Gut Microbes 2:6, 326–334; November/December 2011; © 2011 Landes Bioscience

Cefoperazone-treated mice as an experimental platform to assess differential virulence of *Clostridium difficile* strains

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Keywords: *Clostridium difficile*, antibiotics, cefoperazone, colitis, strains

Abbreviations: CDI, *C. difficile* infection; GI, gastrointestinal; CFU, colony forming unit

The toxin-producing bacterium *C. difficile* is the leading cause of antibiotic-associated colitis, with an estimated 500,000 cases *C. difficile* infection (CDI) each year in the US with a cost approaching 3 billion dollars. Despite the significance of CDI, the pathogenesis of this infection is still being defined. The recent development of tractable murine models of CDI will help define the determinants of *C. difficile* pathogenesis in vivo. To determine if cefoperazone-treated mice could be utilized to reveal differential pathogenicity of *C. difficile* strains, 5–8 week old C57BL/6 mice were pretreated with a 10 d course of cefoperazone administered in the drinking water. Following a 2-d recovery period without antibiotics, the animals were orally challenged with *C. difficile* strains chosen to represent the potential range of virulence of this organism from rapidly fatal to nonpathogenic. Animals were monitored for loss of weight and clinical signs of colitis. At the time of harvest, *C. difficile* strains were isolated from cecal contents and the severity of colitis was determined by histopathologic examination of the cecum and colon. Cefoperazone treated mice challenged with *C. difficile* strains VPI 10463 and BI1 exhibited signs of severe colitis while infection with 630 and F200 was subclinical. This increased clinical severity was correlated with more severe histopathology with significantly more edema, inflammation and epithelial damage encountered in the colons of animals infected with VPI 10463 and BI1. Disease severity also correlated with levels of *C. difficile* cytotoxic activity in intestinal tissues and elevated blood neutrophil counts. Cefoperazone treated mice represent a useful model of *C. difficile* infection that will help us better understand the pathogenesis and virulence of this re-emerging pathogen.

Introduction

Clostridium difficile is an anaerobic, spore-forming, gram-positive bacillus first isolated in 1935.[1] Within the past decade new focus has been put on *C. difficile* due to an increase in the prevalence and severity of infection.[2,3] *C. difficile* infection (CDI) is now the leading cause of hospital-acquired infections, surpassing methicillin-resistant *Staphylococcus aureus*.[4] *C. difficile* accounts for almost all cases of pseudomembranous colitis and 20% of antibiotic-associated diarrhea cases.[5] Antibiotic treatment is a major risk factor for CDI with elevated risk associated with the administration of antibiotics from multiple classes including clindamycin, quinolones, cephalosporins, and aminopenicillins.[6-8] Standard treatment of CDI has traditionally involved the administration of metronidazole or vancomycin. Unfortunately, after initial successful treatment an increasing number of patients experience one or more relapses of disease.[9] Not only is relapse more prevalent, morbidity and mortality per year has increased, where an estimated 15,000 to 20,000 patients die annually in the US from CDI.[10] There has been some success with alternative treatments for patients with reoccurring or severe CDI, however further effort is needed in developing novel treatments for *C. difficile* infection.

The development of tractable animal models greatly aids in understanding the pathogenesis of infectious agents. Syrian hamsters were first used to fulfill Koch's postulates for *C. difficile* in the 1970s and are still being used today.[11-13] Infection of clindamycin treated hamsters with *C. difficile* results in severe colitis and death within 3 d.[11,14] The use of the hamster model has demonstrated a role for the *C. difficile* toxins A and B (TcdA and TcdB) in the pathogenesis of infection.[12,13] More recently, mouse models of CDI have been developed that approximate human *C. difficile* infection. Pretreatment of mice with a cocktail of five antibiotics, followed by an intraperitoneal injection of clindamycin changes the gut microbiota and renders animals susceptible to colonization with *C. difficile* vegetative cells.[15,16] Unlike the

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Submitted: 09/06/11; Revised: 12/16/11; Accepted: 12/21/11
http://dx.doi.org/10.4161/gmic.2.6.19142

uniformly fatal hamster model, disease severity can vary with the size of the bacterial inoculum administered and the strain of *C. difficile* used for infection.[16]

We recently demonstrated that the broad-spectrum cephalosporin cefoperazone is sufficient to make mice susceptible to infection with *C. difficile* strain VPI 10463.[15] This *C. difficile* strain produces high amounts of toxin and experimental infection with this strain is lethal in hamsters and, with increased dose, in mice.[16-18] Since we demonstrated a dose-response to inoculum size with VPI 10463 in cefoperazone-treated mice, we hypothesized that this model could be used as a platform to examine differential virulence of *C. difficile* strains and isolates. As a proof of principle we compared the outcome of experimental infection of cefoperazone-treated mice with four *C. difficile* strains, including those used in past murine models.[16,19,20] In addition to VPI 10463, we also challenged mice with a BI1 strain which is a member of the restriction enzyme analysis (REA) group BI, ribotype 027, from North American isolates NAP1. This strain is an ancestor of the epidemic strain that has appeared in the past decade.[21,22] The 630 strain is a genetically tractable strain that was originally isolated from a clinical case of pseudomembranous colitis in Switzerland.[23,24] Given the essential role of toxin production for pathogenesis in hamsters,[12,13] we obtained a non-toxigenic human isolate (F200) of *C. difficile* as a control. We selected these strains since they represent the potential range of virulence as judged by previous in vitro and in vivo studies. We demonstrate that cefoperazone treated mice exhibit varying degrees of disease when challenged with these different *C. difficile* isolates and thus this represents a model that can be used in future studies to test the relative virulence of different strains.

Results

Varied clinical courses in cefoperazone-treated mice challenged with different *C. difficile* strains. Wild type C57BL/6 mice were made susceptible to infection with *C. difficile* by a 10 d pretreatment with cefoperazone followed by a 2 d period without antibiotics.[15] Mice were orally challenged in multiple trials with the vegetative form of *C. difficile* strains VPI 10463, BI1, 630 and F200. Animals infected with 2×10^5 CFU of *C. difficile* strain VPI 10463 developed clinical signs of CDI (including lethargy, diarrhea and hunched posture) within 24–48 h post infection and lost $\geq$ 20% of their initial body weight by day 2 post infection necessitating euthanasia (**Fig. 1A**). Similarly, animals infected with 6×10^4 CFUs of *C. difficile* strain BI1 lost $\geq$ 20% of initial body weight by day 2 post infection (**Fig. 1B**). Interestingly, decreasing the challenge dose of VPI 10463 to 4×10^4 CFU extended the time to the development of severe

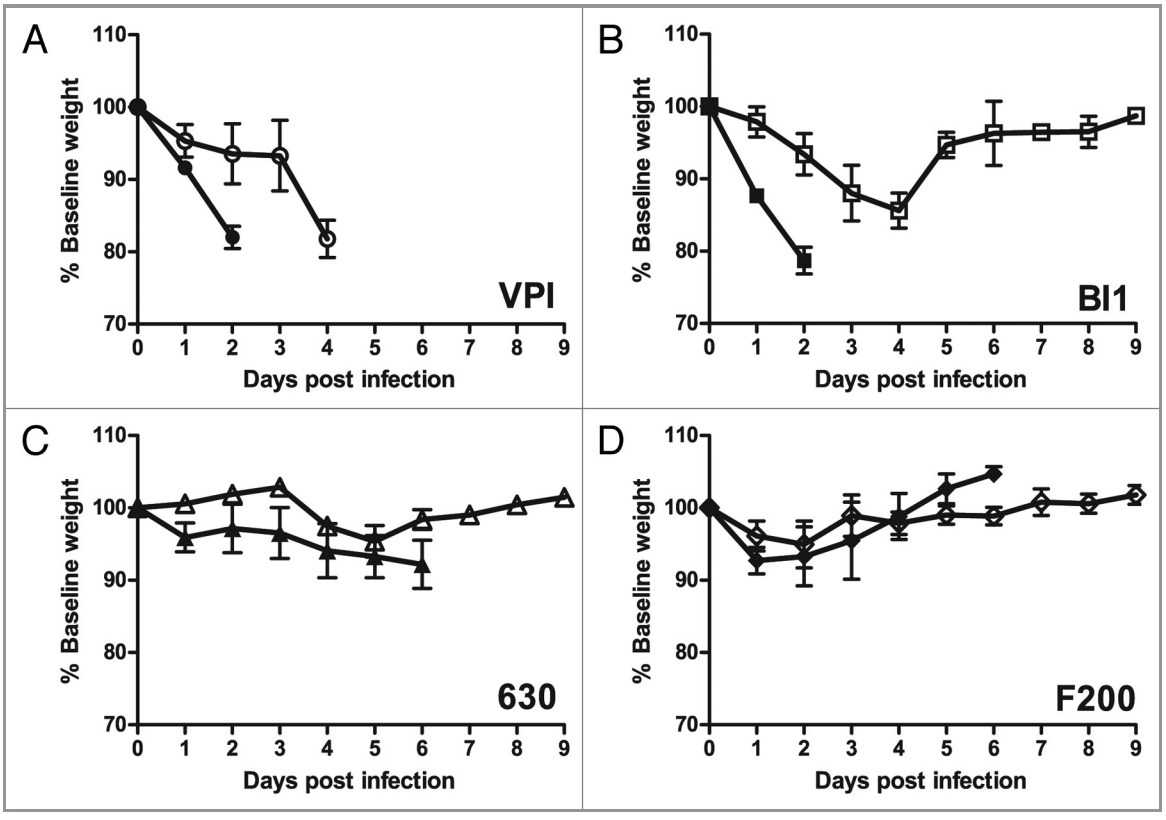

Figure 1. *C. difficile* strains exhibit different clinical outcomes in cefoperazone treated mice. Body weight was measured daily starting from Day 0 or the day of infection. Animals were challenged with *C. difficile* strains (A) VPI 10463 (2×10^5 CFU closed circles and 4×10^4 CFU open circles) (B) BI1 (6×10^4 CFU closed squares and 8×10^3 CFU open squares) (C) 630 (2×10^5 CFU closed triangles and 8×10^4 CFU open triangles) and (D) F200 (4×10^5 CFU closed diamonds and 5×10^3 CFU open diamonds). Black lines represent mean percentage of the baseline weight for animals in each group.

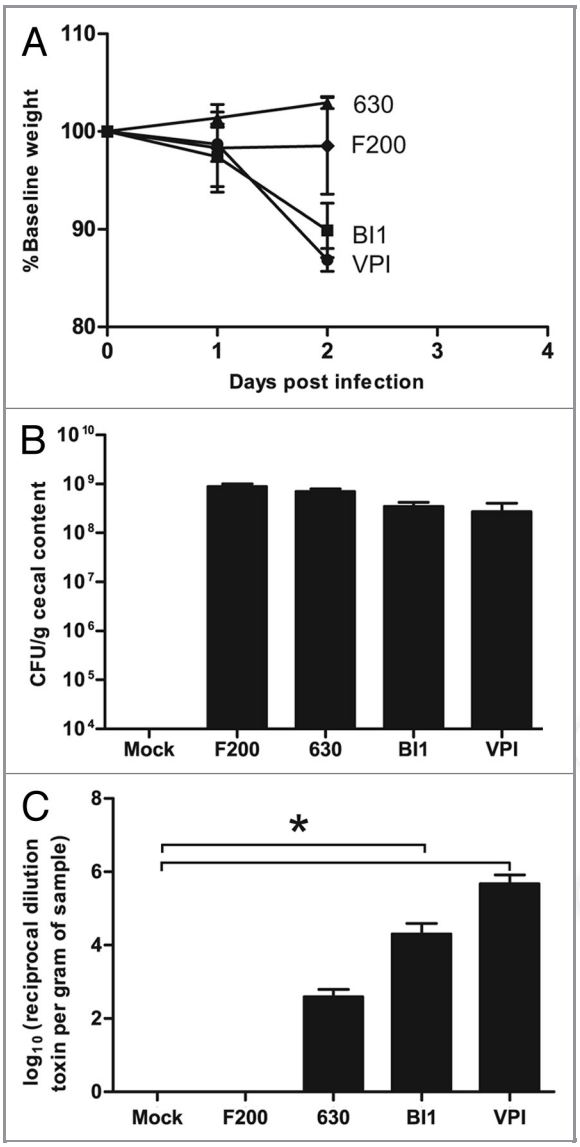

Figure 2. Early murine infection with different *C. difficile* strains. Cefoperazone treated C57BL/6 WT mice challenged with *C. difficile* strains, VPI 10463 (n = 4), BI1 (n = 3), 630 (n = 5), F200 (n = 3) and a mock (n = 3) infected were all sacrificed early during infection, or 48 h post infection. (A) Average body weight was measured daily starting from Day 0 or the day of infection (B) Colonization levels in CFU per gram of cecal content at the time of necropsy (C) Vero cell cytotoxicity assay from cecal content of each mouse in $\log_{10}$ reciprocal dilution toxin per gram of cecal content at the time of harvest (VPI 10463 vs. F200, $p < 0.05$; VPI 10463 vs. mock, $p < 0.05$ Kruskal-Wallis 1way ANOVA).

CDI (**Fig. 1A**). However, decreasing the infectious dose of the BI1 strain to 8×10^3 CFU resulted in the survival of 3 of 5 animals that neither lost weight nor developed signs of severe disease (**Fig. 1B**).

In contrast, cefoperazone treated mice infected with strain 630 or F200 never reached clinical endpoints (20% weight loss or death) regardless of the size of bacterial inoculum (a total of three infection trials were performed). Animals challenged with the 630 strain exhibited minor weight loss, although this did not approach the loss observed in animals infected with VPI 10463 or BI1. While animals infected with the higher doses of VPI 10463 or BI1 required euthanasia by 48 h after infection due to reaching clinical endpoints, animals challenged with 630 or F200 remained well for the duration of the experiment (6 to 9 d post infection) (**Fig. 1C and D**).

Variable severity of disease early in the time course of infection. Since the clinical trajectory of CDI varied with the strain of *C. difficile* used for infection in multiple experiments, additional infections were done to compare disease at a uniform time early in the infectious course. Cefoperazone treated C57BL/ six mice were challenged with the four *C. difficile* strains, VPI 10463, BI1, 630 and F200 at an average dose of 7×10^5 CFU. All animals were harvested by 48 h after challenge. As in the previous experiments, animals infected with *C. difficile* strain VPI 10463 and BI1 exhibited significant weight loss, but animals infected with 630 and F200 remained well (**Fig. 2A**).

By 48 h after experimental challenge, mice infected with each of the four strains had high levels of *C. difficile* colonization with 10^8-10^9 colony-forming units per gram of cecal content (**Fig. 2B**). Despite this uniform colonization, the levels of *C. difficile* cytotoxic activity detected in the intestine varied with the infecting strain. Mice infected with VPI 10463 had the highest levels of cytotoxic activity in the gut followed by animals infected with BI1 and then those infected with 630 (**Fig. 2C**). No cytotoxic activity was detected in gut tissues isolated from animals infected with F200 or in uninfected controls.

Upon histopathological examination of cefoperazone treated mice, the colons of mice infected with VPI 10463 and the BI1 strain had the most severe pathology (**Fig. 3A**). Maximal levels of inflammation, edema and epithelial damage were seen in animals infected with VPI 10463 and BI1 (**Figs. 4A, B and S1**). Minimal inflammation and slight edema without significant epithelial damage was encountered in animals infected with 630 or the F200 (**Figs. 4C, D and S1**). However, one F200 animal with moderate inflammation (score 2) had small multifocal neutrophilic aggregates with mild edema and no epithelial damage. This may represent a background lesion or limited inflammation in response to colonization but without pathological significance, as evidenced by the fact that none of the F200 mice had epithelial damage. Histopathological results had similar statistical significance regardless of whether a rank-ordering or numerical scoring system was used (**Figs. 3A and S1**).

As a correlate of systemic illness induced by CDI, blood was collected from mice at the time of necropsy and the total number of circulating white blood cells and neutrophils were determined. Mice infected with *C. difficile* strains VPI 10463 and BI1 had elevated levels of peripheral neutrophils at the time of necropsy (**Fig. 3B**). In fact, these mice had a reversal of the normal lymphocyte-predominance in peripheral mouse blood. Neutrophil predominance is consistent with a severe inflammatory response. Mice infected with 630 and F200 retained the normal lymphocyte-predominance.

***C. difficile* spores can infect cefoperazone-treated mice.** The previous infection experiments were conducted with the vegetative form of *C. difficile*. It is thought that human clinical

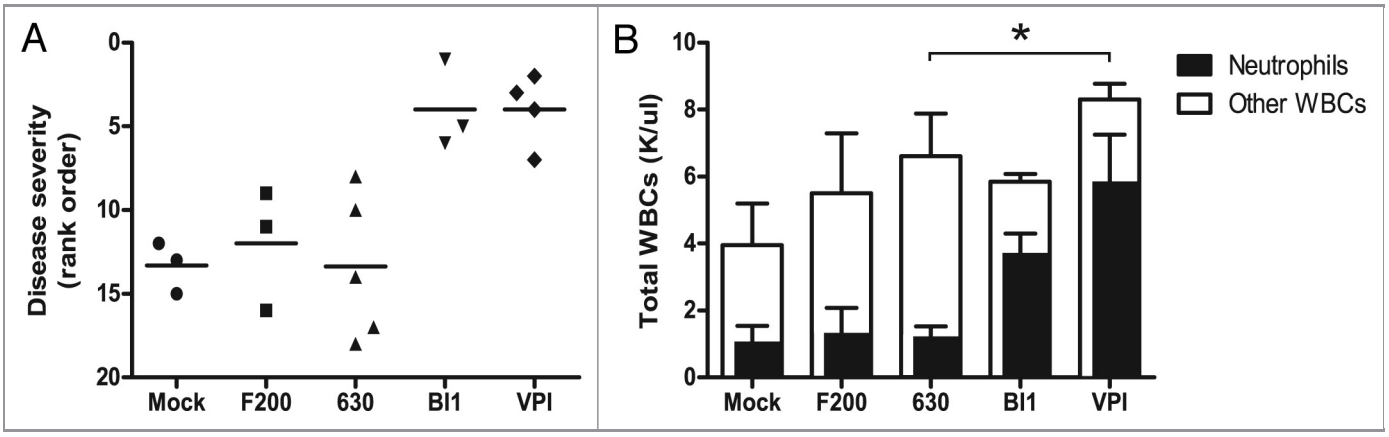

Figure 3. Histopathology and biomarkers of disease severity during early infection with different *C. difficile* strains. (A) Rank order analysis of histology slides from the murine colon showing disease severity in order from 1, most severe histopathological changes, to 18, least severe histopathological changes. (B) Total white blood cell count in thousand (K)/μl. The black shaded boxes indicate the number of neutrophils present out of the total number of white blood cells present (in white) in the blood of infected *C. difficile* mice (VPI vs. 630, $p < 0.05$ Kruskal-Wallis 1way ANOVA).

infection generally arises from the ingestion of the spore form of the organism. To demonstrate that cefoperazone-treated mice could be infected with *C. difficile* spores, antibiotic-treated animals were challenged with the spore form of strains VPI 10463 and BI1. Animals infected with 2×10^5 spores of *C. difficile* strain VPI 10463 developed clinical signs of CDI (including lethargy, diarrhea and hunched posture) within 24–48 h post infection and lost ≥ 20% of their initial body weight by day 2 post infection (**Fig. 5**). Animals infected with the same spore dose of *C. difficile* strain BI1 lost weight, but never reached clinical endpoints requiring euthanasia (**Fig. 5**). After day 4 post infection animals gradually gained weight until day 7 post infection when the experiment was terminated.

Discussion

The clinical picture that results from human infection with *C. difficile* ranges from asymptomatic colonization to fulminant colitis.[25] Chronic, recurrent infection is becoming an increasingly important problem.[26] This wide range of disease manifestations is presumed to reflect differences in the host, the indigenous microbiota, and infecting *C. difficile* strains.[10] While much attention has been focused on host determinants of disease severity (such as age, immunosuppression, co-morbidities), fewer studies have addressed differences among *C. difficile* strains that influence clinical outcome in patients with CDI. However, the emergence of epidemic strains of *C. difficile* with apparently increased virulence (e.g., NAP1/BI/027) has opened new questions about the specific determinants of *C. difficile* virulence in different isolates.[27]

While important insights into the pathogenesis of CDI have been gained from experimental infection of Syrian hamsters,[14,28] one limitation of this model is that animals infected with toxigenic *C. difficile* strains generally have a uniformly fatal course, reducing the ability to detect differences in virulence.[19,20] In this report, we present a model of CDI that should be useful to show relative differences in the pathogenicity of *C. difficile* strains. Animals are made susceptible to colonization with *C. difficile* through the administration of a single antibiotic, cefoperazone. This closely mimics the development of CDI in humans, which can arise following exposure to a single antimicrobial. Cephalosporins, the class of antibiotics which includes cefoperazone, are a key risk for the development of CDI.[29] This model has potential advantages over previously published murine models of CDI in immunocompetent animals, which required multiple antibiotics to make animals susceptible to CDI[16] or were models of colonization without the development of significant clinical or histopathologic disease.[30,31]

The use of inbred mice from a single breeding colony in the current model controls for variation in host genetics. Furthermore, based on our previous studies, cefoperazone treated mice demonstrate reproducible changes to the gut microbiota, limiting microbiota variability as a potential modifier of disease outcome.[32] Therefore, the main variable in determining the clinical outcome in this murine model of CDI is the strain of *C. difficile* used for challenge. Depending on the specific strain of *C. difficile*, infected mice exhibited a variety of clinical courses ranging from asymptomatic colonization to a subacute, resolving histopathologic colitis to rapidly fatal, clinically severe colitis.

The main virulence factors of *C. difficile* are thought to be toxin A (TcdA) and toxin B (TcdB).[33] Both TcdA and TcdB are cytotoxic and trigger inflammatory responses, causing disruption of the actin cytoskeleton of intestinal epithelial cells and disrupting tight junctions.[34,35] In this study we confirmed the essential role of these toxins in mediating colitis as no clinical or histopathologic disease was seen in animals that were challenged with the non-toxigenic strain F200. However, the fact that this strain could colonize to a level equivalent of a fully toxigenic strain demonstrates that toxin A and toxin B are not required to establish *C. difficile* colonization. Our results are also in accord with clinical observations that the severity of CDI is proportional to the in vivo production of these *C. difficile* toxins.[36] The most

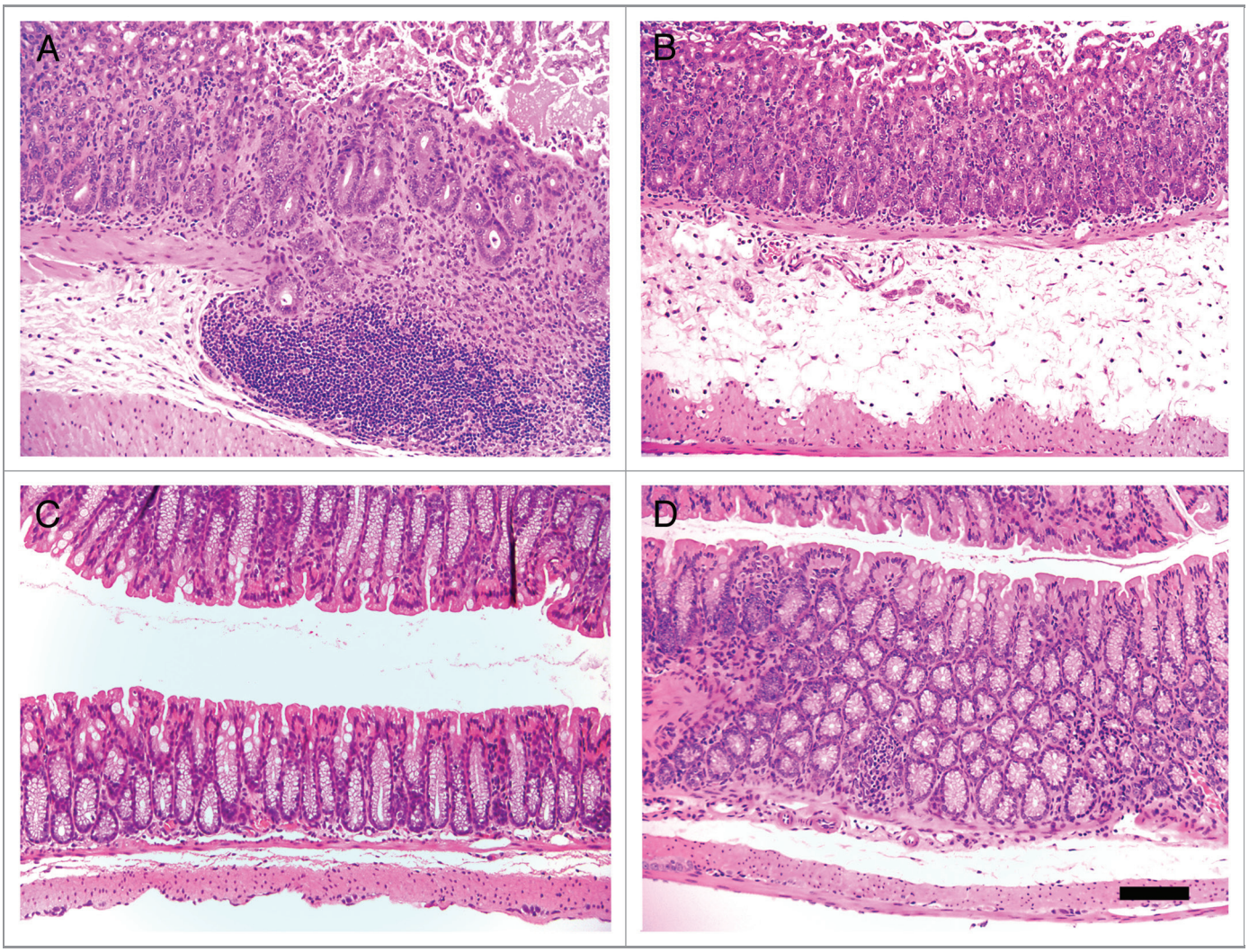

Figure 4. Histopathology in the colon of *C. difficile*-infected animals during early infection. (A) Colon of a mouse with clinically severe CDI infected with VPI 10463. There is severe submucosal edema accompanied by inflammatory cells within the mucosa and invading the submucosal lymphoid tissue. The epithelial surface is also irregular and eroded. (B) Colon of a mouse infected with BI1 that shows significant submucosal edema and scattered inflammatory cells. (C) Colon of a mouse infected with the 630 strain of *C. difficile* shows minimal to no histopathological changes. (D) Colon of a mouse colonized with F200 which shows some inflammation but otherwise no significant histopathological changes. HE. All images were the same magnification (x200). Scale bar = 100 μm.

severe disease encountered in cefoperazone treated mice was seen in animals that were infected with VPI 10463 and BI1. Much greater cytotoxic activity was found in the tissues of animals infected with these strains compared with animals infected with strain 630 during early infection. However it should be noted that a relative difference in the virulence of VPI 10463 and BI1 could be demonstrated by the fact that decreasing the challenge dose of BI1 would result in the survival of a proportion of infected mice while decreasing the infectious dose of VPI 10463 merely resulted in extending the time to the development of a uniformly fatal course.[15]

Our results in cefoperazone treated mice contrasts with the clinical outcome seen in hamsters infected with BI1 and strain 630.[19,20] Infection of hamsters with both of these strains was uniformly fatal, although death occurred more rapidly in animals infected with BI1. Cefoperazone-treated mice infected with a strain 630 have a clinically benign course. Although toxin expression occurred in mice infected with 630, the lower quantities apparently did not induce clinical signs of toxemia (unlike what was encountered in animals infected with VPI 10463 and BI1 strains, which produced high levels of toxin in vivo).

Cefoperazone-treated mice will likely have utility in defining the role of other potential *C. difficile* virulence factors in the pathogenesis of infection. The production of another toxin, known as binary toxin or CDT, has been proposed to be another factor that may influence the virulence of *C. difficile*.[37] CDT is produced by a number of *C. difficile* strains including the recent NAP1/BI/027 epidemic isolates.[3] In spite of the correlation between emergence of this epidemic strain and increased disease prevalence and severity, there is conflicting evidence on the

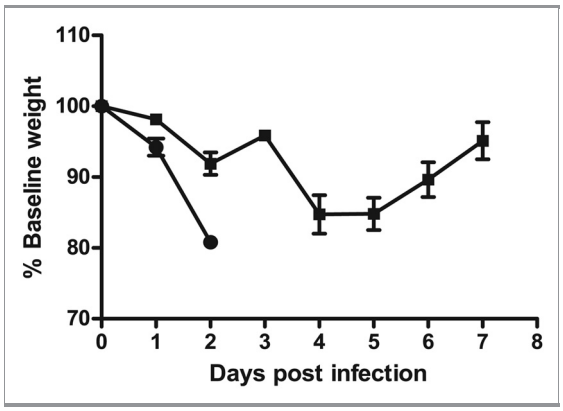

Figure 5. *C. difficile* strains exhibit different clinical outcomes in cefoperazone treated mice when infected with spores. Average body weight was measured daily from Day 0 or the day of infection. Animals were challenged with *C. difficile* strains (A) VPI 10463 circles (2×10^5 spores) (B) BI1 squares (2×10^5 spores). All animals infected with VPI 10463 met their clinical endpoint by day 2 post infection and had to euthanized. Animals infected with BI1 never met the clinical endpoint and were euthanized when the experiment was terminated on day 7 post infection.

specific role of CDT in pathogenesis.[38] The strain BI1 that we employed in the current study is a historic isolate of the current epidemic strain and produces CDT. Our finding that the strain VPI 10463 (which does not produce CDT due to a deletion in the binary toxin genes)[39] produced equivalent disease in cefoperazone treated mice to BI1 underscores the controversial role for binary toxin in disease pathogenesis.[40] To test the role of potential virulence factors such as CDT in the pathogenesis of *C. difficile* infection would require the use of isogenic strains, as has been done to show the essential role of TcdA and TcdB in hamsters.[12,13] The primary goal of the current study was not to obtain new insight into the pathogenesis of *C. difficile* strains that have been previously studied in vitro and in vivo. The toxigenic strains that we employed (VPI 10463, BI1 and 630) have been the subject of multiple other studies. We chose to use these well-characterized strains, in addition to a non-toxigenic strain, so that we could reasonably expect the full range of disease that can result from *C. difficile* infection, ranging from asymptomatic colonization to rapidly fatal colitis. Having demonstrated the utility of this model in potentially distinguishing relative virulence, future studies with isogenic mutants or multiple isolates of genetically related strains (e.g., a collection of NAP1/027 strains) could extend our knowledge of *C. difficile* virulence determinants and mechanisms.

Human infection with *C. difficile* is thought to result primarily from exposure to the spore form of the organism.[10] A potential weakness of the current experiments is that we employed challenge with vegetative forms of *C. difficile* in this study. We and others have used vegetative cells in previous studies to initiate *C. difficile* infection in antibiotic-treated mice.[15,16] Here it is demonstrated that cefoperazone-treated mice are also overtly infected when challenged with the spore form of VPI 10463 and BI1. Similar to the results demonstrated with vegetative cells, spores of VPI 10463 had apparently greater virulence in this model. This was evidenced by the fact that the same inoculum size of VPI 10463 spores that uniformly killed infected mice caused significantly less severe disease when BI1 spores were used for challenge. The ability to utilize what is thought to be the nosocomial and naturally infectious form of the organism opens the possibility of examining the role of spore germination in the pathogenesis of disease. We anticipate that future studies employing this model system of CDI will lead the way to novel means for the prevention and treatment of this significant hospital-acquired infection.

Materials and Methods

Ethics statement. This study was approved by the University Committee on the Care and Use of Animals (UCUCA) at the University of Michigan. The University of Michigan laboratory animal care policies follow the Public Health Service policy on Humane Care and Use of Laboratory Animals. Animals were assessed daily for physical condition and behavior and those assessed as moribund were humanely euthanized by CO_2 asphyxiation. Animal husbandry was performed by trained animal technicians in an AAALAC-accredited facility.

Animals and housing. 5–8 week old C57BL/6 WT mice (male or female) were used from a breeding colony that was established using animals purchased from Jackson Laboratories for the experimental infections. Mice were housed with autoclaved food, bedding and water. Cage changes were performed in a laminar flow hood. Mice had a cycle of 12 h of light and 12 h of darkness.

***Clostridium difficile* strains and growth conditions.** The *C. difficile* strains used in this study include reference strain VPI 10463 (ATCC 43255), BI1 (NAP1/BI/027) which was obtained from Dale Gerding (Hines VA Hospital Loyola University Medical Center Maywood, IL), strain 630 (ATCC BAA-1382) and a non-toxigenic clinical strain (F200) which was obtained by the University of Michigan Archives. VPI 10463 was first isolated from an abdominal wound (http://img.jgi.doe.gov/cgi-bin/w/main.cgi: DOE Joint Genome Institute website) and is grouped in toxinotype 0. The BI1 strain is from isolate 5352 and was recovered from a patient in the surgical intensive care unit on the Minneapolis VA Hospital in February 1993 (personal correspondence, Stuart Johnson).[21] The BI1 strain represents the REA, restriction enzyme analysis; group BI, ribotype 027, from North American isolates NAP1, that is an ancestor of the epidemic strain that has appeared in the past decade. It is a part of the toxinotype III group, with a point mutation in the *tcdC* (negative regulator) that results in expression of a truncated protein in the pathogenicity locus and carries the binary toxin genes.[22] The 630 strain, toxinotype 0, was originally isolated from a clinical case in Switzerland with pseudomembranous colitis and is now genetically tractable.[23,24] A non-toxigenic strain (isolate F200) of *C. difficile* that is a clinical isolate obtained from the University of Michigan hospital archives will also be used in this study as a control. F200 was confirmed as a non-toxigenic strain by PCR and a negative Vero cell cytotoxicity assay (data not shown).

All strains were isolated and grown on brain heart infusion media (BHIS) supplemented with 0.01% L-cysteine (Sigma-aldrich cat# C7352). *C. difficile* vegetative cells were grown in a Coy anaerobic chamber (Coy Industries). When preparing inoculum for *C. difficile* infections, isolates were plated on BHIS agar to isolate single colonies, which were used to inoculate an overnight culture of BHIS broth. The next morning, a back dilution of 1:10 was made with fresh BHIS broth to ensure uniform growth phase of bacteria used for infection. After 4 h of growth, the culture was harvested by centrifugation and washed 3 times with PBS pH 7.4 (Gibco, cat# 10010) that was pre-equilibrated to anaerobic conditions. *C. difficile* cultures were diluted to the appropriate final dose and loaded into 1 ml syringes for gavaging animals. Bacterial enumeration was performed by plating on BHIS agar in order to determine the actual dose.

C. difficile spores were prepared as follows. Strains were grown overnight in BHIS broth. The next day, 100 ul of these overnights was spread onto BHIS plates (four plates per strain). The plated strains were allowed to grow for seven days before being removed from the anaerobic chamber and subjected to oxygen overnight to kill vegetative bacilli. Plates were flooded with 15 ml cold water and bacteria were removed by scraping with a sterile loop. Bacterial suspensions were centrifuged and washed in cold water at least three times. Spore stocks were stored at 4°C in sterile water. The presence of spores was confirmed using phase contrast microscopy and stocks were enumerated by plating for viable CFU. *C. difficile* spores were heat treated for 20 min at 65°C to ensure that all spores were viable prior to gavaging animals. Spores were enumerated by plating dilutions on TCCFA agar in order to determine the actual dose.

Antibiotic administration and infection with *C. difficile*. C57BL/6 WT mice (male or female) ranging from 5–8 weeks in age were used in this study. Mice were given cefoperazone (0.5 mg/ml) (MP Bioworks, cat# 199695) in sterile drinking water for 10 d. Antibiotic water was refreshed every other day in order to prevent the antibiotic from breaking down. After 10 d, mice were switched to regular water (Gibco, cat# 15230) and allowed to recover for 2 d before being infected by oral gavage with *C. difficile* vegetative cells. The actual dose of *C. difficile* vegetative cells administered ranged from 10^3 – 10^5 CFUs of *C. difficile* strains VPI 10463, BI1, 630 and a F200. Cefoperazone treated mice in **Figure 1** were orally gavaged with approximately: VPI 10463: 2×10^5 CFU (n = 4), 4×10^4 CFU (n = 5); BI1: 6×10^4 CFU (n = 3), 8×10^3 CFU (n = 5); 630: 2×10^5 CFU (n = 5), 8×10^4 CFU (n = 5) and F200: 4×10^5 CFU (n = 4), 5×10^3 CFU (n = 3). Mock infected animals were pretreated with cefoperazone but orally gavaged with PBS. Animals in the early infection study were all orally gavaged with an average dose of 7×10^5 CFUs and sacrificed at day 2 post infection. Cefoperazone treated mice infected with the spore form of *C. difficile* were orally gavaged with approximately 2×10^5 spores of VPI 10463 and BI1. Animals challenged with *C. difficile* were monitored for signs of clinically severe CDI including inappetence, diarrhea, and hunching. Animals were euthanized after losing 20% of initial baseline weight or after developing any severe clinical signs listed above.

Necropsy and histological procedures. Mice were euthanized by CO_2 asphyxiation. Contents and tissue from the cecum and colon were collected, flash frozen and stored at -80°C. For infected animals, the cecum and colon were prepared for histology by placing the intact tissue into histology cassettes and stored in 10% buffered formalin for 24 h then transferred to 70% ethyl alcohol. Tissue cassettes were further processed and paraffin embedded then sectioned. Haematoxlyin and eosin stained slides were prepared for histopathological examination (McClinchey Histology Lab Inc.).

Hematologic analysis. Blood from animals was taken at the time of harvest and collected in Microtainer tubes with K_2EDTA (BD, cat# 365974). Blood samples were taken immediately to the ULAM Pathology Core for Animal Research, Animal Diagnostic Laboratory. Samples were processed for complete blood count with automated white blood cell differential.

Histopathological examination. Histological sections were coded, randomized, and scored in a blinded manner by a board-certified veterinary pathologist (ILB). The slides were scored two times using two separate methods. First, a previously published numerical scoring system was used.[15] Edema, cellular infiltration, and epithelial damage were assessed separately in cecal and colonic tissue using numerical severity scores from 0–4 according to previously defined criteria.[15] Edema, cellular infiltration and epithelial damage for the cecum and colon was scored from 0–4 according to the following defined criteria: Edema scores: 0, no edema; 1, mild edema with minimal (< 2x) multifocal submucosal expansion; 2, moderate edema with moderate (2–3x) multifocal sub-mucosal expansion; 3, severe edema with severe (> 3x) multifocal sub-mucosal expansion; 4, same as score 3 with diffuse sub-mucosal expansion. Cellular infiltration scores were graded as follows: 0, no inflammation; 1, minimal multifocal neutrophilic inflammation; 2, moderate multifocal neutrophilic inflammation (greater submucosal involvement); 3, severe multifocal to coalescing neutrophilic inflammation (greater submucosal ± mural involvment; 4, same as score 3 with abscesses or extensive mural involvement. Epithelial damage was scored as follows: 0, no epithelial changes; 1, minimal multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 2, moderate multifocal superficial epithelial damage (vacuolation, apoptotic figures, villus tip attenuation/necrosis); 3, severe multifocal epithelial damage (same as above) +/– pseudomembrane (intraluminal neutrophils, sloughed epithelium in a fibrinous matrix); 4, same as score 3 with significant pseudomembrane or epithelial ulceration (focal complete loss of epithelium).

The slides were then re-scored using a rank-ordering system. Under some circumstances, this method is considered more powerful than traditional numerical or categorical scoring systems.[41,42] In brief, the same histological criteria for edema, inflammatory cell infiltration, and epithelial damage were used as with the categorical scoring method but, rather than grouping slides into numerically defined categories, all slides were simply placed in order of increasing severity of histopathological changes.

Colonization of *C. difficile* from cecal contents. At the time of necropsy, cecal contents were taken from mice and weighed. Cecal

contents were passed immediately into the anaerobic chamber for bacterial enumeration. Cecal contents were serial diluted and plated on TCCFA (Taurocholate Sigma, cat# T4009, D-cycloserine Sigma, cat# C6880, cefoxitine Sigma, cat# C47856, fructose Fisher, cat# L95500 agar) selective media in order to isolate and quantify the *C. difficile* load in the cecum of infected mice.

***C. difficile* cytotoxin assay.** Vero cells were grown and used as described in Reeves et al.[15] Briefly, cells were maintained in DMEM media supplied from (Gibco Laboratories, cat# 11965) with 10% fetal bovine serum (Gibco Laboratories, cat# 16140) and 1% Penicillin streptomycin solution (Gibco Laboratories, cat# 15140). Cells were incubated with 0.25% trypsin (Gibco Laboratories, cat# 25200) washed with 1X DMEM media and harvested by centrifugation 1,000 RPM. Cells were plated at 1×10^5 cells per well in a 96-well flat bottom microtiter plate (Corning, cat # 3596). Luminal content from mice was prepped by weighing final contents and adding 10-fold higher volume of 1X PBS to make a 1:10 initial dilution. Samples were vortexed and then spun at 13,000 rpm for 5 min. Supernatant was collected and put through a 0.2 μm filter membrane. Each sample was titrated in 10-fold dilutions within the wells to a maximum dilution of 20^{-7} and each well had a corresponding control to which both antitoxin (TechLabs, cat# T5000) and sample were added. After an overnight incubation at 37°C, plates were viewed under 200X magnification for Vero cell rounding. The cytotoxic titer was defined as the reciprocal of the highest dilution that produced rounding in 100% of Vero cells per gram of cecal sample. Vero cells treated with purified *C. difficile* toxin and antitoxin (TechLabs, cat# T5000) were used as controls.

Statistical analysis. Prism 5 Graphpad Software was used for statistical analysis. Kruskal-Wallis (1-way ANOVA) test was used for nonparametric analysis with statistical significance set at a p value of < 0.05.

Disclosure of Potential Conflicts of Interest

No potential conflicts of interest were disclosed.

Acknowledgments

The authors would like to thank Angela Reeves, Judy Opp and Nabeetha Nagalingam for helping with the animal studies of *C. difficile*. The authors would also like to thank Dale Gerding (Hines VA Hospital Loyola University Medical Center Maywood, IL) for sending the BI1 strain used in this study. Also, thanks to Seth Walk for the use of the F200 isolate. This work was funded by NIH grants DK070875 (VBY) and AI090871 (VBY, DMA). CMT was funded by training grant AI07528.

Supplemental Material

Supplemental Material can be found at:
http://www.landesbioscience.com/journals/gutmicrobes/article/19142/

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