Infection of neonatal swine with *Clostridium difficile*

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Summary

Clostridium difficile is an established cause of antibiotic-associated diarrhea and pseudomembranous colitis in humans and domestic and laboratory animals. Diagnostic findings support a role for *C. difficile* in neonatal enteritis of pigs. A typical case will occur in a piglet 1–7 days of age, with diarrhea beginning soon after birth. Pathology includes moderate to severe mesocolonic edema, sometimes accompanied by hydrothorax and/or ascites, with scattered foci of suppuration in the colonic lamina propria and accumulation of neutrophils in the mesocolon. Exudation of neutrophils and fibrin into the lumen gives rise to so-called "volcano" lesions. Cultures of affected tissues commonly yield heavy growth of *C. difficile*, and toxin testing almost invariably reveals the presence of toxins A and B. Treatment with antimicrobials and use of probiotics has yielded mainly unsatisfactory results, but bacitracin methylene disalicylate may be useful for prevention. The lack of commercially available immunoprophylactic products may cause producers to turn to autogenous bacterin/toxoids, but their effectiveness is uncertain.

Keywords: *Clostridium difficile*, neonatal pigs, mesocolonic edema, bacitracin methylene disalicylate

Clostridium difficile is a gram-positive to gram-variable, spore-forming anaerobe which is found widely in soil, water, and the intestinal tract of various mammals, birds, and reptiles. It is an established cause of antibiotic-associated diarrhea and pseudomembranous colitis in humans, enterocolitis in foals, nosocomial diarrhea and typhlocolitis in adult horses, and typhlitis in adult hamsters (Table 1).

Germination of *C. difficile* spores in the large intestine is usually uneventful, but if the normal flora has not been established or has been disrupted in some way, *C. difficile* may establish, multiply, and produce toxins. Infections often occur as a result of exposure to antimicrobials, but dietary changes may also potentiate infection.

Clostridium difficile colonizes young animals and human infants, but is rapidly displaced as the flora matures. Despite the presence of high concentrations of stool cytotoxin (toxin B) in human infants, they are resistant to clinical manifestations of disease, apparently because neonatal enterocytes lack toxin receptors.

Clinical manifestations and pathologic lesions

Although it remains difficult to reproduce the disease, diagnostic findings to date support a working hypothesis that *C. difficile* is capable of producing important pathologic changes in the intestines of piglets. Infection of pigs with *C. difficile* was apparently first confirmed by cultural isolation in 1980[1] in gnotobiotic pigs experimentally infected with *Brachyspira hyodysenteriae* and accidentally exposed to *C. difficile*. These pigs excreted mucoid feces containing specks of blood and became dehydrated. Inoculation with *C. difficile* alone produced similar signs and lesions; 1×10^7–1×10^8 colony-forming units (CFU) of *C. difficile* were recovered per g of intestinal content, and toxin could be detected at a dilution $\geq 10^{-4}$. Natural infection was reported in 8-week-old conventional pigs, from which luxuriant growth of *C. difficile* was obtained.[2] A diphtheritic layer covered the colonic mucosa, and stunting of ileal villi was also observed, with evidence of regenerative activity throughout the small intestine.

An excellent recent description of an outbreak of enteritis associated with *C. difficile* is, for practical purposes, the only such account available.[3] Pigs averaging 5 days of age displayed dyspnea, mild abdominal distension, scrotal edema, and occasional diarrhea. Ascites (> 50 mL), conspicuous edema of the ascending mesocolon, hydrothorax, and precipitation of urates in the kidney were common. Microscopic examination revealed severe submucosal and mesocolonic edema in the ascending colon, with multifocal exudation of mucus, fibrin, and neutrophil aggregates. Both *C. difficile* and its toxins were detected, and the latter is considered pathognomonic for *C. difficile*-associated disease. In another outbreak confirmed by toxin detection (Waters E, personal communication, 1999), there was no evidence of predisposition by use of antimicrobials, but two were "high-health" herds, and may have been immunologically naïve. Mortality reached 50% in suckling piglets. In Quebec, outbreak-associated mortality of 16% was mainly the result of respiratory distress (due to ascites and severe mesocolonic edema).

Case reports

Recent cases from the records of the Rollins Animal Disease Diagnostic Laboratory reveal aspects of *C. difficile*-associated enteritis that are useful in diagnosis and development of control strategies.

Case 1

Three piglets were submitted with a history of diarrhea beginning at 2–3 days of age. Herd morbidity was 7%–8%. Sows had received a commercial *Escherichia coli* vaccine. At necropsy, pigs had dark yellow diarrhea, scant contents in the small intestines, and marked edema of the mesocolon. Large intestinal contents were yellow to dark yellow. One pig had milk in the stomach and two did not. Microscopic lesions

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Table 1: Diseases associated with *Clostridium difficile* infection

Species	Condition(s)
Human	Antibiotic-associated diarrhea Pseudomembranous colitis Toxic megacolon Septicemia Myonecrosis
Laboratory rodents	Typhlitis in hamsters Antibiotic-associated diarrhea in mice, rabbits, and guinea pigs
Horses	Hemorrhagic necrotizing enterocolitis in neonatal foals Nosocomial diarrhea and typhlitis in adult horses
Dogs	Chronic diarrhea
Ratites	Enterotoxemia in ostriches
Pigs	Neonatal necrotizing colitis

included limited multifocal suppurative infiltrate in the large intestine. The colonic mucosa was generally intact with the exception of some very focal erosions with loss of superficial epithelium. The submucosa and serosa were edematous and there was infiltration of mixed inflammatory cells, including some mononuclear cells and neutrophils. Large numbers of small rod-shaped bacteria were also observed in the lumen and the mesocolon was edematous. No significant lesions were seen in small intestine, liver, or mesenteric lymph nodes.

Aerobic bacteriologic culture of small intestine yielded moderate growth of a nonhemolytic *E. coli* and anaerobic cultures yielded moderate numbers of *C. perfringens* type A, which was nonenterotoxigenic and did not produce β2 toxin. Neither latex agglutination tests nor direct electron microscopic examination revealed the presence of rotavirus, and examination of frozen sections by a fluorescent antibody test was negative for transmissible gastroenteritis virus (TGEV). Anaerobic cultures of the colon yielded moderate numbers of *C. difficile* and toxins A and B were detected in large intestinal contents examined by an enzyme immunoassay.

Case 2

Three piglets, 4 days old, were submitted with a history of diarrhea beginning at 3–4 days of age. Mortality was low but affected pigs were usually stunted. Necropsy revealed stomachs filled with milk, a dehydrated subcutis, and a small intestine which was flaccid and pale. Colonic contents were yellow and fluid-to-pasty in consistency, and the mesentery was edematous.

There were some damaged villi in the small intestine, and jejunal villus tips were edematous. There was an accumulation of fibrin and some infiltration of mixed inflammatory cells, predominantly neutrophils, in the lamina propria of the apical portion of the villi. The colonic mucosa was generally intact with no significant lesions. There were a few scattered inflammatory cells, including some neutrophils, in the submucosa. The serosa and mesentery were edematous and infiltrated with a scattering of inflammatory cells. Small lymph nodes in the mesentery appeared to be somewhat hyperplastic, and macrophages in the periphery of the nodes contained some hemosiderin. Moderate numbers of large rod-shaped bacteria were found in the colonic lumen.

Bacteriologic culture of the small intestine revealed moderate numbers of nonhemolytic *E. coli* and low to moderate numbers of *C. perfringens* of two colony types. These genotyped as nonenterotoxigenic, β2 toxin-negative type A and nonenterotoxigenic, β2 toxin-producing type C. As in Case 1, tests for rotavirus and TGEV were negative. Heavy growth of *C. difficile* was obtained from the large intestine, and intestinal contents contained toxins A and B as determined by enzyme immunoassay.

Case 3

Three 10-day-old pigs were submitted with a history of diarrhea. The herd morbidity was 10%, and the case fatality rate averaged 35%. Diarrhea began at about day 10 of age. Processing of pigs had included injection with ceftiofur sodium, and the same antimicrobial had been used for treatment with some improvement noted. Necropsy revealed stomachs well filled with milk curd. The small intestine was congested and flaccid, and the spiral colon was edematous. All pigs had pasty yellow feces. Microscopic examination of sections of small intestine revealed congestion of the submucosa and mucosa but no other significant lesions. The colonic mucosa was histologically normal with some areas of mild to moderate congestion. Separation of cells in the serosa indicated edema. These areas contained a scattering of mononuclear inflammatory cells with an occasional neutrophil. There were heavy populations of large rod-shaped bacteria in the lumen of the small and large intestine.

Bacteriologic culture of small intestine yielded heavy growth of β-hemolytic *E. coli*, which genotyping revealed to contain genes for the K88 pilus and toxins STb and LT. A few colonies of *C. perfringens* were isolated from the small intestine, and these genotyped as nonenterotoxigenic, β2 toxin-nonproducing type A. As in Cases 1 and 2, tests for rotavirus and TGEV were negative. Moderate-to-heavy growth of *C. difficile* was obtained from the colon, and intestinal contents contained toxins A and B, as determined by enzyme immunoassay.

Based upon these cases and others, including those described in the literature, the case definition would typically include piglets 1–7 days of age, presenting with a history of diarrhea shortly after birth (Table 2). Litters from both gilts and sows are affected, and respiratory distress and decreased survival rates are common. Gross pathology includes moderate to severe edema of the mesocolon (Figure 1), often accompanied by hydrothorax and/or ascites. Large intestines are frequently filled with pasty to watery yellowish feces. Microscopic lesions in the colon consist of scattered foci of suppuration in the lamina propria, with additional accumulation of neutrophils in the mesocolon. A common lesion is edema involving the colonic serosa

Table 2: Case definition for *Clostridium difficile*-associated disease in neonatal pigs

	Features
History and clinical signs	Piglets (from gilts and sows) 1–7 days of age, with diarrhea beginning shortly after birth; loss of condition, stunting of survivors; respiratory distress, decreased survival rates common
	Morbidity 10%–90%, averaging 20% Case fatality rate up to 50%, averaging 20%
	Possible association with administration of penicillins or cephalosporins at processing
Gross pathology	Moderate to severe mesocolonic edema; hydrothorax and/or ascites occasional; pasty to watery yellowish colonic contents
Microscopic pathology	Scattered suppurative foci in colonic lamina propria; neutrophilic infiltrate in mesocolon; segmental erosion of colonic mucosal epithelium; "volcano" lesions (neutrophil and fibrin exudation into colonic lumen); large rods, sometimes with spores, on mucosal surface, in lumen
	No remarkable lesions in small intestine
Bacteriology and toxin testing	Moderate to heavy growth of *C.difficile*
	Presence of toxins A and B

and mesentery (Figure 2), and there are infiltrations of mononuclear inflammatory cells and neutrophils in the edematous areas. There may be segmental erosion of the mucosal epithelial surface of the colon. Exudation of neutrophils and fibrin from these inflamed mucosal segments into the lumen give rise to so-called "volcano" lesions (Figure 3). Gram stains of smears or sections may reveal large numbers of gram-positive rods. There are usually no remarkable lesions in the small intestines of pigs with uncomplicated *C. difficile* infection. Villous blunting, congestion of mucosal vessels, atonicity, and pale contents in the small intestine are often accompanied by concurrent infection with *C. perfringens* or *E. coli*; the possible presence of these or viral agents should be documented.

At the time of processing, neonatal pigs are commonly treated with penicillins or cephalosporins for prophylaxis. The sensitivity of the developing gut flora and the possible resistance of *C. difficile* to these antimicrobials may be potentiating factors in the incidence of *C. difficile*-associated enterocolitis. Currently available data warrant no firm conclusions, but anecdotal evidence suggests increased incidence on those premises where such processing is practiced.

At the present time, we do not have access to data obtained via processing of a random or representative sample from the population at risk. Thus, accurate estimates of incidence and prevalence are not available. However, widespread occurrence of *C. difficile*-associated disease in pigs is implied by its diagnosis in laboratories throughout swine-producing areas of the United States. Data from one of these laboratories suggests that annual incidence peaks in January through March, and that cases involving *C. difficile* as an etiologic agent represent approximately 52.8% of the total neonatal pig enteritis accessions; more than 36% of these cases involved *C. difficile* as the only pathogen of interest. Subjectively, this may place the importance of *C. difficile*-associated disease above that of *E. coli*, *C. perfringens* type A, and viral agents in at least some parts of the United States.

Bacteriologic culture and toxin testing

Cultures of affected tissues (Table 3) commonly yield heavy growth of *C. difficile*, and toxin testing by neutralization in Chinese hamster ovary (CHO) cells or commercial enzyme immunoassay almost invariably reveal the presence of toxins A and B. Results of our preliminary studies suggest a 93.7% correlation between clinical, pathologic, and bacteriologic diagnosis, on the one hand, and toxin detection on the other. Toxin detection in rectal swabs is also an acceptable protocol, in that results are 92.8% correlated with those from examination of intestinal contents. A protocol for the CHO cell assay, a list of manufacturers of enzyme immunoassays, and procedures for bacteriologic culture are available.[4]

Pathogenesis

Virulence attributes may include pili, capsule, and degradative enzymes, but production of toxins is essential. Toxins A (307 kDa) and B (270 kDa) of *C. difficile* are the largest bacterial toxins described to date, and are members of the family of so-called "large clostridial toxins." The former is an enterotoxin which causes fluid accumulation in the intestine, and the latter is a cytotoxin which is highly cytopathic for cultured cells. Both toxins are internalized by target cells and disrupt the cytoskeleton by enzymatic attack on intracellular targets. Cessation of protein synthesis and cell division is followed by exfoliation of enterocytes. Degranulation of mucosal mast cells and release of inflammatory mediators causes an influx of granulocytes, resulting in substantial tissue damage.[5]

In the only study addressing pathogenesis of *C. difficile* infection in pigs, crude preparations of toxins A and B had minimal effect when administered into colonic loops of pigs.[6] However, inadequate toxin concentration in the inoculum, dilution of toxin in the colonic segment, or interference with toxin action by flora or other

Figure 1: Four-day-old piglet with gross lesions suggestive of *Clostridium difficile* infection, with prominent edema of the mesocolon

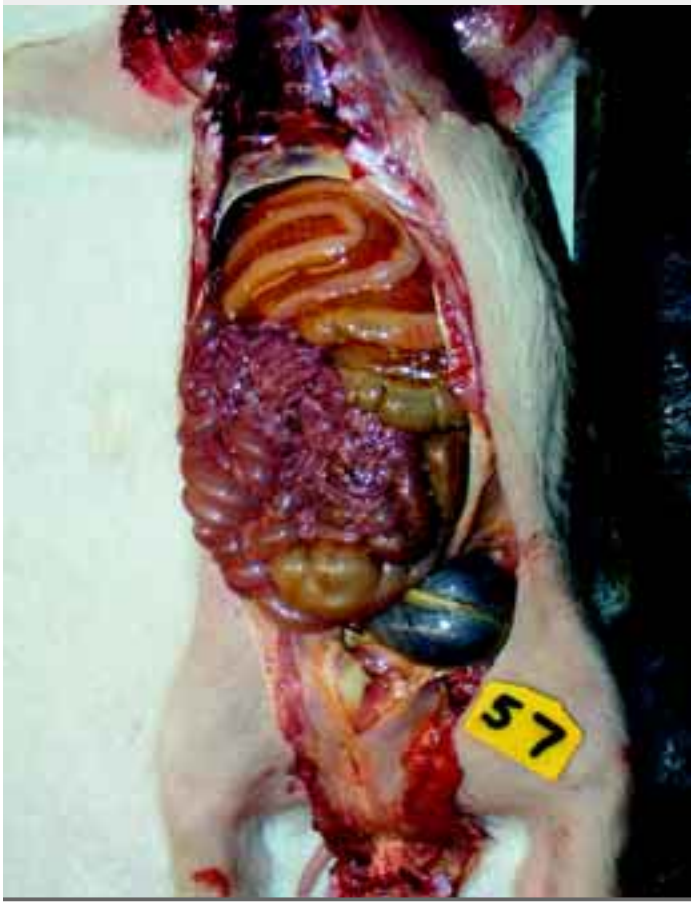


Figure 2: Microscopic appearance of the mesocolon in *Clostridium difficile* infection

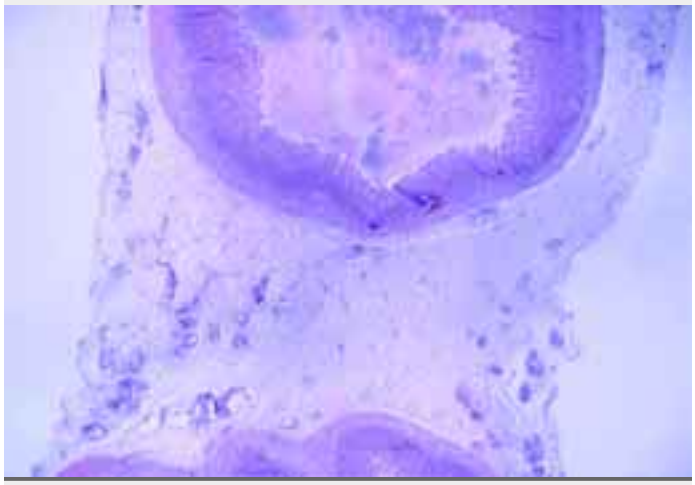
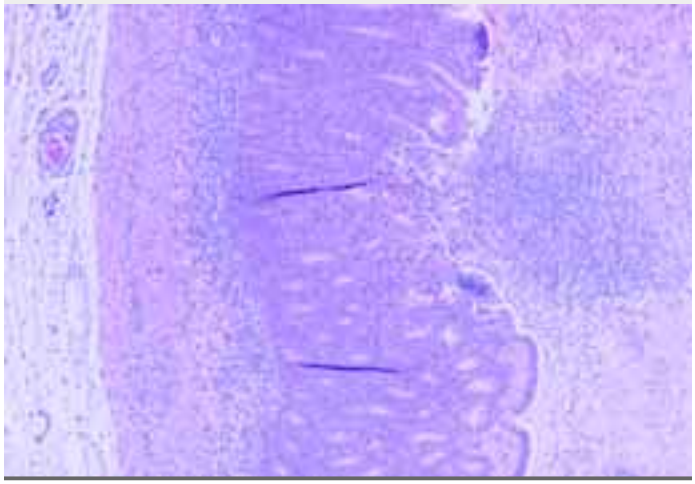


Figure 3: Suppurative focus ("volcano" lesion) in the colonic mucosa of a piglet infected with *Clostridium difficile*. Exudation of neutrophils into the colonic lumen is a common finding.



materials may have influenced the results. There is clearly a need to reexamine the effect of toxins A and B on the pig intestine, using high-titer crude and purified toxins and cultures of *C. difficile*.

Prophylaxis and therapy

Due to the relative lack of information on *C. difficile*-associated enteric disease in swine, one must look to other species for prophylactic and therapeutic options. Metronidazole, bacitracin zinc, and vancomycin have been used to treat *C. difficile* infections in the horse[7,8] and the last is the antimicrobial of choice for the treatment of typhlitis in hamsters.[9] Metronidazole and vancomycin are not approved for use in food animals, but bacitracin methylene disalicylate (BMD®, Alpharma; Fort Lee, New Jersey), is approved to prevent and treat enteritis due to *C. perfringens* in pigs. It is typically administered to pregnant females at 250 g per ton of feed for 2 weeks prefarrowing and in the lactation ration at the same concentration for 3 weeks postfarrowing.[10] Anecdotal evidence in some North Carolina outbreaks indicate that this may be effective. In some outbreaks in pigs, treatment with antimicrobials and use of probiotics has produced inconsistent, and mainly unsatisfactory, results (LaRochelle D, personal communication, 1999).

Clostridium sordellii toxins are antigenically related to toxins A and B, and *C. sordellii* antitoxin has been administered orally to prevent enteritis in the hamster model of *C. difficile* infection.[11] A nonpathogenic yeast, *Saccharomyces boulardii*, can suppress intestinal overgrowth by *C. difficile* after antimicrobial therapy.[12,13] Live, nontoxigenic strains of *C. difficile* can fill niches opened in the gut of the hamster by antimicrobial therapy, as evidenced by the subsequent protection against challenge with

Table 3: Specimen selection for diagnosis of *Clostridium difficile* infection in neonatal pigs

Specimen	Comments
Piglets	Live, typical clinical signs, untreated Ligated loops of small intestine and colon, shipped on wet ice (not frozen)
Feces	Gut contents or rectal swabs (in appropriate anaerobic transport medium* if shipment requires >24 h), shipped on wet ice

* See reference 4

toxigenic strains.[14] Parenteral and mucosal immunization with formalin-inactivated strains and with toxoids has been reported,[15,16] and protection is apparently associated with high serum concentrations of toxin-neutralizing antibodies. Finally, antibodies to recombinant toxins A and B have been used to treat infections.[17] Knowledge of the structure and function of toxins A and B, derived from studies directed at understanding of the human disease, will likely be useful in designing methods for immunoprophylaxis of *C. difficile*-associated disease in pigs.

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