibiotic administration and concurrent hospitalization, and environmental sources of *C. difficile* predispose hamsters to antibiotic-associated diarrhea (Madewell, Tang et al. 1995; Baverud, Gustafsson et al. 1997; Baverud, Franklin et al. 1998). In neonatal pigs, however, antibiotics probably do not directly predispose the individuals to CDAD because they develop the disease at a very young age, possibly before they would receive antibiotics (Waters, Orr et al. 1998; Songer, Post et al. 2000).
Epidemiologic investigations of human CDAD have employed a variety of phenotypic and genetic methods (Brazier 2001). PCR-ribotyping has been used extensively due to its reported ability to discriminate among a large number of strains, its reproducibility and its relative simplicity (Bidet, Lalande et al. 2000). The technique uses specific primers complementary to portions of the 16s and 23s rRNA genes, such that the sequence separating the two genes is amplified. *Clostridium difficile* possesses multiple copies of the rRNA genes, always in the same orientation relative to the intergenic sequence (IS) (Gurtler 1993). In addition, different strains of *C. difficile* possess different numbers of rRNA genes and the intergenic sequences vary in size, yielding distinct banding patterns for each designated ribotype (Figure 1). The U.K. Anaerobe Reference Unit in Cardiff, Wales, has used this technique extensively. Beginning in 1993, this research group has provided a typing service to hospitals throughout England and Wales. The isolates defined by this service, as well as a number of mostly human isolates from a variety of other sources, have been compiled in an extensive database containing 116 different ribotypes (Stubbs, Brazier et al. 1999).

Strain typing has been used extensively in the epidemiology of human CDAD, but very few studies have typed isolates from animals or environments where they are housed. Restriction enzyme analysis (REA) and restriction fragment length polymorphism (RFLP) have been used to demonstrate that isolates from dogs and cats are different from those associated with human CDAD (O'Neill, Adams et al. 1993). However, there is a broad deficit in our knowledge of the *C. difficile* strains prevalent in
other species and the relationship, if any, of such animal strains to the strains occurring in human cases of CDAD.

MATERIALS AND METHODS

*Clostridium difficile* Isolates

Two-hundred-thirty-five isolates from 4 host species were analyzed in this study. One-hundred-forty-four porcine isolates, 33 bovine isolates, 22 equine isolates, and 13 canine isolates were examined. Porcine isolates were derived from pigs in Iowa, Ohio, Montana, North Carolina, and Utah. Twenty equine isolates were from horses seen at the Ontario Veterinary College. Two of the equine isolates were from Kansas and Arizona, but the source of the last was not known. All bovine isolates were collected from dairy calves in Arizona; however, these were bull calves shipped from multiple states. All 13 canine isolates were from patients of the Ontario Veterinary College. Twenty-three human isolates from two hospitals in Colorado and one hospital in Louisiana were also examined. Primary isolations were made by culturing stool samples or rectal swabs directly on taurocholate, cycloserine, cefoxitin, fructose agar (TCCFA) and *C. difficile* selective agar (CDSA; brain heart infusion, *C. difficile* antimicrobial supplement CC, sodium taurocholate and bovine blood) plates as previously described (George, Sutter et al. 1979; Wilson, Kennedy et al. 1982). Suspicious colonies were passed to brain heart infusion (BHI) agar with cysteine (0.05%), yeast extract (0.5%) and defibrinated bovine blood (2%) (BHI-CYE).
Screening for Toxin Genes

All isolates were analyzed for the presence of intact toxin genes, \textit{tcdA} and \textit{tcdB}, using an established multiplex PCR technique (Gumerlock, Tang et al. 1993; Tang, Gumerlock et al. 1994). The primers specific for toxin A were CdAF (GCATGATAAGGCAACTTCAGTGG) and CdAR (GAGTAAGTTTCCTCCTGCTCCATCAA), amplifying the 3' and 5' ends of \textit{tcdA}, respectively. Toxin B was amplified by the primers CdBF (GGTGGAGGTTGAATTGGAGAG) and CdBR (GTGTAACCTACTTTCCATAACACCAG), respectively specific for the 3' and 5' ends of \textit{tcdB}. Isolated colonies were selected from pure cultures grown on BHI-CYE and emulsified in 100 µl sterile distilled water. The emulsification was boiled for 5 minutes and centrifuged for 2 minutes at 17,000 X g. Five µl of supernatant was used as template in a PCR reaction including 50 pmol of each primer. PCR reaction mixtures contained 5 U Taq polymerase (Promega) and 1.5 mM MgCl$_2$ and were made up to a total volume of 50 µl. PCR products were visualized by electrophoresis in 1.5% Metaphor agarose at 120 mV for approximately 20 minutes. A 600 bp product was consistent with amplification of \textit{tcdA}; \textit{tcdB} was indicated by a 399 bp product.

PCR-Ribotyping

PCR-ribotyping was performed based on previously described techniques (O'Neill, Ogunsola et al. 1996; Stubbs, Brazier et al. 1999). Primers RiboF (CTGGGGTGAAGTCGTAACAGG) and RiboR (CGGCGCTTTTTGAGCTTTGACC)
were used to amplify the intergenic sequences. Each 100 μl reaction mixture contained 5 U of *Taq* polymerase (Promega), 1.5 mM MgCl₂ and 100 pmol of each primer. Crude template DNA was prepared by emulsification of isolated colonies in 100 μl of 5% Chelex 100 (Bio-Rad) followed by boiling for 10 min. Cell debris was removed by centrifugation (10 min at 17,000 g) and 10 μl of template were added to each reaction mixture. PCR products were separated by electrophoresis in a gel bed of 3% Nusieve agarose at 150 mV for 6 hr. A 100 bp ladder was placed at 5 lane intervals for accurate characterization of the bands. Gels were photographed under UV illumination and the band patterns analyzed with GelCompar image analysis software (Applied Maths, Kortrijk, Belgium).

RESULTS

Twenty-six ribotypes were identified from among the 235 *C. difficile* isolates examined in this study. Bovine and swine isolates were much less diverse than those of dogs, horses or humans (Figure 2). Only 3 ribotypes were present among calves and 4 occurred in pigs. Furthermore, a single ribotype, 078, was by far the most prevalent, representing 94% of bovine and 83% of swine isolates (Figure 3). This ribotype did not occur in any of the 13 dog strains examined and represented only 4.6 and 4.4% of equine and human isolates, respectively.

Canine isolates consisted of 6 ribotypes, the most common of which was AG representing 38% of all isolates. Ribotypes 020, AB, and AD each accounted for 15% of the isolates. Ribotypes AC, AD, and AG were found only among the canine isolates.
The most common ribotype of the equine isolates was X, with a 27% prevalence rate. This isolate did not occur among any of the other host species. Overall, however, there was considerable overlap of the ribotypes occurring in horses and other species. Five of the 12 ribotypes occurring in horses were not present in other species.

Among the 23 human isolates examined, 11 ribotypes were identified. The most common of these was 020 (22%) which also occurred in horses (5%) and dogs (15%). Five of the 11 isolates did not occur in the other host species examined.

All isolates within a single ribotype had the same expression profile of toxins A and B. However, rare isolates were negative by the PCR method used to detect the toxin genes, even though functional toxin was produced. Furthermore, isolates of distinct colony morphology could be identified within a particular ribotype. Two colony morphologies were easily differentiated. The most common was an opaque, white to beige colony with a slightly rough surface. The second colony type was white, but slightly translucent, and tended to spread out into larger colonies with irregular or stellate margins. The colony morphologies were obvious when age matched cultures were compared and were consistent through serial passages.

DISCUSSION

The limited number of ribotypes observed among pigs and calves contrasts significantly with the findings from horses, dogs, and humans. Though greater numbers of isolates were examined from pigs and calves, both species had an absolute number of ribotypes smaller than any of the other three species. The limited diversity among porcine
isolates is even more remarkable when we consider that they were collected from a very large geographic area. In contrast, canine and equine isolates were all from Ontario and the human isolates came from only three hospitals in the United States.

The same ribotype, 078, was predominant in both pigs and calves, though it is almost certain that there was no interspecific contact. The only factor common to both species is close contact with human caretakers. However, ribotype 078 was not common (4% of isolates) among the human isolates examined in this study and was even more rare (0.64%) in a much larger collection of human isolates from the United Kingdom (Stubbs, Brazier et al. 1999). This ribotype did not occur among the 13 canine isolates and represented only 5% of the equine isolates. Therefore, it must be assumed that 078 is the most common ribotype circulating independently among pigs and calves and among numerous populations within those species.

Human *C. difficile* isolates are also over-represented by a single ribotype. Of the 116 ribotypes described in humans, ribotype 001 represented 55% of the isolates from clinical cases. In the much smaller sample of human isolates examined in this study, ribotype 001 represented 13% of the isolates. The predominant ribotype, with a prevalence rate of 22%, was 020. It is uncertain whether the disparities in the prevalence of ribotypes among the two surveys is real or if it is a reflection of error imparted by the much smaller sample size in this study. However, there is a much more distinct difference in the ribotypes occurring among animal isolates and the human isolates examined in both studies. Only 17% of the human isolates had a ribotype that was also described in the group of porcine isolates. Similarly, 13% of the human isolates had a ribotype present
among the bovine isolates. Only 15% of the canine isolates were a ribotype that occurred in the human isolate group. The ribotype common to dogs and humans was 020, the most prevalent ribotype among the U.S. sample.

*Clostridium difficile* strains that cause clinical disease in animals are generally distinct from those strains associated with clinical disease in humans. This host specificity was most obvious in the comparison of bovine, canine, and porcine isolates to human isolates. The reasons for such a host preference are unclear, but could involve host-specific adhesion factors, differences in the micronutrients or other aspects of intestinal environment, or intraspecific interactions among host normal flora. Perhaps the most difficult of these findings to comprehend is not a difference in isolates, but the remarkable consistency of ribotypes occurring among pigs and calves, two very dissimilar species with no intraspecific contact. However, outward differences of the two species may be confounded by age-specific physiology. All animals sampled for this study were neonates. Therefore, the calves were functional monogastrics and subsisted on a milk diet. Milk, even from two different species is more similar than the feed rations given to adult hogs and cows. Furthermore, the microbial environment of the two species, as adults, undoubtedly contributes to a great disparity in the normal flora and microbial food chain. It would be interesting to survey *C. difficile* isolates from adults of the 2 species to compare the prevalence of individual ribotypes to those occurring in the neonates.

Even though a single ribotype was predominant in the majority of pigs and calves it is a mistake to assume that there is no heterogeneity among these isolates. Very distinct
differences in colony morphology were noted among isolates of ribotype 078 from both species and may reflect some underlying strain differences. PCR-ribotyping is a useful technique but its discriminatory power is not absolute. Ribotype 001, the most common ribotype occurring in humans can be subtyped using other methods including random amplified primer-DNA, ribospacer PCR, and pulsed field gel electrophoresis (Fawley, Freeman et al. 2003). It cannot be assumed that isolates of ribotype 078, from pigs and calves are identical, but this technique does demonstrate that they differ from the most common ribotypes occurring in all other species examined. The *C. difficile* isolates in humans are consistently different from those of animals. This is particularly important to concerns of zoonotic acquisition of isolates by susceptible humans.
Figure 1. PCR-ribotype band patterns. Detail of a gel demonstrating the varied banding pattern produced by PCR amplification of the intergenic sequences occurring on the genome of different *C. difficile* ribotypes. A 100 bp marker is present at lanes 4 and 9. All other lanes contain PCR-ribotyping products from different ribotypes.
Figure 2. Ribotype diversity by host species. The Y-axis values represent the total number of *C. difficile* isolates for each host species divided by the number of ribotypes identified among that pool of isolates. Swine and bovine isolates were much less diverse than either canine or equine isolates. A significant number of different ribotypes were also present among the human isolates in the strain collection at the Songer Lab (Human-SL). The collection of human isolates from the Anaerobe Reference Laboratory in Wales (Human-ARL) had much less diversity. However, that strain collection was much more expansive with 2030 isolates and 116 ribotypes, greatly reducing error introduced by chance.
Figure 3. Prevalence of ribotypes in pigs and calves. A single ribotype, 078, was predominate between both species. Legend: □ Pigs (n=144) □ Calves (n=33).
Figure 4. Prevalence of porcine and bovine ribotypes in dogs and horses. Ribotypes of *C. difficile* from pigs and calves were rare in horses (1/22) and never occurred in dogs.

For illustration purposes all ribotypes not occurring in calves and pigs are pooled into one group. Legend: ■ Pigs (n=144), □ Calves (n=33), ■ Horses (n=22), ■ Dogs (n=13).
Figure 5. Prevalence of porcine and bovine ribotypes in humans. Ribotype 078, the most common in pigs and calves, was very rare among human isolates. Ribotypes 002, 003 and 126 had a similar prevalence among pigs, calves, and humans. For illustration purposes, all ribotypes not occurring in pigs and calves are pooled into one group. Those ribotypes were much more common among the two collections of human isolates.

Legend: ■ Pigs, □ Calves, □ Humans (Songer Lab strain collection), ■ Humans (Anaerobe Reference Laboratory strain collection).
REFERENCES


APPENDIX B.

The Susceptibility of Neonatal Pigs to Toxin A and Toxin B of *Clostridium difficile*

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ABSTRACT

*Clostridium difficile*-associated disease (CDAD) has become a common diagnosis in neonatal pigs in many swine-producing areas, in North America and elsewhere. We examined the etiopathology of porcine CDAD through intragastric treatment of pigs with *C. difficile* toxins or exposure of porcine intestinal explants to toxins. Day-old pigs gavaged with culture supernatant (750 μg or 250 μg total protein per pig) from a toxigenic strain of *C. difficile* developed diarrhea within 3 h and became moribund in 10 h or less. Post mortem examination revealed effusion into the lumen of the small intestine and variable mesocolonic edema, hydrothorax, ascites, and/or subcutaneous edema. Diffuse necrosis of the superficial mucosa was present in the small and large intestine. Purified toxin A (TcdA) and toxin B (TcdB) caused the same type of lesions when applied independently or in combination. In contrast to previously described experiments, TcdB alone was sufficient to cause severe lesions. However, the two toxins together resulted in much more severe lesions than either toxin alone. TcdA (0.25 to 2.5 mg/ml) was applied to colon explants, and a dose-dependent response was documented by light microscopic examination. Colon explants, however, were not sensitive to TcdB alone. These results confirm that pigs are very sensitive to the effects of *C. difficile* toxins and provide additional evidence that *C. difficile* is a significant etiologic agent of gastrointestinal disease in neonatal pigs. The effects of TcdB, administered by gavage, dispute previous research and the dogma that TcdB, alone, is ineffective in the host
INTRODUCTION

*Clostridium difficile* is a Gram-positive to Gram-variable, spore-forming bacillus and an obligate anaerobe. It is a leading cause of antibiotic-associated diarrhea and colitis in humans and is often encountered as a nosocomial infection among elderly hospital patients (Harbarth, Samore et al. 2001). It is also an important cause of disease of laboratory animals and many other domestic species. Hamsters are especially sensitive to the disease and develop fatal typhlitis with the administration of just one colony forming unit (CFU) of *C. difficile*, after treatment with clindamycin (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 gastrointestinal lesions 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).

Recent evidence has implicated *C. difficile* as a common cause of typhlocolitis in suckling pigs (Waters, Orr et al. 1998). The disease occurs at one to seven days of age with diarrhea that often begins almost at birth, though obstipation is an occasional sign, as well (Waters, Orr et al. 1998; Songer, Post et al. 2000). Mortality is low, with a case fatality rate of approximately 20%. Survivors, however, generally represent an economic loss due to prolonged weaning times and, ultimately, reduced slaughter weights.
Morbidity varies from 10% to 90% (0 = 20%) in affected farrowing units (Songer, Post et al. 2000). The actual prevalence of the disease may be higher than is recognized due to the typical mildness of the clinical signs and low mortality rates.

The principal virulence factors of *C. difficile* are two large exotoxins, toxin A (TcdA) and toxin B (TcdB). They belong to a family of bacterial toxins, the large clostridial cytotoxins, which 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 disregulation of actin filaments with cell rounding and eventual cell death. TcdB has 1000X greater catalytic activity and is much more toxic to cells in culture monolayers than TcdA (Chaves-Olarte, Weidmann et al. 1997). Most research has indicated that TcdB is much less active in vivo and requires the presence of TcdA, or some initial trauma to mucosal epithelium, to exert its effects (Lyerly, Saum et al. 1985).

Pathogenesis of *C. difficile*-associated disease (CDAD) is very complex. It begins with the inactivation of GTPases in the intestinal epithelium, but *C. difficile* toxins also have direct and indirect effects on mast cells, monocytes, local neurons, and smooth muscle. A complex cascade of inflammatory mediators are integrated with mucosal and smooth-muscle functions to create the clinical and lesional characteristics of the disease (Pothoulakis and Lamont 2001). Diarrhea can be severe even though lesions are mild.
The lesions are typified by mucosal necrosis and ulceration and intense infiltration of neutrophils. In spontaneous cases of CDAD, in most species, the lesions begin as multifocal ulcers with exudation of neutrophils and protein-rich fluid. Such lesions may progress to the formation of pseudomembranes and, in extreme cases, transmural necrosis. The lesions described in pigs are most often limited to multifocal, superficial necrosis of cecum and colon with infiltrates of neutrophils and pinpoint ulcers characterized by the egress of large numbers of neutrophils (Waters, Orr et al. 1998; Songer, Post et al. 2000).

*Clostridium difficile* was first isolated from pigs in 1983, and the authors noted that previous attempts to isolate the bacteria from this species were unsuccessful (Jones and Hunter 1983). Based on the results of other experiments, pigs were not believed to be susceptible to *C. difficile* toxins (Sisk, Cole et al. 1998). Intestinal loops in pigs did not develop lesions when purified *C. difficile* toxins were injected into the lumen. Such results would seem to refute the diagnosis of CDAD in neonatal pigs, but the disease has been reproduced by the oral inoculation of colostrum deprived pigs with viable spores (Post, personal communication). Nonetheless, the characteristics of the disease in pigs and their propensity to develop CDAD remain to be fully investigated. This research assessed the susceptibility of pigs to purified *C. difficile* toxins A (TcdB) and B (TcdB), and the dose dependent responses of colon explants to the toxins.
METHODS

Bacterial Culture

Fecal samples from all animal subjects were swabbed, and streaked for isolation, directly on taurocholate, cycloserine, cefoxitin, fructose agar (TCCFA) and C. difficile selective agar (CDSA; brain heart infusion, C. difficile antimicrobial supplement CC, sodium taurocholate and bovine blood) as previously described (George, Sutter et al. 1979; Wilson, Kennedy et al. 1982). After 48-72 h, suspect C. difficile colonies were picked and transferred to brain heart infusion agar with yeast extract (0.5%), cysteine hydrochloride (0.05%) (BHI-CYE) and bovine blood (2%). Identification of C. difficile was based on colony morphology, odor, weak α-hemolysis, and production of L-proline aminopeptidase (Remel, Lenexa, Kansas, U.S.A.). For toxin production, cultures were maintained for 5-7 days in dialysis tubing (molecular weight cut off of 10,000) suspended in BHI-CYE broth.

Toxin Purification

Supernatant from dialysis bag cultures of C. difficile strain VPI 10463 (ATCC, Manassas, VA) was harvested by centrifugation at 15,000 X g for 20 min. Toxins were purified from the supernatant by ion-affinity chromatography on a DEAE-sepharose column (Amersham Biosciences, Uppsala, Sweden). Fractions of 5 ml were collected on a GradiFrac™ rotary fraction collector (Amersham Biosciences, Uppsala, Sweden) and protein elution was monitored by a UV-1 ultraviolet monitor (Amersham Biosciences, Uppsala, Sweden). 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. After elution of TcdA, TcdB remained on the column and was recovered with a linear gradient of 0.25-0.6 M NaCl in 50 mM Tris-HCl at a pH of 7.4. Fractions of interest were pooled and concentrated by centrifugal filtration (molecular weight cut off = 100,000 daltons; Millipore, Bedford, MA). Toxin concentration was determined by a Bradford assay per the manufacturer’s instructions (BioRad, Hercules, CA). Toxin activity was determined by CHO-cell assay and purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE).

**Cell Culture**

Activity of *C. difficile*-toxin samples was tested by overlay on Chinese hamster ovary cells (CHO cells). CHO cells were maintained in Iscove’s modified Dulbecco’s Medium (IMDM; Cambrex, Walkersville, Maryland, U.S.A.) with 10% fetal bovine serum (FBS; Intergen Co., Purchase, New York, U.S.A.) and 100 mg/ml gentamicin (Sigma-Aldrich, St. Louis, Missouri, U.S.A.). For toxicity assays, cells were trypsinized, washed, and suspended at a concentration of $2 \times 10^5$ cells / ml; 100 ml of suspension was dispensed into each well of a 96-well tissue culture plate. Cells were incubated for 4 h at 37°C in 5% CO$_2$, and 100 µl aliquots of serial dilutions of toxin preparations were added to each well. Cells were incubated, as before, for an additional 18 h. The endpoint was the dilution that caused 50% cytopathic effect (CPE).

**Animals.**
Clinically normal, 12-24 h old pigs were selected based on negative culture results for *C. difficile* and negative ELISA results for TcdA and TcdB (Techlab®, Blacksburg, Virginia, U.S.A). Three separate trials involved intragastric administration of *C. difficile* supernatant, or purified toxins, to naive pigs (Table 1). All pigs used in the toxin gavage experiment were initially treated with 20 ml 1 M Na₂HCO₃ using a 3.0 endotracheal tube passed to the stomach. Two and one-half h later, principals were gavaged with 750 µg supernatant, 250 µg supernatant, 50 µg TcdA, 50 µg TcdB or a combination of 25 µg TcdA and 25 µg TcdB. For all treatments, toxin was dissolved in 10 ml 1 M Na₂HCO₃ and controls were sham treated with 10 ml 1 M Na₂HCO₃. Except for spontaneous deaths, pigs were euthanized by compressed CO₂ at 12 h post-treatment or when they became moribund. Postmortem examinations were immediately performed on all pigs and samples of stomach, duodenum, jejunum, ileum, cecum, ascending colon, descending colon, and pancreas were immersed in 10% neutral buffered formalin. Samples of feces were collected from the colon using aseptic techniques and retained for toxin ELISA and *C. difficile* culture.

Intestinal explants were initially collected in wash media consisting of DMEM/ F-12 (Cambrex, Walkersville, Maryland, U.S.A.) with amikacin (40 µg/ml; Fisher Scientific, Fairlawn, New Jersey, U.S.A.), kanamycin (0.6 mg/ml; Sigma-Aldrich, St. Louis, Missouri, U.S.A.), streptomycin (2 mg/ml; Fisher Scientific, Fairlawn, New Jersey, U.S.A.) and vancomycin (100 µg/ml; Sigma-Aldrich, St. Louis, Missouri, U.S.A.). They were then maintained in nutrient media, IMDM / F-12 with amikacin (20
μg/ml), streptomycin (2 mg/ml) and vancomycin (50 μg/ml). All media were pre-warmed to 37°C and pre-gassed with 5% CO₂ before initiation of experiments.

Pigs used for tissue explants were euthanized by CO₂ and were placed in dorsal recumbency in a laminar flow hood. The abdomen was disinfected with surgical scrub and the abdomen opened by a midline incision; the ascending colon was freed using sterile technique. The colon was placed in a sterile tray, irrigated with wash media, linearized by alternate blunt and sharp dissection, and opened by a longitudinal incision on the antimesenteric surface. Fecal material was gently flushed from the mucosal surface with wash media and 0.5-cm square sections were trimmed with a razor blade. The tissue samples were maintained in wash media until they were added to 6-well tissue culture plates, 25 to 30 min after euthanasia. Only sterile instruments were used, to insure that no exogenous bacteria were introduced to the explants. After incubating the samples for the designated period of time, they were removed from the media and placed in 10% neutral buffered formalin.

Tissue Processing.

Within 48 h of placement in formalin, tissue samples were dehydrated by serial baths of graded alcohol and, finally, by xylene. The dehydrated samples were embedded in paraffin and 5-μm-thick sections were stained by hematoxylin and eosin (H&E).
Lesion Scores

H&E-stained sections were examined by light microscopy and were assigned lesion scores based on the degree of necrosis (Table 2). Tissues were randomized and lesions scored in a blinded manner.

RESULTS

Intragastric inoculation of pigs with C. difficile-culture supernatant was fatal within 2-10 h, regardless of dosage (250 μg or 750 μg total protein). The most severe gross lesions were present in the group receiving the lower dosage of culture supernatant (Figure 1). Small intestine hyperemia was the most common lesion, affecting 3 of the 4 pigs in that group. Fifty percent of the 250 μg treatment group also had small-intestine distension by red-tinged to frankly hemorrhagic fluid (Figure 2). Fluid accumulation in a potential space or edema in tissues was a common finding in both groups. Seventy-five percent of pigs in the low dosage group and 60% of pigs in the high dosage groups had subcutaneous edema, ascites, hydrothorax and/or hydropericardium. Subcutaneous edema was localized to the inguinal and submandibular regions. Pleural effusion was often associated with hyperemia of the visceral pleura and deposition of small amounts of fibrin on pleural surfaces (Figure 2). On cut surfaces, the pulmonary parenchyma appeared normal and no lesions were microscopically evident. None of the uninoculated controls, in the supernatant gavage trial, developed clinical signs or gross lesions. Microscopically, all pigs in both treatment groups had the same type of lesions. Throughout the small intestine, villus enterocytes were necrotic and most had sloughed
into the lumen. Necrosis of epithelial cells extended into the superficial portion of the crypts. The lamina propria mucosa was markedly congested. In the cecum and colon, epithelial necrosis was limited to the layer of cells at the mucosal surface. In severe lesions this necrosis was diffuse and the entire layer of the superficial epithelium was lifted from the lamina propria by eosinophilic (protein-rich) fluid (Figure 4). Tissues with very mild lesions often had small amounts of karyorhectic debris in the superficial lamina propria.

Principals of toxin-gavage trials 2 and 3 received purified TcdA, TcdB, or a combination of the two. Gross lesions were similar to those in the supernatant-gavage trial (Figure 3), but small intestinal effusion was more common, affecting all pigs receiving 50 µg TcdA, and two of four dosed with 25 µg TcdA. TcdB administered alone also resulted in significant lesions; small intestine effusion, colon effusion and mesocolonic edema each affected two of three pigs in the 50 µg group and two of five pigs in the 25 µg group. Collectively, 88% of pigs receiving TcdB developed at least one of these lesions. Both TcdB dosage groups had a greater prevalence of mesocolonic edema than either of the TcdA inoculation groups.

Microscopically, more severe mucosal necrosis was consistently evident in the small intestine, in both trials 2 and 3 (Figures 4 and 5). The only exception was that the pigs treated with 50 µg TcdB, in trial 2, had slightly more severe lesions in the ascending colon than in the jejunum (Figure 4). The lesions associated with both toxins were similar to those of trial 1, differing only in their severity. Necrosis in the small intestine occurred at the villus tips in the mildest lesions, but the most severe lesions involved
crypt epithelium as well. As in trial 1, necrosis of the cecum and colon was limited to the most superficial epithelial cells (Figure 6). The superficial lamina propria had scattered karyorhectic debris consistent with remnant apoptotic bodies.

TcdB was generally associated with more severe mucosal-necrosis scores than TcdA. The two toxins together elicited a significant synergistic response; the severity of necrosis associated with dual inoculation was much greater than the anticipated sum of lesions produced by the individual toxins.

The lesional scores from the first trial, associated with the inoculation of 25 μg TcdA or TcdB, are greater than the scores in tissues inoculated with 50 μg TcdA or TcdB in the second trial. This may be due to the use of different lots of toxin for the two trials. Within each trial the trends are consistent and trial 2 clearly indicates a synergism between TcdA and TcdB.

Organ culture experiments were limited to explants of the ascending colon. Lesion scores in the small intestine were too variable, even among the controls, to yield meaningful data. The villus epithelium of small intestinal explants was apparently fragile and prone to acquire traumatically-induced lesions during collection. Control negative colon explants, however, did not develop any lesions after two h incubation in organ culture media (Figure 6).

TcdB had no significant affect on colon explants, but a dose dependent response was observed for tissues inoculated with TcdA (Figure 7). Necrosis produced by the combination of TcdA and TcdB was not significantly different than for the same dosage of TcdA applied alone. As in the toxin gavage experiments, necrosis in the colon
explants was limited to the superficial layer of epithelial cells (Figure 7). Cells were partially extruded from the epithelial layer or entire sheets of cells were slightly elevated above the lamina propria.

**DISCUSSION**

Treatment of pigs and colon explants with *C. difficile* toxins demonstrated that pigs are very sensitive to the effects of culture supernatant and to purified TcdA and TcdB. Supernatant of *C. difficile* cultures caused the most severe lesions and clinical disease *in vivo*. All inoculated principals develop severe, acute diarrhea and died quickly. The greater severity of lesions in the small intestine of pigs treated by toxin gavage might be explained by several mechanisms. The toxin was applied directly into the stomach; therefore tissues in the anterior gastrointestinal tract were exposed to toxin longer than distal segments and had a longer period of time to develop lesions. It is also probable that the concentration of toxins delivered to the distal portions of the gastrointestinal tract were decreased by the effects of effusion and possibly by adsorption of toxin to tissues in the proximal portions. IHC experiments indicated that TcdA has a greater ability to bind to the brush border of the small intestine, particularly the jejunum, than to the cecum and colon (M. K. Keel, unpublished results). Therefore, the small intestine may be more prone to develop lesions by virtue of more efficient binding of TcdA. The distribution of toxin binding in those IHC experiments correlates very well with the cells most affected in the toxin gavage experiments. In the organ culture experiments, as well, the
distribution of necrotic cells corresponded closely with the cells labeled by TcdA in IHC assays.

Lesions associated with spontaneous cases of CDAD are more often multifocal than those in the toxin gavage and organ culture experiments. Infiltrates of neutrophils are also more localized, with intense infiltrates associated with small ulcers and large numbers of neutrophils migrating across the mucosal surface to the lumen of the intestine. The multifocal nature of mild, spontaneous cases of CDAD may be associated with bacterial adhesion and local production of toxin. Adhesion factors of C. difficile have been described and were claimed to represent significant virulence factors (Hennequin, Porcheray et al. 2001; Tasteyre, Barc et al. 2001; Calabi, Calabi et al. 2002).

Apoptosis of cells in the lamina propria was common in treated tissues, even at low dosages. The identification of these cells was not confirmed but their location, and the appearance of other mesenchymal cells, suggests they were leucocytes. TcdB causes apoptosis of macrophages, T cells, and eosinophils. TcdA causes death of macrophages by undetermined mechanisms but directly interacts with T cells to cause death by apoptosis. The mechanism of T-cell recognition by TcdA is uncertain. IHC did not demonstrate binding of any leucocyte populations in the lamina propria (M. K. Keel, unpublished data).

The ability of TcdB to cause lesions when applied alone by gavage was surprising. Other experiments reported that TcdB alone has minimal or no effect when introduced by gavage, unless there is incidental trauma to the epithelium. However, there are some conflicting reports. Other experiments (unpublished data, our lab) confirmed
that TcdB was just as active as TcdA when injected into intestinal loops of calves. Such intestinal loops, however, are associated with trauma at the site of the ligature. A prevalent hypothesis is that TcdB receptors are on the basolateral aspect of the cells and that trauma provides access to the receptors on damaged cells; a cascade of cell damage by TcdB and access to additional binding sites would then ensue. However, in vivo susceptibility of pigs to TcdB is supported by reports of TcdA-negative, TcdB-positive, C. difficile strains producing human CDAD (Komatsu, Kato et al. 2003; van den Berg, Claas et al. 2004). Other reports also describe enterotoxic activity of TcdB on human intestinal mucosa (Savidge, Pan et al. 2003).

The lack of TcdB-induced lesions in the colon explants also counters current dogma. These were very small explants (approximately 0.5 cm diameter) with cut surfaces contributing a major portion of the surface area. Damaged cells would have been readily available for exposure to TcdB. The pathogenesis of the disease caused by TcdB is still uncertain, but it is postulated that the in vivo Rho-glucosylating activities of TcdB may be more significant than those of TcdA (Chaves-Olarte, Weidmann et al. 1997). As with TcdA, receptor mediated endocytosis of TcdB may be a necessary first step in its intracellular pathogenesis. However, no receptor for TcdB has been identified. After decades of research, the initial events in the pathogenesis of TcdB remain elusive.
<table>
<thead>
<tr>
<th>Trial</th>
<th>Treatment</th>
<th>Number of Pigs</th>
</tr>
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<tr>
<td>1</td>
<td>250 µg supernatant</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>750 µg supernatant</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>Negative controls</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>50 µg TcdA</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>50 µg TcdB</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td>25 µg TcdA and 25 µg TcdB</td>
<td>4</td>
</tr>
<tr>
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<td>Negative controls</td>
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</tr>
<tr>
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<td>25 µg TcdA</td>
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</tr>
<tr>
<td></td>
<td>25 µg TcdB</td>
<td>4</td>
</tr>
<tr>
<td></td>
<td>Negative controls</td>
<td>4</td>
</tr>
</tbody>
</table>

Table 1. Treatment groups in toxin gavage experiments. Trial 1 was an initial experiment to determine the extent of lesions caused by *C. difficile* toxins and to assess the sensitivity of neonatal pigs. Trials 2 and 3 used purified toxins but different lots of toxins were used for the 2 experiments.
<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 permissible.</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>

Table 2. Lesion scores for histopathology. H&E stained sections were read in a blinded manner and scored by this scheme. Mean lesional scores were then computed for each group.
Lesions

Figure 1. Prevalence of gross lesions – toxin gavage trial 1. Lower dosages of toxin were associated with a higher prevalence of all lesions.

Legend:  ■ 250 μg supernatant. □ 750 μg supernatant.
Figure 2. Gross lesions resulting from the intragastric inoculation of pigs with partially purified *C. difficile* supernatant. A, Pleural effusion, ascites and/or subcutaneous edema were seen in some pigs. The thoracic cavity in this image is expanded by serous fluid and fibrin strands coat the pleural surfaces. The pericardial sac contains a small amount of fluid as well. Pulmonary surfaces were hyperemic but cut surfaces appeared normal and no lesions were microscopically evident in the parenchyma. B, The small intestine of this pig was very hyperemic and contained abundant red-tinged fluid. Histologically, the jejunum had severe mucosal necrosis with sloughing of nearly all epithelial cells.
Figure 3. Prevalence of gross lesions associated with intragastric treatment of neonatal pigs with varying concentrations of TcdA or TcdB. Similar lesions were seen in all treatment groups, but mesocolonic edema and colon effusion were most prevalent in pigs receiving only TcdB. Legend: □ 50 μg TcdA, ■ 25 μg TcdA, □ 50 μg TcdB, ■ 25 μg TcdB, ■ 25 μg TcdA and TcdB.
Figure 4. Toxin gavage trial 2 – mean necrosis scores. The small intestine was consistently the most severely affected tissue. TcdB caused more severe necrosis than TcdA in all tissues except the ascending colon. No significant lesions were present in tissues from any of the negative controls.

Figure 5. Toxin gavage trial 3 – mean necrosis scores. TcdB caused more severe necrosis than TcdA administered alone. The 2 toxins together had a synergistic effect on the tissues. No significant lesions were present among the negative controls.

Figure 6. Hematoxylin and eosin stained sections from toxin-gavage and colon-explant-inoculation experiments. A, Jejunum from a pig treated intragastrically with 25 μg TcdA and TcdB. Most epithelial cells are necrotic and have sloughed into the lumen. B, Colon from a pig treated intragastrically with 25 μg TcdA and TcdB. Only the cells lining the lumen are necrotic and they are elevated from the lamina propria as a sheet. C, Control-negative, colon explant maintained for two h in tissue culture media. D, Colon explant inoculated with 1 μg/ml TcdA for two h.
Figure 7. Organ culture assay – mean necrosis scores. A dose dependent response to TcdA occurred. TcdB did not cause significant lesions when inoculated alone. Necrosis scores associated with the combined toxins were not significantly different from the scores of the same concentration of TcdA applied alone.

Legend: ◆ TcdA and TcdB Combined, □ TcdA, ▲ TcdB.
REFERENCES


APPENDIX C.

The Distribution and Density of *Clostridium difficile* Toxin Receptors on the Intestinal Mucosa of Neonatal Pigs

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ABSTRACT

*Clostridium difficile* is an enteric pathogen affecting a variety of mammals and has recently been diagnosed as the cause of neonatal typhlocolitis in pigs. Toxin A (TcdA) is a large AB-like exotoxin and an important virulence factor of *C. difficile*. It has been clearly established that the effects of TcdA on host tissues are dependent upon the receptor-mediated endocytosis of the intact toxin. One hypothesis to explain the resistance of most species as neonates (e.g. humans and hamsters) is that they may lack significant numbers of toxin receptors. The susceptibility of neonatal pigs suggests cells of the gastrointestinal mucosa express sufficient numbers of toxin receptors for lesion development. Immunohistochemical (IHC) assays documented specific binding of toxin A, but not toxin B, to epithelium of the small and large intestine. The carbohydrate Galα1-3β1-4GlcNAc-R, has been described as an important receptor for TcdA. However, IHC indicated a distribution on cell surfaces much different than that of TcdA binding, suggesting a specific interaction of toxin with an alternative receptor.
INTRODUCTION

_Clostridium difficile_-associated disease (CDAD) is a major health problem among human hospital patients and a growing concern for the husbandry of many other mammals. The etiologic agent is a Gram-positive bacillus that is an obligate anaerobe and forms environmentally-hardy spores. Recently it has been identified as an etiologic agent of diarrhea in neonatal pigs and represents a significant concern to the pork industry (Waters, Orr et al. 1998; Songer, Post et al. 2000).

CDAD is associated with the elaboration of two large exotoxins by _C. difficile_, toxin A (TcdA) and toxin B (TcdB). The toxins have similar structures with 3 distinct domains (von Eichel-Streiber, Laufenberg-Feldmann et al. 1992). The carboxy-terminus includes a series of tandem repeats and, in the case of TcdA, is associated with receptor binding. This portion of the molecule is very different for the 2 toxins and no receptor molecules have been identified for TcdB. The amino terminus, however, is very well conserved between TcdA and TcdB; it is the enzymatic portion and is responsible for mono-glucosylation of a specific class of host-cell signal-transduction molecules, the rho-subtype GTPases (Just, Fritz et al. 1994; Dillon, Rubin et al. 1995). The mid-portion of the two toxins includes hydrophobic regions hypothesized to be associated with membrane insertion and/or translocation (von Eichel-Streiber, Laufenberg-Feldmann et al. 1992; Just, Hofmann et al. 2000).

Receptor-mediated endocytosis is an essential feature of toxin activity. A number of receptors for TcdA have been proposed or positively identified, including Galα1-3β1-4GlcNAc-R (α-Gal epitope or α-Galactosyl), Galβ1-4(Fuα1-3)GlcNAc (Lewis X),
Galβ1-4GlcNAcβ1 (Lewis Y), Galβ1-14GlcNAcβ1-3Galβ1-4(Glc) (Lewis I), and sucrose-isomaltase (Lowe, Fox et al. 1980; Krivan, Clark et al. 1986; Tucker and Wilkins 1991). Mutations in the receptor-binding portion of the molecule, or the presence of antibodies specific for the receptor-binding portion, are sufficient to protect susceptible cells (von Eichel-Streiber, Laufenberg-Feldmann et al. 1992; Barroso, Moncrief et al. 1994; Frisch, Gerhard et al. 2003). Lysosomal acidification is essential for conformational changes, in both toxins, that preclude enzymatic activity and/or translocation (Florin and Thelestam 1983; Henriques, Florin et al. 1987; Qa'Dan, Spyres et al. 2000). Following endosomal acidification of TcdB, a functional subunit of the toxin is present in the cytosol of host cells (Pfeifer, Schirmer et al. 2003).

The α-Gal epitope has received much of the attention with regard to TcdA pathogenesis but the other receptors are fully functional and are important to toxin activity. This is clearly illustrated by the conspicuous absence of the α-Gal epitope from tissues of humans and all Old-World primates, even though these species are fully susceptible. Pigs, however, have a greater abundance of the α-Gal epitopes than do most other species (Galli, Gyr et al. 1994; Tanemura, Maruyama et al. 2000). This carbohydrate is one of the most troublesome antigens leading to graft rejection in xenotransplantation of porcine tissues into humans and Old-World primates (Galli, Gyr et al. 1994). Because pigs are known to have a great deal of this antigen on cell surfaces, we hypothesized that this might be the most significant receptor of TcdA in porcine tissues. If the α-Gal epitope is expressed in significant concentration at an early age it might also explain why pigs are susceptible as neonates, unlike most other species.
MATERIALS AND METHODS

Animals

Eleven, clinically normal, 12-24 h old pigs were selected based on negative culture for *C. difficile* and negative ELISA for TcdA and TcdB using a commercially available ELISA kit (Techlab®, Blacksburg, Virginia, U.S.A.). Five pigs were immediately euthanized by CO2 asphyxiation and served as a source of normal tissues. Six pigs were gavaged with 20 ml 1 M Na2HCO3 using a 3.0 endotracheal tube. Two and one-half h later, three pigs were gavaged with 50 mg TcdA diluted in 10 ml 1 M Na2HCO3; the remaining three were sham treated with 10 ml 1 M Na2HCO3. Pigs were euthanized by CO2 asphyxiation at 12 h post-treatment or when they became moribund.

Five, clinically-normal, adult, female, Syrian hamsters were euthanized by CO2 asphyxiation. It was confirmed that they did not carry *C. difficile* by negative cultures and toxin ELISA. Sixteen hamster pups, approximately 12 h old were also euthanized by CO2 asphyxiation.

Tissue Processing

All animals were dissected immediately after euthanasia and samples of stomach, duodenum, jejunum, cecum, ascending colon, and descending colon were fixed in 10% neutral buffered formalin. Samples of liver were also collected from the five, untreated pigs and the untreated hamsters. A midline incision was made on neonatal hamsters and they were fixed whole in formalin. Within 48 h of placement in formalin, tissue samples were dehydrated by passage through serial baths of graded alcohol and xylene. The
dehydrated samples were embedded in paraffin and 5-μm-thick sections stained by hematoxylin and eosin. Unstained, serial sections were retained for immunohistochemistry.

**Bacterial Culture**

Fecal samples from all animal subjects were streaked for isolation of *C. difficile* on taurocholate, cycloserine, cefoxitin, fructose agar (TCCFA) and *C. difficile* selective agar (CDSA; brain heart infusion, *C. difficile* antimicrobic supplement CC, sodium taurocholate and bovine blood) as previously described (George, Sutter et al. 1979; Wilson, Kennedy et al. 1982). After 48-72 h, suspect *C. difficile* colonies were picked and transferred to brain heart infusion agar with yeast extract (0.5%), cysteine hydrochloride (0.05%) (BHI-CYE) and bovine blood (2%). Identification of *C. difficile* was based on colony morphology, odor, weak α hemolysis and production of L-proline aminopeptidase (REMEL, Lenexa, Kansas, U.S.A.). For toxin production, cultures were maintained for 5-7 days in dialysis tubing (molecular weight cutoff, 15,000 daltons) suspended in BHI-CYE broth.

**Bacterial Strains**

TcdA and TcdB were isolated from *C. difficile* strains VPI 10463 (ATCC, Manassas, Virginia, U.S.A.) and JGS 890, from a field case of CDAD in a neonatal pig. Strain JGS 692, a non-toxigenic isolate from a pig, was the source of non-TcdA and non-TcdB proteins used in negative controls for immunohistochemistry (IHC).
Cell Culture

Activity of *C. difficile* toxins was assessed as toxicity for Chinese hamster ovary (CHO) cells. CHO cells were maintained in Iscove’s modified Dulbecco’s Medium (IMDM; Cambrex, Walkersville, Maryland, U.S.A.) with fetal bovine serum (FBS; 10%) and 100 mg/ml gentamicin. For toxicity assays, CHO cells were trypsinized, washed, and suspended at a concentration of 2 X 10^5 cells / ml; 100 ml of suspension was dispensed into each well of a 96-well tissue culture plate. Cells were incubated for four h at 37°C in 5% CO₂ and serial dilutions of toxin preparations, in 100 µl aliquots, were added to each well. Cells were incubated, as before, for an additional 18 h. The endpoint was determined as the dilution that caused 50% cytopathic effect (CPE).

Toxin Purification and Biotinylation

Supernatant from dialysis bag cultures of *C. difficile* was harvested by centrifugation at 15,000 X g for 20 min in a Sorvall RC-5B centrifuge (Kendro Laboratory Products, Asheville, North Carolina, U.S.A.). Toxins were purified from the supernatant by ion-affinity on a DEAE-sepharose column (Amersham Biosciences, Upsalla, Sweden). 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. After elution of TcdA, TcdB remained on the column and was recovered with a linear gradient of 0.25-0.6 M NaCl in 50 mM Tris-HCl at a pH of 7.4. Toxin activity was determined by CHO-cell assay and purity was confirmed by gel electrophoresis.
Biotin (DAKO Corporation, Carpinteria, California, U.S.A.) was conjugated to purified TcdA or TcdB by addition of 25 μg biotin to 1.5 mg/ml toxin. After four h of incubation at 25°C, 0.2 μl 1 M NH₄Cl was added for every 25 μg biotin in the conjugation mixture. The mixture was incubated for 10 min at 25°C and the biotinylated toxin was dialyzed overnight in PBS. CHO-cell assay demonstrated full activity of biotinylated and freeze-thawed toxins. Biotinylated TcdA and TcdB were diluted to 150 μg/ml and stored at −4°C.

Immunohistochemistry

Deparaffinized slides of normal tissues were blocked by avidin (DAKO, Corporation, Carpinteria, California, U.S.A.) then biotin for 15 min each. Biotinylated TcdA or TcdB was added at a dilution of 1:8 and incubated on the slides for 30 min. Streptavidin-horseradish peroxidase and diaminobenzidine (DAKO, Corporation, Carpinteria, California, U.S.A.) were used to indicate the location of bound toxin. Negative controls consisted of biotinylated “non-toxic” proteins eluted from the ion-affinity columns under the same conditions used to purify TcdA and TcdB.

A traditional IHC assay was used to detect non-biotinylated TcdA or TcdB localized on sections of normal tissues. Sections were blocked by avidin and biotin as before. Untreated TcdA was added at a dilution of 1:100 and incubated for 30 min. Rabbit polyclonal anti-TcdA (Lee Labs, Grayson, Georgia, U.S.A.) was incubated on the slides at a dilution of 1:500 for 30 min. Alternatively a mouse monoclonal anti-TcdA, BDI515 (Biodesign International, Saco, Maine, U.S.A.), was used at a dilution of 1:20 for
60 min. Both the rabbit and mouse antibodies were detected by the Envision reagent (Dako), followed by diaminobenzidine. The distribution of the α-Gal epitope was also determined by a standard IHC procedure using a mouse monoclonal antibody, M86, with known specificity for this carbohydrate. The Envision reagent and diaminobenzidine were also used to demonstrate tissue localization of M86. All incubations were at 25°C, washes were included prior to each treatment, and all slides were counterstained with hematoxylin.

Sections were examined by light microscopy and staining intensity was scored from − (no signal visible) to +++ (intense signal deposition) for each tissue and for surface epithelium and crypts or glands. Values from all individuals were averaged for each site. Tissues from neonatal pigs were examined by each of the aforementioned methods. Direct IHC was performed on adult hamster tissues using the biotinylated TcdA and TcdB. Indirect IHC was performed on neonatal hamsters using the polyclonal antibody specific for the TcdA.

RESULTS

Direct IHC with biotinylated TcdA demonstrated extensive binding of toxin throughout the mucosa of the neonatal porcine gastrointestinal tract. Specific binding to mucosal epithelial cells was limited to the brush border; the cytoplasm and nuclei of labeled cells were unstained. Brush border binding was differentiable from any edge effect because individual cells exhibited intense staining while rare cells in the midst of positively stained cells were negative (Figure 1). In addition some regions of the intestinal tract had
superficial cells that were stained but those lining the lumen of crypts or glands were unstained (Figures 2B and 2D). The most intense binding of TcdA was in the jejunum (Figure 2A), where cells of the villi and crypts were all intensely stained. Duodenal crypt cells were variably stained, but generally staining was more notable on villus surfaces of the duodenum or on superficial cells of the gastric mucosa. Strong binding to villus enterocytes in the ileum was also seen, but binding in the crypts was moderate (Figure 2C). The cecum slightly bound TcdA on cells of the glands and at the surface. Moderate staining (++) occurred in the ascending and descending colon but only on the brush border of cells lining the lumen or in the superficial portion of colon glands (Figure 2B). The only cell type, other than those of the gastrointestinal mucosa, to which TcdA specifically bound was gall bladder epithelium (Figure 1D). Intense specific staining of all the cells occurred, but only in the cytoplasm, not at the cell surface. Biotinylated TcdB did not stain any of the tissues (Figure 2D).

IHC using polyclonal antibodies to identify unlabeled toxin bound to tissue sections generated the same results as did the direct IHC experiments using biotinylated toxins. The same relative intensity of toxin binding was demonstrated throughout the gastrointestinal tract. IHC with a monoclonal antibody against TcdA failed to stain any of the bound toxin.

A monoclonal antibody against the α-Gal epitope specifically stained the brush border of enterocytes, but the stain distribution was significantly different than that of TcdA binding (Table 1 and Figure 3). Most cells labeled by TcdA, detected by either direct or indirect IHC, were not labeled by the α-Gal antibodies. The only cells labeled
by both methods were villus enterocytes in the jejunum and ileum. Staining of these cells was less intense (++) than that observed with the TcdA techniques and staining of the crypts did not occur. The α-Gal antibodies did positively stain endothelium (+) (Figure 3A) and cells of the pancreatic duct (+++) (Figure 2D). Neither of these cell types bound TcdA.

Hamster tissues, both neonatal and adult, exhibited binding characteristics similar to those of pig tissues (Table 2). The duodenum of the adults had more intense staining (++++) than noted for neonatal pigs, with equal staining along the villi and in the crypts. Staining was more intense on the surface of the cecal (++) and colonic (+++) mucosa but the glands were not stained at all. Gall bladder epithelium was stained just as it was in the pigs. Neonatal hamsters had analogous staining but the most intense signal was (++). There was also some staining of cecal and colon glands, unlike in the adult hamsters. Gall bladder epithelium was not examined in the neonates.

DISCUSSION

Specific staining for TcdA was achieved by the use of biotinylated TcdA or polyclonal antibodies against TcdA. Intensity of toxin binding does not correlate with the distribution of lesions occurring in spontaneous cases of porcine CDAD. Naturally-occurring lesions are generally limited to the cecum and colon, but toxin binding was much more intense in the small intestine, especially the jejunum. Experimental gavage of pigs with TcdA, however, generated lesions which were much more severe in the small intestine than in the rest of the gastrointestinal tract (M. K. Keel, unpublished data). This
distribution of lesions could reflect the greater density of receptors on those tissues but unintended effects of anterograde administration of the toxin cannot be ruled out.

The limitation of lesions, in spontaneous cases, to the cecum and colon may reflect the natural distribution of vegetative *C. difficile* and production of toxin, rather than specific susceptibility of those tissues. Inoculation of naive hamsters with *C. difficile* led to greater concentrations of the bacteria in the distal small intestine, cecum, and colon (Rolfe and Iaconis 1983). Highly virulent strains of *C. difficile* most readily adhered to the cecum of hamsters, the typical site of lesions in hamsters (Borriello, Welch et al. 1988). Within each segment of the intestine, initial lesions are limited to those cells possessing receptors, as indicated by toxin binding (M. K. Keel, unpublished data). Lesions in naturally-occurring cases of disease may progress to much deeper tissues. However, the pathogenesis of CDAD is multifactorial and much of the damage to deeper epithelial cells, lamina propria, and even submucosa, probably results from inflammation.

TcdA binding to tissue sections could not be documented by an IHC employing a monoclonal antibody specific for TcdA. However, the antibody may have recognized epitopes in or near the receptor-binding portion of the protein. Such sites would have been masked in the bound toxin. The monoclonal antibody did not cross react with TcdB. This also is consistent with antibody binding near the receptor portion of the molecule because other regions of the two toxins are well-conserved.

The well-documented abundance of the α-Gal epitope on pig tissues, and its identification as a receptor for TcdA, naturally led to the hypothesis that it is the dominant receptor on porcine tissues. However, there is significant disparity in the
binding of TcdA to mucosal epithelium and the distribution of this carbohydrate; toxin binding was much more extensive than the distribution of the α-Gal epitope. IHC indicated α-Gal was limited to the surface of villus enterocytes in the jejunum and ileum only. Specific binding was also present on endothelial cells and ductal epithelial cells of the pancreas. TcdA, in contrast, bound to neither of those cell types. In addition, TcdA labeled the cytoplasm of gall bladder epithelial cells though M86 did not (Figure 3).

Neonates of most species are resistant to CDAD even though *C. difficile* readily colonizes their large intestine. This has prompted the hypothesis that newborns of those species lack adequate toxin receptors until later in life. Neonatal pigs seem to have abundant toxin receptors and are known to be susceptible to the disease. The IHC assay was repeated with newborn hamsters, who do not develop CDAD as neonates. Significant binding of toxin occurred throughout the intestine, suggesting that resistance to the effects of *C. difficile* colonization may be due to other factors. Very young hamsters, less than 4 days of age, are resistant to colonization by *C. difficile*, possibly providing some protection from CDAD (Rolfe and Iaconis 1983). Neonatal pigs, however, are readily infected with *C. difficile*. IHC of tissues from adult hamsters indicated a slightly more intense binding of TcdA. As in neonatal pigs, TcdB did not bind to any tissues in neonatal or adult hamsters.

A receptor for TcdB has never been identified on the luminal surface of intestinal epithelial cells, and there is evidence that an intact mucosa is resistant to the effects of TcdB (Lyerly, Saum et al. 1985). Only with mucosal trauma or concomitant exposure to TcdA was TcdB reportedly able to exert a significant effect. A prominent hypothesis to
explain this scenario is that the receptor for TcdB is restricted to the basolateral aspect of
the cell. After damage to epithelial cells or loss of intracellular attachments due to TcdA
action, the receptors are exposed to TcdB. However, in the IHC experiment, TcdB had
access to all aspects of the cell because they were sectioned. Still, no binding was
detected even though the biotinylated toxin was fully active in cell culture assays. TcdB
may gain access to the cell through another route, perhaps pinocytosis. It is also possible
that fixation modified the receptor in such a manner that toxin binding was blocked.
Further research is needed to investigate this aspect of the disease.

Demonstration of abundant widespread receptors on intestinal surfaces of
neonatal pigs provides additional evidence of their specific susceptibility to *C. difficile*
toxins. The receptors for TcdA in the pig remain to be identified, but the \( \alpha \)-Gal epitope
does not seem to be significant to its binding.
<table>
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<tr>
<th>Porcine Tissues</th>
<th>Biotinylated TcdA</th>
<th>Polyclonal α-TcdA Ab’s</th>
<th>Monoclonal α-TcdA Ab’s</th>
<th>Biotinylated TcdB</th>
<th>Anti-α-Galactosyl Monoclonal Ab’s</th>
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Table 1. IHC of *C. difficile* toxin attachment and receptor sites in pig tissues. Relative intensity of IHC signal is indicated for mucosal epithelial cells in each segment of the gastrointestinal tract and in additional, select tissues. No binding is indicated by a score of −. Maximal binding is indicated by a score of +++.
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Table 2. IHC of *C. difficile* toxin attachment and receptor sites in hamster tissues.

Relative intensity of IHC signal is indicated for mucosal epithelial cells in each segment of the gastrointestinal tract and in additional, select tissues. No binding is indicated by a score of -. Maximal binding is indicated by a score of +++.
Figure 1. Specific staining of intestinal mucosa by biotinylated TcdA. A. The brush border of epithelial cells in the descending colon is generally stained very heavily. However, individual colonocytes are unstained. B. Ileal brush border is variably stained with dense staining of individual cells and no staining of cells immediately adjacent.

Bars = 10 μm
Figure 2. IHC of pig mucosa with biotinylated TcdA (A-C) and TcdB (D). A, Jejunum is heavily stained with deposition of TcdA along the brush border of the entire villi, extending to the bottom of the crypts. B, Descending colon has moderate adherence of TcdA to the surface of superficial epithelial cells with only those in the most superficial portion of the glands exhibiting any staining. C, TcdA stains villus enterocytes in the ileum but staining extends only a short distance into the crypts. D, None of the tissues were stained by biotinylated TcdB; colon is depicted here. Bars = 25 μm.
Figure 3. IHC for the galactosyl epitope compared to TcdA binding. In figures A (duodenum) and C (gall bladder) the galactosyl epitope was stained by a specific monoclonal antibody. Only endothelial cells are stained in both sections. Figures B (duodenum) and D (gall bladder) were stained with a technique to demonstrate TcdA binding. The brush border of duodenal enterocytes and the cytoplasm of gall bladder epithelial cells are stained but endothelial cells are not. Bars = 25 μm
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APPENDIX D.

The Attachment, Internalization and Time-dependent, Intracellular Distribution of

*Clostridium difficile* Toxin A

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ABSTRACT

Toxin A (TcdA), secreted by toxigenic strains of *Clostridium difficile*, produces lesions typical of *C. difficile*-associated disease (CDAD) in susceptible mammal species. Colon explants maintained for two h with TcdA developed severe lesions characterized by cell swelling, swelling of mitochondria and other organelles, distension of cytoplasmic vesicles, expansion of paracellular spaces, apoptosis and necrosis. Severity of lesions was proportional to the dosage of toxin. No lesions were present in uninoculated control tissues after two h. Receptor-mediated endocytosis is the keystone event in the pathogenesis of the toxin and susceptibility of a given species is thought to depend on the presence of receptors on intestinal epithelial cells. The fate of TcdA applied to viable colon explants was determined by transmission electron microscopy in an anti-toxin-labeled gold assay. At five min post-inoculation, the presence of TcdA was indicated at the membrane of microvilli or in the cytoplasm of epithelial cells. TcdA was also indirectly observed within endosomes or attached at their margin. A 30-min inoculation period was associated with many more gold particles labeling structures inside the cell, though some were still attached to microvilli. Within the cell most TcdA was associated with mitochondria of epithelial cells but some gold particles decorated the nuclei. Endothelial cells of the lamina propria had evidence of TcdA at both their lumenal and basal aspects, as well as in the cytoplasm and, occasionally, nuclei. Gold particles also labeled the lumen of such vessels as well as leucocytes in blood vessels and the lamina propria.
INTRODUCTION

*Clostridium difficile* is an anaerobic, Gram-positive, spore-forming bacillus associated with diarrhea and enterocolitis in a variety of mammals and is the most common bacterial cause of antibiotic-associated diarrhea in humans (Jobe, Grasley et al. 1995; Hurley and Nguyen 2002). The principal virulence factors, toxins A and B (TcdA and TcdB) are large, AB-like toxins. They belong to a group of toxins with similar topology and function, the large clostridial toxins (LCT's). TcdA and TcdB share significant sequence homology and have similar domains, including an amino-terminal enzymatic domain, a hydrophobic region, and a carboxyl-terminal domain characterized by a series of repetitive peptides (von Eichel-Streiber, Suckau et al. 1989; von Eichel-Streiber and Sauerborn 1990; von Eichel-Streiber, Laufenberg-Feldmann et al. 1992). The carboxyl-terminus is the receptor binding portion and differs significantly between the two toxins. The carboxyl terminus of TcdA recognizes several carbohydrate receptors on the plasma membrane of susceptible cells. These include Galα1-3β1-4GlcNAc-R (α-Gal epitope), Galβ1-4{Fuα1-3}GlcNAc (Lewis X), Galβ1-4GlcNAcβ1 (Lewis Y), Galβ1-14GlcNAcβ1-3Galβ1-4(Glc) (Lewis I), and sucrose-isomaltase (Krivan, Clark et al. 1986; Tucker and Wilkins 1991; Pothoulakis, Galili et al. 1996; Pothoulakis, Gilbert et al. 1996). Receptor binding is followed by endocytosis, and acidification of the endosome is essential for full toxin activity (Florin and Thelestam 1983; Henriques, Florin et al. 1987; Qa'Dan, Spyres et al. 2000). A receptor for TcdB has never been identified but is hypothesized to exist on the basolateral aspect of intestinal epithelial cells.
The amino-termini of TcdA and TcdB contain the enzymatic domain and exhibit similar activity and substrate specificity (von Eichel-Streiber, Meyer zu Heringdorf et al. 1995; Chaves-Olarte, Weidmann et al. 1997; Faust, Ye et al. 1998). Both inactivate Rho-subtype GTPases by glucosylating a threonine residue at 35/37 (Just, Selzer et al. 1995; Just, Wilm et al. 1995; Genth, Aktories et al. 1999). These GTPases are a group of signal transduction molecules essential for the regulation and maintenance of actin filaments. Dysregulation of actin filaments leads to loss of tight junctions and cell rounding, both directly associated with the loss of mucosal function as an osmotic barrier.

TcdA is also associated with mitochondrial damage of epithelial cells and colocalizes with mitochondria within 5 min of cell entry (He, Hagen et al. 2000). Mitochondrial colocalization, and damage by TcdA, occur prior to the blockade of Rho-dependent pathways. Disruption of mitochondria is associated with decreased production of ATP, generation of reactive oxygen intermediates, and production of cytokines such as IL-8 (He, Sougioulitzis et al. 2002). Though the effects of TcdA on mitochondrial function have been catalogued, the direct interactions of TcdA with the mitochondria remain uncertain. TcdA-induced apoptosis is associated with the generation of reactive oxygen species by the mitochondria and activation of pro-apoptotic caspases but it was also dependent upon the inactivation of Rho-subtype GTPases (Brito, Fuji et al. 2002). Both caspase 8 and 9 were involved in TcdA-induced apoptosis, incriminating both the extrinsic and intrinsic apoptosis pathways.

A number of intestinal cell types other than mucosal epithelial cells are directly affected by TcdA, including neutrophils, mast cells, monocytes, and neurons (Calderon,
Torres-Lopez et al. 1998; Xia, Hu et al. 2000; Brito, Sullivan et al. 2002). Pathogenesis of the disease is complicated by the interactions of affected cell types by inflammatory mediators and release of cytotoxic inflammatory compounds that can contribute directly to damage of host tissues (Pothoulakis and Lamont 2001).

Proinflammatory mediators are associated with vascular changes in vivo and much of the vascular leakage is supposedly leukocyte dependent (Kurose, Pothoulakis et al. 1994). However, both TcdA and TcdB are directly toxic to endothelial cells and such toxicity could contribute to the effusion and edema, associated with C. difficile-associated disease (CDAD), if the toxins gain access to endothelial cells (Muller, von Eichel-Streiber et al. 1992). Virtually all cell types are somehow involved in the complex cascade of events associated with CDAD. Which of these associations are due to the direct interaction of the toxins with the target cells and which are due largely to the actions of intermediate participants is often difficult to elucidate.

Typhlocolitis and neonatal diarrhea due to C. difficile were only recently reported in pigs (Waters, Orr et al. 1998). Previous attempts to isolate C. difficile from pigs were unsuccessful, though it was eventually cultured from 8-week old pigs co-infected with Salmonella typhimurium (Jones and Hunter 1983). The susceptibility of pigs to CDAD was further cast in doubt by intestinal loop studies in which C. difficile toxins directly injected into the lumens did not result in significant lesions (Sisk, Cole et al. 1998). There have been additional reports of CDAD in pigs and experimental inoculation of colostrum-deprived pigs resulted in disease and lesions identical to those of spontaneous cases (Songer, Post et al. 2000). Nonetheless, there is a need for better characterization
of the susceptibility of pigs to CDAD and the host-tissue response to infection. This is important as a potential model for better understanding the disease in humans and other species and to better explore prevention strategies for the swine industry.

MATERIALS AND METHODS

Antibodies

A mouse, monoclonal antibody, BDI515 (Biodesign International, Saco, Maine), was used in the immunoaffinity chromatography of TcdA. Its isotype was IgG and it was specific for TcdA without recognizing any of the related toxins. An immunoaffinity-purified, rabbit, polyclonal antibody, M-C663-50L (Lee Laboratories, Grayson, GA, U.S.A.), raised against TcdA, was used for all immunoelectron microscopy procedures. Goat, anti-rabbit antibodies, linked to 6-nm colloidal gold beads (Aurion, Wageningen, The Netherlands) were also used.

Animals

Clinically normal, 12-24 h old pigs were selected based on negative culture for C. difficile and negative ELISA for TcdA and TcdB (Techlab®, Blacksburg, Virginia, U.S.A.). Intestinal explants collected from the pigs were initially placed in wash media consisting of DMEM/ F-12 (Cambrex, Walkersville, Maryland, U.S.A.) with amikacin (40 μg/ml; Fisher Scientific, Fairlawn, New Jersey, U.S.A.), kanamycin (0.6 mg/ml; Sigma-Aldrich, St. Louis, Missouri, U.S.A.), streptomycin (2 mg/ml; Fisher Scientific, Fairlawn, New Jersey, U.S.A.) and vancomycin (100 μg /ml; Sigma-Aldrich, St. Louis,
Missouri, U.S.A.). They were then maintained in nutrient media, DMEM / F-12 (Cambrex, Walkersville, Maryland, U.S.A.) with amikacin (20 μg/ml), streptomycin (2 mg/ml) and vancomycin (50 μg/ml). Both types of media were prewarmed to 37°C and pregassed with 5% CO₂ before initiation of experiments.

Pigs were euthanized by CO₂, and were placed in dorsal recumbency in a laminar flow hood. The abdomen was disinfected with surgical scrub and opened by a midline incision; the ascending colon was freed using sterile technique. The colon was placed in a sterile tray, irrigated with wash media, linearized by alternate blunt and sharp dissection, and opened by a longitudinal incision on the antimesenteric surface. Fecal material was gently flushed from the mucosal surface with wash media and 0.5-cm square sections were trimmed with a razor blade. The tissue samples were maintained in wash media until they were added to 6-well tissue culture plates, 25 to 30 min after euthanasia. Sterile instruments were used to insure that no exogenous bacteria were introduced to the explants. After incubating the samples for the designated period of time, they were removed from the media and placed in glutaraldehyde with formalin.

**Tissue Processing**

Tissue samples used exclusively for ultrastructural studies were fixed in 4% formalin with 1% glutaraldehyde in PBS (4F1G). After approximately 24 h the tissues were rinsed in 0.1% PBS (pH 7.4) and post-fixed in 1% osmium tetroxide. They were then rinsed in distilled water and dehydrated through graded alcohol. They were incubated in propylene oxide followed by 50% propylene oxide with 50% epon. Tissues
were embedded in epon and cured at 75°C for 8 h. Thin sections were post-stained with saturated aqueous uranyl acetate and Reynold’s lead citrate.

Samples generated for immunostaining were fixed in 1% formalin with 0.1% glutaraldehyde in PBS (1F0.1G). Tissues were fixed for 45 min, rinsed in distilled water, and stained with 2% aqueous uranyl acetate for 30 min. They were then dehydrated through graded alcohol and embedded in LR-white (Ted Pella Inc., Redding, CA, U.S.A.). Thin sections were placed on nickel grids which had been immersed in 1.25% Formvar (Ted Pella Inc., Redding, CA, U.S.A.) in chloroform, such that grid bars were coated but the open area was uncoated. This optimized adhesion of sections to the grids but eliminated the problem of background staining of colloidal gold on the hydrophilic plastic coating. Grids were blocked by immersion in 0.1% BSA with 0.1% Tween 20 (Electron Microscopy Sciences, Ft. Washington, PA, U.S.A.) in PBS (pH 7.4) for 10 min. They were then immersed in a 1:50 dilution of the rabbit polyclonal anti-TcdA and incubated at room temperature for two h. Labeled grids were washed 3 times with PBS, pH 7.4 and labeled with the secondary antibody (1:20) linked to colloidal gold. After another wash step they were fixed in 0.5% glutaraldehyde. The grids were washed in distilled water and post-stained for 10 min with saturated uranyl acetate. At each step grids were completely immersed to insure that both sides of the sections were labeled and washed.

All sections were examined with a Jeol transmission electron microscope (Japan Electron Optical Laboratories, Peabody, MA, U.S.A.) with an accelerating voltage of 60-80 kV.
Experimental Protocol

Five groups were used in the ultrastructural study of TcdA-associated lesions. These groups were treated with 2.5, 1, 0.5, 0.25 or 0 μg/ml of TcdA, respectively. Organ culture media was prewarmed to 37°C and pregassed with 0.5% CO2. TcdA was added to separate aliquots of toxin, to yield the required concentration, and 7.5 ml of media was added to the tissue culture wells of the corresponding treatment groups. Colon explants were added to each well within 25-30 min of euthanasia of the donor pig. Tissues were incubated for two h, then removed for fixation in 4% glutaraldehyde.

The immunoelectron microscopy study was performed in the same manner, with minor variation in the treatment groups. One treatment group consisted of samples incubated in 5 μg/ml TcdA for 5 min and 3 additional treatment groups consisted of samples incubated in 10 μg/ml TcdA for 5, 30 or 60 min. Control tissues were incubated in organ culture media, without toxin, for 5 min. After the requisite incubation period, tissues were placed directly in 1% glutaraldehyde.

Bacterial Culture

Fecal samples from all animal subjects were swabbed, and streaked for isolation, directly on taurocholate, cycloserine, cefoxitin, fructose agar (TCCFA) and *C. difficile* selective agar (CDSA; brain heart infusion, *C. difficile* antimicrobial supplement CC, sodium taurocholate and bovine blood) as previously described (George, Sutter et al. 1979; Wilson, Kennedy et al. 1982). After 48-72 h, suspect *C. difficile* colonies were picked and transferred to brain heart infusion agar with yeast extract (0.5%), cysteine
hydrochloride (0.05%) (BHI-CYE) and bovine blood (2%). Identification of *C. difficile* was based on colony morphology, odor, weak α hemolysis and a positive L-proline aminopeptidase assay (REMEL, Lenexa, Kansas, U.S.A.). For toxin production, cultures of *C. difficile* strain VPI 10463 (ATCC, Manassas, VA) were maintained for 5-7 days in dialysis tubing (molecular weight cutoff, 10,000) suspended in BHI-CYE broth.

**Cell Culture**

Activity of *C. difficile*-toxin samples was tested by overlay on Chinese hamster ovary cells (CHO cells). CHO cells were maintained in Iscove’s modified Dulbecco’s medium (IMDM; Cambrex, Walkersville, Maryland, U.S.A.) with 10% fetal bovine serum (FBS; Intergen Co., Purchase, New York, U.S.A.) and 100 mg/ml gentamycin (Sigma-Aldrich, St. Louis, Missouri, U.S.A.). For toxicity assays, cells were trypsinized, washed, and suspended at a concentration of $2 \times 10^5$ cells / ml; 100 ml of suspension was dispensed into each well of a 96-well tissue culture plate. Cells were incubated for 4 h at 37°C in 5% CO₂, then serial dilutions of toxin preparations, in 100 μl aliquots, were added to each well. Cells were incubated, as before, for an additional 18 h. The endpoint was determined as the dilution that caused 50% cytopathic effect (CPE).

**Toxin Purification**

Supernatant from dialysis bag cultures of *C. difficile* was harvested by centrifugation at 15,000 X g for 20 min. Toxins were purified from the supernatant by ion-affinity chromatography on a DEAE-sepharose column (Amersham Biosciences,
Fractions of 5 ml were collected on a GradiFrac™ rotary fraction collector (Amersham Biosciences, Uppsala, Sweden) and protein elution was monitored by a UV-1 ultraviolet monitor (Amersham Biosciences, Uppsala, Sweden). 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. Fractions of interest were pooled and the column eluate was concentrated by centrifugation in a centrifugal filter device with a molecular weight cut off of 100,000 daltons (Millipore, Bedford, MA). Concentration of the toxin was determined by a Bradford assay per the manufacturer’s instructions (Bio-Rad, Hercules, CA). Toxin activity was determined by CHO-cell assay and purity was confirmed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE). TcdA collected by ion-affinity chromatography was further purified by immunoaffinity chromatography using the monoclonal antibody, BDI515. The antibody was immobilized to an agarose column (Carbolink Gel, Pierce, Rockford, IL, U.S.A.) by coupling the carbohydrate groups in the Fc region to terminal hydrazide groups on the column matrix. Binding and elution of TcdA from the column were performed as per the manufacturers instructions. Purity of the toxin was determined by SDS-PAGE, using a 7.5% polyacrylamide gel and an electrophoretic current of 200 mV. The gel was stained with 0.2% coomassie brilliant blue, R-250 (Pierce, Rockford, IL, U.S.A.).

RESULTS

Untreated control tissues were maintained in organ culture media, at 37°C, for two h with minimal development of lesions. Rare, isolated, necrotic cells were present,
but the majority of the epithelial cells were normal. Microvilli were of uniform length and cells remain tightly apposed with no obvious loss of intracellular adhesion complexes and little expansion of the potential space between cells (Figure 1).

Tissues treated with as little as 0.25 μg/ml TcdA developed significant lesions in all cells. The most common changes included rarefaction of the cytoplasm, expansion of paracellular spaces, swelling of organelles, accumulation of autophagic vacuoles and blebbing of microvilli (Figure 2). In most sections, the tight junctions appeared to be intact. Individual cells with more serious lesions were often interspersed among more moderately affected cells, or were partially extruded from the mucosal epithelium (Figures 3). Moderately affected cells had more prominent cytoplasmic rarefaction at the basal aspect of the cells; organelles in the apical cytoplasm were closely packed.

A dosage of 0.5 μg/ml TcdA was correlated with more severe swelling of organelles, a greater frequency of necrosis, and extrusion of necrotic epithelial cells at the lumenal aspect of the mucosa (Figure 4). Tight junctions and desmosomes were still visible, even at the margins of some necrotic cells. Most cells exhibited features of coagulative necrosis, including cell swelling and karyorhexis, but some cells had features of apoptosis, such as formation of apoptosomes and fragmentation of nuclei with chromatin crescents (Figure 4).

At TcdA dosages of 1 and 2.5 μg/ml, cell swelling increased in severity and was primarily due to enormous distension of cytoplasmic vesicles (Fig. 4). Such severe changes affected all cells remaining in the mucosa. Surprisingly, microvilli often persisted on such cells, though there was abundant blebbing. Only in cells that were in
advanced stages of apoptosis or necrosis, were microvilli consistently lost, significantly blunted, or severely misshapen (Figure 4). As with low dosage groups, extrusion of necrotic cells at the lumenal surface was common. Cells were present at various stages of necrosis or apoptosis; apoptosis was more common than in tissues at lower dosages (Figure 4).

Immunoelectron microscopy specifically indicated TcdA bound to the surface of microvilli and within the cytoplasm of epithelial cells after 5 min (Figure 5). Particles attached to microvilli specifically labeled TcdA at the plasmalemma. Transverse sections of microvilli demonstrate that all beads are associated with microvilli and were in proximity to the surface (Figure 6). A small number of gold beads were visible in endosomes, but most of the intracellular particles at this time point were free in the cytoplasm (Figure 7-A). A significant number of beads were attached at the periphery of endosomes (Figure 7-B). These particles could indicate toxin molecules still attached to the membrane or recently translocated to the cytoplasm. Blood vessels in the superficial lamina propria had a moderate number of beads labeling the lumen, as well as the surfaces and cytoplasm of endothelial cells.

There was too much variability within each section to objectively enumerate the beads attached to cells, but a cursory assessment suggested that the tissues incubated with 10 µg/ml toxin had more attached beads than tissues incubated with 5 µg/ml toxin.

At 30 min post inoculation, a moderate number of beads still labeled microvilli but far fewer than at 5 min. The majority of the beads were intracellular and most of those were associated with mitochondria (Figure 10). The majority of mitochondria-
associated particles were attached at the margin but occasionally they appeared in the mitochondrial matrix. At this time point, TcdA was also localized to the nuclei of epithelial cells and other cell types in the lamina propria. TcdA was rarely associated with the nuclear membrane and seemed to be randomly dispersed in the interior. Rarely, beads in the vicinity of the nuclear membrane were associated with nuclear pores (Figure 10). Small vessels at the 30 min post-inoculation interval were again labeled by beads localized to the cytoplasm of endothelial cells, but they also occurred in the lumen and at the basal aspect of endothelial cells (Figure 11).

Colloidal gold did not label control tissues as it did the TcdA treated samples (Figure 12). Only rare beads adhered to the control tissues and these occurred singly, or occasionally in pairs, whereas beads in treated tissues most often occurred in clusters consistent with the multiple epitopes available on toxin molecules and primary antibodies.

DISCUSSION

_Clostridium difficile_ toxins ultimately cause necrosis of epithelial cells in intestinal mucosa. However, this is a complex reaction, involving many host cell types and a complex interplay of intercellular mediators. The seminal event in toxin pathogenesis is recognition of receptors on the luminal surface of susceptible enterocytes and expression of such receptors is an important factor in the susceptibility of a given animal to CDAD. This report demonstrates the susceptibility of pig colon explants to TcdA and documents the associated ultrastructural lesions. Also described are the
specific attachment of toxin A to epithelial cells, and the endocytosis and intracellular translocation of the toxin. This is the first time the intracellular localization of the toxin has been reported in intact mucosa.

*In vivo* studies demonstrate that glucosylation of Rho-subtype proteins leads to disorganization of tight junction structural proteins (Nusrat, von Eichel-Streiber et al. 2001). This perturbation of tight junction function is thought to be the premise for altered epithelial barrier function (Hecht, Pothoulakis et al. 1988; Hecht, Koutsouris et al. 1992). The increased paracellular accumulation of fluid in tissues inoculated with TcdA in this study is consistent with permeability of the apical aspect of the mucosa. However, there was no clearly defined loss or degeneration of tight junctions, even in cells with severe lesions. Observation of the tight junctions in transverse sections might not reveal focal damage to the structures, or functional changes could occur in tight junctions that are not easily resolved by the techniques used here. Ultrastructural studies of polarized intestinal epithelial cells treated with TcdA yielded the same results described here (Hecht, Pothoulakis et al. 1988). There was no visible difference in the ultrastructure of tight junctions though transepithelial resistance was nearly abolished and dissolution of F-actin rings was demonstrated by an immuno-assay with fluorescence.

Other ultrastructural lesions correlated with TcdA administration in colon explants were severe and dose dependent. Though not specific for TcdA they can, nonetheless, be attributed to its cumulative effects in the cells. Alteration of cytoskeletal structure is an early and consistent consequence of TcdA and TcdB (Thelestam and Bronnegard 1980; Wedel, Toselli et al. 1983; Hecht, Pothoulakis et al. 1988; Thelestam
and Chaves-Olarte 2000). Rate of cell rounding due to TcdB is dose dependent and the severity of ultrastructural lesions probably is, as well (Fiorentini, Fabbri et al. 1998). The disorganization of membrane-associated actin filaments is directly correlated with cell swelling. Since the organization of F-actin in the apical cytoplasm is negatively affected by TcdA, it is likely involved in the cell swelling seen in the colon explants.

The fate of cells in this study included apoptosis and necrosis, either with condensation of cell contents or, rarely, cell lysis. Both necrosis and apoptosis were most severe at higher dosages of toxin. Apoptosis due to TcdB is also known to be dose dependent in cultured intestinal epithelial cells (Fiorentini, Fabbri et al. 1998). It may involve direct effects by TcdA as well as loss of signals due to elevation from the basement membrane (Mahida, Makh et al. 1996; Mahida, Galvin et al. 1998). TcdA has also been associated with apoptosis of T cells, but not enough were observed in the tissues examined in this study to qualify their involvement (Mahida, Galvin et al. 1998). Severity of effects were not always uniform in the epithelial layer and necrotic or apoptotic cells were frequently attached to less severely deranged cells. This may reflect a greater susceptibility of individual cells due to age, differentiation, metabolic activity or a greater density of toxin receptors. Immunohistochemistry assays demonstrated significant heterogeneity among the ability of individual cells to bind toxin (M. K. Keel, unpublished data). This greater density of bound toxin is presumably due to a greater density of receptors. As cell lesions due to _C. difficile_ toxins are dose dependent, a greater density of TcdA receptors may contribute to the susceptibility of individual cells.
Microfilament arrays or derangement of microfilaments were not evident. The only exception was the disorganization of microfilaments, associated with the terminal web, coeval with the loss or derangement of microvilli. The presence of filaments forming in the perinuclear cytoplasm and within the nucleus due to TcdA are reported in CHO cells but were not evident here (Kushnaryov and Sedmak 1989). There were differences in fixation, however, and the significance of the absence of the filaments in this study is uncertain.

Specificity of the antibody was demonstrated by the absence of gold particles in the supracellular portions of the grid sections, and the specific binding of particles at the cell membrane as visible in transverse sections of microvilli. Negative control tissues, incubated for the same period of time as principals but in the absence of TcdA, did not display significant binding of colloidal-gold labeled secondary antibody. Translocation of an N-terminal subunit of TcdB occurs after fusion of the endosome and lysosomes (Pfeifer, Schirmer et al. 2003). In the colon explants examined here the translocated fragment was also labeled by the immunogold technique, employing a primary polyclonal antibody specific for TcdA.

Specific binding of TcdA to the brush border of porcine enterocytes is consistent with reports of the binding of TcdA to the brush border of other species of known susceptibility to *C. difficile* (Krivan, Clark et al. 1986; Pothoulakis, Gilbert et al. 1996). At 5 min post-inoculation, TcdA was bound to microvilli but some was seen in endosomes and even more was translocated to the cytoplasm. Five min is sufficient time for internalization of toxin in viable cells (Kushnaryov and Sedmak 1989; He, Hagen et
al. 2000). Many beads were also present just adjacent to endosomes and these may indicate toxin that is partially translocated. There was no evidence of accumulated C-terminal particles in the endosomes, possibly because of degradation. TcdA internalization in CHO cells was reportedly due to pinocytosis and association of bound toxin with coated pits (Kushnaryov and Sedmak 1989). Beads were not observed in coated pits in this study but the fixation methods were probably not optimal for their observation.

After 30 min, fewer particles were adhered to microvilli and more beads decorated intracellular structures. Most particles were localized to the margins of mitochondria but there was insufficient resolution to determine whether the particles were adhered to the outer membrane or other structures. Rarely, beads did appear to be within mitochondria, but again, it could not be determined if they were in the intermembrane space, attached to cristal membranes, or if they were in the internal matrix. Using confocal microscopy and fluorescent antibody techniques, other researchers also observed internalization of TcdA and localization to mitochondria (He, Hagen et al. 2000). TcdA specifically caused early mitochondrial dysfunction including the production of reactive oxygen intermediates, decreased cellular concentration of ATP and release of cytochrome C. Such changes occurred prior to and independent of the glucosylation of GTPases and TcdA exerted its effects directly on isolated mitochondria. Other reports confirmed involvement of TcdA in mitochondrial damage and cytochrome C release but also found that the proapoptotic mechanism was dependent upon inactivation of Rho, caspases 3, 6, 8 and 9, and Bid (Brito, Fujji et al. 2002).
The direct effects of TcdA on mitochondria could be involved in the apoptosis or necrosis characteristic of TcdA intoxication on a variety of cell types (Fiorentini, Fabbri et al. 1998; Mahida, Galvin et al. 1998). A number of bacterial pathogens are known to induce apoptotic cell death by directly interfering with mitochondrial function. Another LCT, lethal toxin (LT) of *Clostridium sordellii*, is included among these (Petit, Breard et al. 2003). Like TcdA, LT induced apoptosis by direct mitochondrial association, and this was apparent irrespective of GTPase inactivation. The ability of leucotoxin (Ltx), produced by *Actinobacillus actinomycetemcomitans*, to induce apoptosis was also associated with mitochondrial damage (Korostoff, Yamaguchi et al. 2000). The *Helicobacter pylori* vacuolating cytotoxin (VacA) also localizes to mitochondria and drives apoptotic pathways. It was believed that the mechanism of VacA activity was formation of an anion selective channel in the mitochondrial membrane (Willhite and Blanke 2004). Though TcdA localizes to mitochondria very soon after cell entry and is directly associated with mitochondrial derangement, no molecular target of TcdA has been identified in association with TcdA, and the nature of TcdA interaction with this organelle remains to be described.

TcdA was also detected, by immunoelectron microscopy, inside nuclei. Rarely, beads were localized to nuclear pores but the frequency of association with nuclear pores was not great enough to draw any conclusions about their involvement in entry of the toxin. Nuclear localization has been described for a number of other viral proteins and bacterial toxins. These use a variety of mechanisms such as transport vesicles derived from endocytosis, association with a host protein that has a nuclear localization sequence
or expression of a domain within the alien protein, analogous to host nuclear localization sequences (Zhao and Padmanabhan 1988; Pollard, Michael et al. 1996; Bild, Turkson et al. 2002).

Vascular changes are very important to the pathogenesis of CDAD in the host, and endothelial cells are known to be directly influenced by the effects of C. difficile toxins as well as cytokines released by local inflammatory cells (Muller, von Eichel-Streiber et al. 1992; Kurose, Pothoulakis et al. 1994; Aepfelbacher, Essler et al. 1997; Hippenstiel, Tannert-Otto et al. 1997). TcdA was labeled at the basal aspect of endothelial cells, in vesicles and in the cytoplasm, consistent with uptake of TcdA from the lamina propria and transport into endothelial cells. Aggregates of colloidal gold also indicated the presence of TcdA in the lumen of vessels. TcdA within blood vessels could have been transferred by endothelial cells or it may have entered postcapillary venules or lymphatic vessels along an osmotic gradient. All blood vessels examined were located in the superficial lamina propria.

Spontaneous cases of CDAD in pigs, as well as pigs experimentally intoxicated with TcdA or TcdB, often developed systemic lesions including hydrothorax, pleural effusion and subcutaneous edema (Waters, Orr et al. 1998). The fluid accumulating in body cavities was a high protein effusion with abundant fibrin suggesting a direct effect of C. difficile toxins on regional blood vessels (M. K. Keel, unpublished data). The generation of proinflammatory mediators by circulating leucocytes is unlikely to lead to such regional vascular leakiness. The general outcome is hypotensive shock. The
demonstration of TcdA localizing to endothelial cells further supports the hypothesis that a direct interaction is important to the pathogenesis of such lesions.

Leucocytes including macrophages, monocytes, mast cells, and neutrophils were also labeled by colloidal gold, indicating TcdA in the cytoplasm and in nuclei. A direct link between TcdA and leucocytes has been confirmed in multiple reports. TcdA-treated macrophage cultures increased production of IL-1 and lamina propria macrophages increase production of TNF-α and substance P in response to TcdA (Miller, Pothoulakis et al. 1990; Castagliuolo, Keates et al. 1997). Isolated human neutrophils responded to TcdA with increased expression of L-selectin and decreased expression of Mac-1; in contrast to the proinflammatory responses, their migratory abilities and oxidative responses were impaired (Brito, Sullivan et al. 2002). Purified rat mast cells initially responded to TcdA by increased expression of TNF-α, but longer incubation times hampered function and survival (Calderon, Torres-Lopez et al. 1998). No molecular targets of TcdA have been identified on the surface of leucocytes. Within the cells they presumably have the same activity as in epithelial cells and alterations in actin filaments have been reported in neutrophils (Brito, Sullivan et al. 2002). The immunoreactivity of these cells for TcdA confirms the same types of interactions seen in epithelial cells.

This report confirms the specific susceptibility of porcine colonic mucosa to TcdA. Severe intracellular lesions were associated with inoculation with TcdA, though they were generally nonspecific. Immunoelectron microscopy studies confirmed the specific binding of TcdA to the apical plasmalemma of enterocytes. As indicated by indirect assays, bound TcdA was internalized within endosomes and rapidly transferred
to the cytoplasm. Within the cytoplasm, TcdA localized primarily to mitochondria. The specific association of the two is uncertain, but it is consistent with reports of mitochondria-mediated apoptosis in TcdA-treated cells. TcdA also occurred in blood vessels of the lamina propria as well as various leucocytes. These reports confirm, by ultrastructural visualization, the reports of various functional assays as to the nature of the interaction between TcdA and intestinal epithelial cells, as well as various cells of the lamina propria.
Figure 1. Untreated control tissues maintained for two h in organ culture media. A, Low power magnification of 3 colon epithelial cells. No significant lesions are evident in the cells and cell margins remain tightly apposed. (Bar = 0.5 μm). B, Detail of cell junctions at the brush border. A tight junction (arrow) is visible. The potential space between the two cells is not expanded. (Bar = 0.25 μm).
Figure 2. Organ explants treated with 0.25 μg/ml TcdA for two h. A, Most cells have intracellular edema, swollen organelles, and autophagic vacuoles. (Bar = 1 μm). B, Autophagic vacuoles are prominent (arrowheads); fluid expands the intercellular space. (Bar = 1 μm). C, A necrotic cell bordering another with less severe lesions. Microvilli are blebed (left) or absent (right) A tight junction is visible (arrowhead) (Bar = 0.5 μm). D, Tight junctions (arrowhead) and desmosomes (arrow) persist in many cells. Mitochondria are swollen and misshapen. (Bar = 250 nm).
Figure 3. Organ explants treated with 0.5 mg/ml TcdA demonstrating the range of effects on individual cells. A, Necrotic cells are partially extruded from an intact mucosal epithelium. Surviving cells have markedly swollen vesicles and are separated by paracellular spaces expanded by fluid. (Bar = 1 μm). B, A lysed cell remains attached to an intact but damaged cell by junctional complexes. (Bar = 0.75 μm).
Figure 4. Colon explants treated with 0.5 mg/ml TcdA. Cell swelling is more severe than in tissues treated with less concentrated toxin and large vesicles have formed in all cells (B & D). Microvilli persist on all but even the most severely damaged cells (B and D). Some cells have condensed nuclear chromatin or chromatin crescents indicative of apoptosis (A and C). Blebbing of nuclear membranes (arrowheads) (A and C) was present in apoptotic cells. Bars = 0.75 µm (A and C), 1 µm (B) and 2 µm (D).
Figure 5. Immunoelectron microscopy of colon explants after 5 min inoculation with 10 μg/ml TcdA. Colloidal gold attached to microvilli and the cell’s cytoplasm indicates the location of toxin molecules. The average diameter of gold beads is 6 nm. (Bar = 100 nm)
Figure 6. Transverse section of microvilli. Gold beads indicate the localization of TcdA to the cell membrane. (Bar = 100 nm).
Figure 7. Colloidal gold localizes TcdA to multiple sites in the cell. A, Beads are visible at the margins of microvilli, within the cytoplasm and in endosomes. (Bar = 200 nm). B, Beads often labeled TcdA at the margins of endosomes. (Bar = 100 nm).
Figure 8. Endothelial cell with multiple aggregates of gold beads in the cytoplasm. One group of beads appears to label TcdA in a vesicle adjacent to the lumen (arrowhead). (Bar = 200 nm).
Figure 9. Mitochondria in a colon epithelial cell are labeled by colloidal gold. Beads generally decorate the margins of mitochondria. The size of the beads and steric effects of the attached proteins do not permit the precise localization of TcdA with respect to the individual mitochondrial membranes or internal structures. (Bar = 250 nm).
Figure 10. Mitochondria and cell nuclei labeled for TcdA at 30 min post-inoculation. A. TcdA is localized to mitochondrial margins of colon epithelial cells and occasionally to the interior (arrowheads). A cluster of beads also lie in the nucleus (arrow). (Bar = 200 nm). B. A detail of the cell nucleus from “A”. (Bar = 100 nm). C. An endothelial cell nucleus from the superficial lamina propria. Beads are present in the antrum of a nuclear pore (arrow). (Bar = 100 nm).
Figure 11. Gold beads label TcdA, indicating its presence in a small vessel from the lamina propria of a colon explant. TcdA is present at the basal margin of endothelial cells (dark arrowheads), in the cytoplasm (white arrowhead) and the lumen (arrow). (Bar = 250 nm).
Figure 12. Uninoculated, negative control tissue. Negative controls were treated the same as all other tissues except TcdA was withheld from the organ culture media. A single bead is present in this section (arrow). Beads were rarely seen in control negative tissues and were almost always solitary. (Bar = 100 nm).
REFERENCES


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