Piglet Models of Acute or Chronic Clostridium difficile Illness

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We examined the piglet model of Clostridium difficile illness (CDI) in humans, because swine are naturally susceptible to C. difficile. The piglet is a reproducible model of acute or chronic CDI with characteristic pseudomembranous colitis. Germ-free piglets were consistently and extensively colonized after oral challenge with the human strain 027/BI/NAP1, establishing an infectious dose–age relationship. This allowed a demarcation between acute fatal and chronic models. The clinical manifestations of disease inclusive of gastrointestinal and systemic symptoms and characteristic mucosal lesions of the large bowel (including pseudomembranous colitis) are described. Additionally, we demonstrate the presence of toxins in feces, body fluids, and serum and a significant elevation in interleukin 8 levels in animals with severe disease. We conclude that piglets infected with C. difficile mimic many of the key characteristics observed in humans with CDI and are suitable animals in which to investigate the role played by virulence attributes, drug efficacy, and vaccine candidates.

Clostridium difficile is a gram-positive, anaerobic, spore-forming bacterium and a major cause of antibiotic-associated diarrhea in many countries worldwide [1–3]. It is the etiologic agent of pseudomembranous colitis in humans, but infection can result in a range of sequelae, from asymptomatic carriage to toxic megacolon and death [2]. C. difficile illness (CDI) has reached epidemic proportions in several countries since the year 2000, and emerging hypervirulent strains are causing increased morbidity and mortality among patients [4]. One group of these hypervirulent strains has been characterized as ribotype 027, restriction enzyme analysis type BI, North American pulsed field type 1 (027/BI/NAP1). These strains produce the 3 known toxins: toxin A (TcdA), toxin B (TcdB), and binary toxin. TcdA and TcdB are known to be important virulence factors that affect intestinal epithelial cells directly and promote inflammatory reactions, leading to the recognized signs of disease [5].

In addition to gastrointestinal disease, systemic complications of infection—such as ascites [6, 7], pleural effusion [8, 9], cardiopulmonary arrest [10, 11], hepatic abscess [12], abdominal compartment syndrome [13], acute respiratory distress syndrome [14], multiple organ dysfunction syndrome [15], and renal failure [16]—have been reported in human cases. The mechanisms by which C. difficile causes these systemic effects are not entirely understood, but the toxins produced by the bacterium (especially TcdA and TcdB) are likely involved. A better understanding of the systemic effects of infection with C. difficile and why they occur in some patients but not in others is important, because these effects are often life-threatening in nature.

Many species have been evaluated as models of CDI, but the hamster has been the classic model because of extreme sensitivity to infection after antibiotic administration [17]. Hamsters develop the clinical signs of
severe diarrhea, weakness, and lethargy, and death usually occurs within 2–3 days of infection. Other laboratory animals (such as mice, rats, and rabbits) have also been used but are not as sensitive to infection as hamsters [5, 17]. Although hamsters do provide a valuable model of acute CDI, the model does have limitations. Few commercial assays and immune reagents are available, and their extreme sensitivity precludes the study of many of the clinical and pathological conditions observed in humans with CDI.

Infection with C. difficile commonly occurs in swine, and in piglets it causes enteritis during the first week of life [18–20]. C. difficile outbreaks on swine farms usually include pasty, yellowish diarrhea, sometimes with respiratory distress and death [20], and C. difficile has become the most commonly diagnosed cause of enteritis in neonatal pigs [18]. CDI has been reproduced in pigs inoculated with pure cultures [18]; however, questions still remain regarding pathogenesis, immune response to infection, and treatment and prevention strategies. The similarities to human disease and availability of reagents make pigs an attractive model for C. difficile studies, and we describe here the development and characterization of the gnotobiotic piglet as a model of acute or chronic CDI.

METHODS

Animals. Gnotobiotic piglets, derived via cesarean section, were housed in sterile isolators and fed Similac milk replacer (Abbott) 3 times daily [21]. Thirty-five piglets derived from 9 litters were divided into 8 uneven groups and inoculated as summarized in Table 1. Eleven piglets from 1 litter were used for evaluation of the relationship between systemic manifestations of disease and toxemia. Two piglets from this litter were inoculated with a nontoxigenic C. difficile strain as controls, and the remaining 9 piglets were inoculated with 1 × 10^1 spores of a toxigenic strain.

Fecal samples were collected daily for the duration of each experiment. After inoculation, piglets were monitored for signs of disease, including diarrhea, dehydration, dyspnea, weakness, lethargy, and anorexia. Piglets were euthanized at a predetermined experimental end point (postinoculation day 15 or 21) or sooner if they displayed severe symptoms, such as weakness, lethargy, or anorexia. Blood was collected after deep sedation before euthanasia. Gross gastrointestinal and systemic lesions were noted during necropsy, and tissues were collected for histologic examination. Tissue sections were collected from the duodenum, jejunum, ileum, cecum, colon, mesenteric lymph nodes, pancreas, spleen, kidneys, liver, and lungs and fixed in formalin. If present, pleural effusion and ascites samples were also collected, using aseptic technique. This study received Institutional Animal Care and Use Committee approval.

Preparation of inoculum. The nontoxigenic strain CD37 was used to inoculate the 2 control piglets. For all other animals, C. difficile strain UK6, which belongs to the 027/BII1/NAP1 type and produces TcdA, TcdB, and binary toxin, was used. Vegetative cells for inocula were grown anaerobically overnight in prereduced brain-heart infusion (BHI) broth at 37°C. The concentration was adjusted to contain 1 × 10^8 colony-forming units (CFUs) per 2 mL per piglet.

Spores were grown on prereduced BHI agar plates anaerobically at 37°C for 48 h. Colonies, scraped off the plates, were suspended in BHI broth and left in flasks for 7–10 days in an anaerobic chamber at 37°C to induce sporulation. The suspension was centrifuged, the supernatant was discarded, and cells were washed with sterile phosphate-buffered saline (PBS) twice. The suspension was then heated at 70°C for 20 min, to kill vegetative cells. The spore suspension was stored at 4°C, and spore concentration was determined by serial dilution before each experiment.

Bacterial culture. Daily fecal and necropsy samples of the gut, blood, pleural effusion, and ascites were cultured for bacterial growth immediately after necropsy. Samples were streaked

| Table 1. Summary of Relationship between Inoculum Dose and Age in the Piglet Model of Clostridium difficile Illness |
|---|---|---|
| Age, inoculating dose (no. of piglets) | Clinical signs | Case outcome |
| 1 day, 1 × 10^8 vegetative cells (n = 2) | Severe diarrhea, weakness, dyspnea | Fatal systemic disease^b |
| 2 days, 1 × 10^8 spores (n = 3) | Severe diarrhea, weakness, anorexia | Fatal systemic disease^b |
| 3 days, 5 × 10^7 spores (n = 2) | Severe diarrhea, weakness, anorexia | Fatal systemic disease^b |
| 5 days | | |
| 1 × 10^7–1 × 10^8 spores (n = 11) | Moderate to severe diarrhea, weakness, anorexia | Fatal systemic disease^b |
| 1 × 10^7 vegetative cells (n = 1) | Chronic diarrhea | Chronic GI disease^c |
| 1 × 10^8 spores (n = 14) | Moderate diarrhea, with or without weakness or anorexia | Range: chronic GI^c (8 piglets) to fatal systemic^b (6 piglets) disease |

NOTE. GI, gastrointestinal.

^a Age at inoculation, in days after birth.

^b Piglets either died or were euthanized because of severe disease.

^c Piglets experienced chronic diarrhea for at least 2 weeks and survived until the experimental end point.
on *C. difficile* taurocholate-cefoxitin-cycloserine-fructose agar (TCCFA) selective-media plates and incubated anaerobically at 37°C for 48 h. *C. difficile* was confirmed using the Pro Disk test (Remel) [22]. Samples were also streaked on MacConkey agar plates and incubated aerobically at 37°C for 24–48 h to determine presence of contaminant bacteria.

**Cytotoxicity assay.** The presence of *C. difficile* toxins in samples was measured using murine macrophage RAW264.7 cells incubated overnight in a 96-well plate before addition of samples or recombinant toxins [23] and then incubated overnight before evaluation for cell rounding. Samples tested for cytotoxicity included feces, serum, pleural effusion, and ascites. In some cases, the standard assay was inadequately sensitive to detect toxin in serum; consequently, we used an ultrasensitive assay recently developed in our laboratory [24] for the detection of toxin in serum and other body fluid. In this assay, the mRG1-1 cell line, which expresses the FCγR1-α chain, was used [25]. The cells were incubated overnight in 96-well plates before sample or toxin addition. Samples were passed through a 0.45-μm syringe filter before addition to cell culture in serial dilutions. In addition to sample dilutions alone, samples were mixed with a saturating dose of A1H3 antibody before addition to cell culture. A1H3 is a mouse anti-TcdA monoclonal antibody of immunoglobulin G2a isotype generated by our laboratory. A1H3 increases the sensitivity of cells to TcdA [25], allowing detection of low concentrations of TcdA in samples. Goat antiserum against TcdA and TcdB was used for blocking toxin activity in the assays (TechLab). Recombinant TcdA was used as a positive control. After addition of samples or toxins, the cells were incubated overnight at 37°C before being evaluated for cell rounding.

**Intestinal bacterial counts.** Intestinal contents were collected from the small intestine (jejunum) and large intestine (cecum and colon) to determine regional bacterial counts. Contents were collected during necropsy, and serial 10-fold dilutions were plated in quadrants on either BHIS (BHI broth, 5 g/L yeast extract, and 0.1% l-cysteine) plus 0.1% taurocholate agar plates or TCCFA plates and grown anaerobically for 24–48 h at 37°C to determine bacterial counts.

**Cytokine measurement.** Cytokine concentrations were determined in the large intestinal contents for interleukin 1β, interleukin 4, interleukin 6, interleukin 8 (IL-8), interleukin 10, interleukin 12, tumor necrosis factor α (TNF-α), transforming growth factor β, and interferon γ, using commercially available porcine cytokine enzyme-linked immunosorbent assay kits (Invitrogen and R&D). Samples were stored at −20°C until use. Contents were diluted 1:2 to 1:10 (depending on the consistency of the sample) in sterile PBS, thoroughly mixed using a vortex, and centrifuged, and the supernatant was added to reagent wells in the assay. The assay was performed in accordance with the manufacturer’s instructions, and cytokine concentration was determined on the basis of the standard curve generated from absorbance measured at 450 nm. Statistical analysis of cytokine measurements among piglet groups was performed with SPSS (version 16.0).

**RESULTS**

**Clinical symptoms.** Piglets inoculated with the nontoxic strain displayed no signs of disease, having normal feces throughout the experiment. Regardless of dose, all piglets inoculated with the toxigenic strain developed diarrhea within 48 h of inoculation. Diarrhea typically progressed from yellow-brown and pasty to yellow and watery, typical of CDI. Overall severity of disease depended on the type (vegetative or spore forms), dose, and age at inoculation. Higher doses of spores given to younger piglets generally produced more severe and often fatal disease. By altering the dose and age, we were able to induce in piglets either acute and severe systemic disease or milder chronic gastrointestinal disease (Table 1).

The most severe disease occurred in piglets inoculated with 1 × 10⁸ vegetative cells 24 h after birth, which displayed serious signs of dyspnea. Severely affected piglets began to show signs of weakness, lethargy, and anorexia beginning on postinoculation day 3–7 and quickly progressed to near death within 24 h, when they were euthanized. In the group receiving the lowest inoculum dose (1 × 10⁷ spores), 8 (57%) of 14 piglets developed clinically mild to moderate chronic diarrhea that lasted until the experiment was terminated on postinoculation day 15. One piglet inoculated with 1 × 10⁹ vegetative cells at 5 days of age also developed chronic diarrhea that lasted until the end of the experiment (postinoculation day 21).

**Necropsy finding.** Piglets inoculated with the nontoxic strain did not have gross gastrointestinal or systemic lesions (Figure 1A). Piglets with chronic diarrhea had mild to moderate mesocolonic edema and inflammation and dilation of the large intestine, which was mostly confined to the spiral colon (Figure 1B). These piglets did not have lesions of the small intestine or any apparent systemic lesions. Piglets with severe acute disease had more profound large intestinal lesions, including extensive mesocolonic edema, severe dilation and inflammation of the large intestine, and pseudomembrane and colonic mucosal hemorrhages extending from the ileocecocolic junction to the rectum (Figure 1C and 1D). Additional gastrointestinal lesions in the most severely affected piglets were profound thickening of the wall of the descending colon and rectum (Figure 1D), and one piglet had a perforation of the spiral colon. In some cases, gross lesions of the gastrointestinal tract from the acutely affected piglets appeared similar to those in the chronically affected animals. However, those with severe clinical disease also developed the extraintestinal lesions of ascites, pleural effusion, and cranial ventral lung consolidation.
which we attribute to the toxins because no bacteria were ever cultured from any of these sites.

**Histopathologic lesions.** Piglets inoculated with the nontoxigenic strain did not have microscopic gastrointestinal or systemic lesions (Figure 2A and 2B). Histologic examination provided the best way to fully differentiate the effects of infection on the gastrointestinal tract in chronically versus acutely affected pigs. Those that were chronically affected had extensive submucosal and mesenteric edema but had only mild focal neutrophilic inflammation and mucosal erosions (Figure 2C and 2D). These piglets did not have pulmonary lesions, and some had nonspecific vacuolar hydropic change in the liver. The piglets with acute critical symptoms had extensive and severe large intestinal lesions. Severe typhlocolitis was present in the most severe cases (Figure 2E), and extensive submucosal and mesenteric edema were present from the ileocecal junction to the rectum. Mucosal lesions ranged from severe erosions and ulcerations to nearly complete loss of mucosal lining with exposed submucosa in the most severe cases (Figure 2F). The colonic lumen was filled with a combination of neutrophils, bacteria, and necrotic debris, which formed a pseudomembrane over the surface of the mucosa in many areas of the colon and cecum (Figure 2F and 2G). In the most severe cases, neutrophilic infiltration was present in the mesenteric lymph nodes. Severely affected piglets also had systemic lesions of the lungs consisting of regional atelectasis, occasional macrophage infiltrate, alveolitis, and interstitial thickening (Figure 2H). No evidence of pneumonia (such as neutrophilic infiltrate or bacteria) was noted in any of the piglet lung sections. Some severely affected piglets also had nonspecific lesions, including vacuolar hydropic changes in the liver and reduced periarteriolar lymphoid sheath diameter in the spleen.

**Fecal and blood cultures.** All cultures of fecal samples collected daily, including the control, grew the respective strain between 24 and 48 h after oral challenge. There was no bacterial growth in any of the aerobic fecal cultures on MacConkey agar, indicating the absence of contaminants. Pleural effusion, ascites, or blood cultured on either TCCFA or MacConkey agar yielded no bacterial growth. Culture of blood from 1 piglet was positive on TCCFA with growth of 3 colonies, which is suspected to be the result of skin contamination.

**Presence of toxin in feces and body fluid.** Feces typically became positive for the presence of toxin 24 h after the first positive fecal culture result. All the pleural effusion and ascites samples from the severely affected piglets were positive by the standard cytotoxicity assay. Several of the serum samples were also positive, and those that were negative by the standard assay were positive by the ultrasensitive assay described above. None of the serum samples from the mildly affected pigs or control piglets caused any degree of cell rounding by either the standard or ultrasensitive assay.

**Intestinal bacterial counts.** Bacterial counts in the large intestine were greater than those in the small intestine for all animals, but there was no significant difference in counts between groups of piglets based on the size of the inoculum and severity of disease (Table 2). For the control piglets, counts in the small intestine ranged from $1 \times 10^4$ to $1 \times 10^5$ CFU/mL, and counts in the large intestine were $1 \times 10^5$ CFU/mL. Piglets that developed a chronic course of disease had counts in the small intestine ranging from 0 (no growth) to $1 \times 10^7$ CFU/mL and counts in the large intestine ranging from $1 \times 10^7$ to $1 \times 10^{12}$ CFU/mL. Piglets that developed an acute severe course of disease had counts in the small intestine ranging from 0 (no growth) to $1 \times 10^7$ CFU/mL and counts in the large intestine ranging from $1 \times 10^7$ to $1 \times 10^{12}$ CFU/mL.

**Cytokine analysis.** The cytokine measurements for all piglets were organized into groups on the basis of severity of disease: acute, chronic, or control. The mean concentration was compared using the Kruskall-Wallis test to evaluate significant differences between groups for each cytokine. Only IL-8 achieved a statistical level of significance ($P = .036$), with acutely affected piglets having significantly greater levels than either chronically affected or control piglets (Figure 3).
**DISCUSSION**

We have described the response of gnotobiotic piglets orally challenged with *C. difficile* in which a spectrum of clinical symptoms and pathological abnormalities largely depending on the age of the animal and the size of the infectious dose were induced. The nature and outcome of disease in these animals mimic many of the characteristics observed in human patients with CDI. Using the hypervirulent strain 027/BI/NAP1, this animal model offers reproducible results, with 100% colonization occurring within 48 h of inoculation and disease severity that can be tailored according to need. Disease ranging from profoundly acute and lethal to chronic diarrhea is readily induced in controlled laboratory settings. The range of clinical signs, including systemic complications, are similar to those observed in human cases [1, 6–8, 11–13, 15, 26], making gnotobiotic piglets an attractive alternative model to the hyperacute hamster model. The piglet model offers flexibility to meet specific research requirements, such as studies of pathogenesis, evaluation of virulence attributes, testing of the efficacy of therapeutics, and evaluation of vaccine candidates for eliminating existing infections or protecting against infections. For this purpose, animals can be immunized orally, intranasally, or systemically at 1 week of age, followed by repeated boosting at ~2 weeks thereafter. Immunized animals can then be monitored for adverse advents or symptoms in the case of live attenuated vaccines. Serum and fecal samples can be analyzed for bacterial excretion and toxins, protective mucosal and systemic antibodies, T cell responses, cytokine responses, and so on. After immunization, animals can then be challenged orally with wild-type strains. For testing vaccine candidates, the model offers several important parameters for measurements before and after challenge, including clinical symptoms; degree of mucosal injury, if any; extent of bacterial colonization in the gut; level of toxin production; cytokine responses; and so on. Essentially, the model can provide most of the required information for preclinical evaluation of vaccine candidates.

Although pigs are naturally susceptible to infection with *C. difficile* and conventional pigs could be used, gnotobiotic piglets was a similar trend for TNF-α, but it was not statistically significant.

**Table 2. Intestinal Bacterial Counts**

<table>
<thead>
<tr>
<th>Group</th>
<th>Small intestine</th>
<th>Large intestine</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>$1 \times 10^5$–1 $\times 10^6$</td>
<td>$1 \times 10^{12}$</td>
</tr>
<tr>
<td>Chronic diarrhea</td>
<td>0–1 $\times 10^7$</td>
<td>1 $\times 10^{12}$–1 $\times 10^{13}$</td>
</tr>
<tr>
<td>Acute disease</td>
<td>0–1 $\times 10^{11}$</td>
<td>1 $\times 10^{12}$–1 $\times 10^{13}$</td>
</tr>
</tbody>
</table>

**NOTE.** Data are ranges of bacterial counts, in colony-forming units per milliliter.

* Contents collected from the jejunum.

* Contents collected from the cecum and colon.
Figure 3. Cytokine production in *Clostridium difficile*-infected piglets. The piglets were grouped according to severity of disease: severe, mild, and uninfected (control). Data are mean cytokine concentrations, by disease severity. The Kruskall-Wallis test was used to determine the statistical significance of differences between groups. Interleukin 8 (IL-8) was significantly elevated in the severe-disease group after infection with *C. difficile*.

IFN-γ, interferon γ; IL-1β, interleukin 1β; IL-4, interleukin 4; IL-6, interleukin 6; IL-10, interleukin 10; IL-12, interleukin 12; TGF-β, transforming growth factor β; TNF-α, tumor necrosis factor α.

were chosen for the models because they have several advantages over conventional animals. Gnotobiotic piglets, delivered via cesarean section, do not nurse from the sow; therefore, interfering maternal antibodies against *C. difficile* are absent in studies involving evaluation of the immune response. They lack normal as well as potentially pathogenic gut flora and consequently do not require starvation and treatment with antibiotics before inoculation to enhance susceptibility, as is required with other animals. The absence of normal gut microflora as a consequence of prolonged antibiotic treatment is the hallmark of CDI and is considered one of the most important risk factors for this disease [1, 2, 4], and gnotobiotic piglets mimic this state without a need for antibiotic preconditioning. Because gnotobiotic piglets are maintained in sterile isolators for the duration of the study, the possibility for the introduction of other pathogens from the sow or human caretakers—including *C. difficile*, which is common in swine—is also eliminated. Because of these traits, inoculation of gnotobiotic piglets produces very consistent and reliable results, which can be modified according to need by manipulating the dose and/or age of the animal. This is particularly useful in the study of vaccine candidates, where several immunizations (with monitoring of immune and clinical responses followed by challenge) can be accomplished independently in each animal.

The major objective of this study was to establish a dose and age relationship for the model with the 027/BI/NAP1 strain. In general, younger piglets inoculated with higher doses experience more severe clinical signs of disease. In addition to the range of clinical signs induced by varying the dose and age at inoculation, we also observed that differences in disease severity could be observed even between individual animals from the same litter given the same dose at the same age. For the strain used in these experiments, a dose of $1 \times 10^5$ spores given at 5 days of age induced acute disease in approximately half of the piglets and chronic disease in the other half. Those experiencing acute disease developed systemic lesions of ascites, pleural effusion, and lung consolidation, whereas those with chronic disease developed only gastrointestinal lesions.

The finding that the severity of disease varies among piglets resembles the situation in human cases. Humans too develop a range of systemic consequences of infection with *C. difficile*—such as ascites, pleural effusion, cardiopulmonary arrest, liver abscess, and multiple organ dysfunction syndrome [6–12, 15]—which result in severe and even fatal disease. The reasons for the case differences observed among human patients, as well as those among the piglets in this study, are not well understood. Hopefully, the piglet model will prove to be a useful tool to delineate the relative role played by each virulence attribute in contributing to the systemic and gastrointestinal disease observed in humans. Immune response is likely to play a deciding role in disease severity, and in this study we analyzed cytokine levels in the large intestine. IL-8 concentration was statistically significantly higher in the piglets that developed acute severe disease than in the chronically affected or control piglets. IL-8 is a component of the inflammatory response, mediating neutrophil migration, and elevated levels have also been observed in in vitro experiments with human cells [27, 28] and in feces of human patients [29]. Our findings suggest that IL-
8 may be a detrimental component of the inflammatory response, because significantly elevated levels of this cytokine correlate with more severe disease.

This study supports the hypothesis that C. difficile toxins, rather than the bacteria, are responsible for the systemic complications we have observed. The presence of toxin in the serum, pleural effusion, and ascites of systemically affected piglets demonstrates the ability of toxins to reach circulation and disseminate to extraintestinal sites and suggests that they play a systemic role, which hopefully the piglet model will help address more precisely in the future. We were unable to culture bacteria from body fluids or serum samples from severely affected animals, and no bacteria were noted on histologic examination of tissues outside the gastrointestinal tract, indicating that dissemination of bacteria through damaged gut mucosa was not responsible for the systemic effects. The finding of toxin in the serum of systemically affected piglets but not in the serum of mildly affected or control piglets is especially important, because to our knowledge toxemia has not been previously documented in human or animal cases. Although toxin concentration in the feces of infected individuals is quite high and may cause cultured cell rounding in a matter of hours, the concentration in body fluids is much lower and requires a more sensitive assay for detection. The ultrasensitive cytotoxicity assay developed in our laboratory [24] has considerably increased the ease and speed of toxin detection in body fluids, especially serum.

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References