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Case Study

Outbreaks of Typhlocolitis Caused by Hypervirulent Group ST1 *Clostridioides difficile* in Highly Immunocompromised Strains of Mice

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Clostridioides difficile **is an enteric pathogen that can cause significant clinical disease in both humans and animals. However, clinical disease arises most commonly after treatment with broad-spectrum antibiotics. The organism's ability to cause naturally occurring disease in mice is rare, and little is known about its clinical significance in highly immunocompromised mice. We report on 2 outbreaks of diarrhea associated with *C. difficile* in mice. In outbreak 1, 182 of approximately 2, 400 NOD.Cg-*Prkdc*^{scid} *Il2rg*^{tm1Wjl}/SzJ (NSG) and related strains of mice became clinically ill after cessation of a 14-d course of 0.12% amoxicillin feed to control an increase in clinical signs associated with *Corynebacterium bovis* infection. Most mice had been engrafted with human tumors; the remainder were experimentally naïve. Affected animals exhibited 1 of 3 clinical syndromes: 1) peracute death; 2) severe diarrhea leading to euthanasia or death; or 3) mild to moderate diarrhea followed by recovery. A given cage could contain both affected and unaffected mice. Outbreak 2 involved a small breeding colony (approximately 50 mice) of NOD.CB17-*Prkdc*^{scid}/NCrCrl (NOD-*scid*) mice that had not received antibiotics or experimental manipulations. In both outbreaks, *C. difficile* was isolated, and toxins A and B were detected in intestinal content or feces. Histopathologic lesions highly suggestive of *C. difficile* enterotoxemia included fibrinonecrotizing and neutrophilic typhlocolitis with characteristic 'volcano' erosions or pseudomembrane formation. Genomic analysis of 4 isolates (3 from outbreak 1 and 1 from outbreak 2) revealed that these isolates were closely related to a pathogenic human isolate, CD 196. To our knowledge, this report is the first to describe naturally occurring outbreaks of *C. difficile*-associated typhlocolitis with significant morbidity and mortality in highly immunocompromised strains of mice.**

Abbreviations: CDI, *C. difficile* infection; FMT, fecal microbiota transplantation; MLST, multilocus sequence typing; PaLoc, pathogenicity loci

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Clostridioides difficile (formerly *Clostridium difficile*) is a gram-positive, anaerobic, spore-forming, and possibly zoonotic pathogen that causes clinical illness resulting from the effects of toxin production in humans and animals.[33,43,56,83] First identified in 1935, *C. difficile* is a leading cause of healthcare-associated infection in the United States and can also be acquired in a community setting.[28,44] Disease severity ranges from mild diarrhea to severe pseudomembranous colitis, toxic megacolon, septicemia, and occasionally death.[3,44,57]

Various strains of *C. difficile* that range in pathogenicity and genetic composition have been identified. Differences in pathogenicity have been attributed to complementary or inhibitory actions of expressed genes, many of which have unknown functions.[45] The pathogenicity loci (PaLoc) of clinically significant *C. difficile* strains encode toxins primarily responsible for their pathologic features.[3,69,73] Two major exotoxins, consistently produced in toxigenic strains, include toxin A, an enterotoxin, and toxin B, an inflammatory cytotoxin.[3,40,41,50,81] The pathogenic and clinical importance of these toxins is further corroborated by the observation that their deletion attenuates pathogenicity in vivo.[40,41]

A recent study found approximately 65% of *C. difficile* infections (CDI) were healthcare-associated, with the remaining 35% community-acquired.[44] The major predisposing factor for *C. difficile* overgrowth and subsequent toxin production is antibiotic treatment, resulting in gastrointestinal dysbiosis with a concomitant decrease in the normal protective microbiota.[70,73] Proliferation of toxigenic, vegetative forms in colonized subjects or exposure to *C. difficile* spores with subsequent germination and rapid proliferation can result in disease. Asymptomatic colonization with *C. difficile* further complicates diagnosis.[73] Diagnosis relies on 'multistep algorithms' that involve testing patients with clinical symptoms (at least 3 unformed stools in 24 h), performing histopathology on endoscopy samples, and isolation of toxigenic *C. difficile* in feces through any of a variety of enzyme immunoassays or PCR analyses.[5,54] Treatment of severe

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cases includes the provision of antibiotics (for example, vancomycin, fidaxomicin, or metronidazole) to control the bacteria, although recurrence is possible.[54,57] Recently, fecal microbiota transplantation (FMT) has been recommended in severe cases or when patients relapse.[5,12,46,71]

Various experimental animal models of *C. difficile*-associated enterocolitis were used to elucidate pathogenesis, study virulence and explore treatments of human clinical isolates.[30,45] Experimental infection in immunocompetent mice requires pretreatment with antibiotic cocktails (metronidazole, vancomycin, kanamycin, gentamicin, and colistin), cefoperazone or clindamycin, or using axenic animals, followed by the oral administration of *C. difficile* spores.[22,30] Only a few reports describe naturally occurring *C. difficile*-associated disease in laboratory mice. *C. difficile* toxins A and B were detected in a pooled fecal sample during an outbreak of obstipation and ulcerative proliferative colitis in C57BL/6 mice after induction of experimental autoimmune encephalomyelitis.[39] In another report, *C. difficile* was associated with soft stool and reduced reproductive performance in an experimentally naïve, breeding colony of C3H-*scid* mice.[37] More recently, the offspring of C57BL/6J mice fed a methyl donor supplementation diet experienced unexpected deaths due to *C. difficile*-induced typhlocolitis.[53] None of the mice in these previously reported cases were exposed to antibiotics.

We here report 2 outbreaks of naturally occurring C. *difficile*-associated disease in mice. Outbreak 1 occurred in naïve and xenograft-implanted NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ (NSG) and related strains (NOD.Cg-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}Tg[CMV-IL3,CSF2,KITLG]1Eav/MloySzJ [NSGS], NOD.Cg-*Hc*^{1}-*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ [NSG-Hc], NOD.Cg-*B2m*^{tm1Unc}*Prkdc*^{scid}*Il2rg*^{tm1Wjl}/SzJ [NSG B2m], and C;129S4-*Rag2*$^{tm1.1Flv}$*Il2rg*$^{tm1.1Flv}$/J [Rag2$^{-}\gamma c^{-}$]) after amoxicillin administration. Outbreak 2 affected a NOD.CB17-*Prkdc*^{scid}/NCrCrl (NOD-*scid*) breeding colony. We describe the clinical presentation, diagnostic approach, and pathologic findings linking *C. difficile* to these outbreaks. Genomic analysis of representative *C. difficile* isolates was conducted to determine the source(s) of infection and examine the isolates' virulence.

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Outbreak 1. During a 1-mo period, approximately 182 NSG and NSG-background mice presented with diarrhea and lethargy. Affected mice were maintained in 2 multiple-investigator (investigators A through C) rooms (nos. 1 and 2) and a single-investigator (investigator D) holding room. In addition to the affected NSG and NSG-background strains, the rooms housed other unaffected, immunocompromised (e.g., C.B-17/IcrHsd-*Prkdc*^{scid}*Lyst*$^{bg-J}$ [SCID beige], Nu/J [athymic nude]) and immunocompetent (e.g., C57BL/6) strains. Affected mice from the multiple-investigator rooms originated from different breeding colonies at a single vendor (The Jackson Laboratory, Bar Harbor, ME) and arrived over a 3-mo period. Affected mice from the single-investigator room had been bred inhouse. The majority of mice were used in oncology studies and were engrafted with human cell lines or primary human xenografts. At the time of the outbreak, the multiple- and single-investigator rooms housed 405, 559, and 193 cages, respectively. All mice housed in these rooms had been treated for 14 d with 0.12% amoxicillin-impregnated feed to control an increase in the incidence of *Corynebacterium*-associated hyperkeratosis caused by *Corynebacterium bovis*, which is endemic in these colonies. Amoxicillin had been used without issue for more than 7 y to manage *Corynebacterium*-associated hyperkeratosis in these and other colonies at our institution.[11] Prior to treatment initiation, multiple mice were confirmed to be culture-positive for *C. bovis*, and the isolates sensitive to ampicillin, the surrogate for amoxicillin (data not shown). After cessation of therapy, the food hoppers in the multiple-investigator rooms were topped off with standard diet, and no additional amoxicillin-supplemented feed was provided. In contrast, in the single-investigator room, all amoxicillin-containing feed was removed completely and replaced with the standard diet.

Within 2 to 3 d after cessation of amoxicillin-containing feed, unexpected illness and death initially was observed among 5 NSG mice in a single cage in a multiple-investigator room. Sick mice appeared thin and hunched, as described retrospectively by the investigator; diarrhea was not observed. The sick mice in this cage were euthanized by the investigator. At 22 and 26 d after cessation of amoxicillin feed in the multiple-investigator and single-investigator rooms, respectively, the first cases of diarrheic feces were observed (Figures 1 and 2). In these new cases, mice presented with soft stool or perineal fecal staining (multiple-investigator rooms, $n = 3$; single-investigator room, $n = 2$) or were found dead (multiple-investigator rooms, $n = 2$; single-investigator room, $n = 1$; Figures 1 and 2).

The peak of the outbreak presented 25 and 30 d after cessation of therapy in the multiple-investigator (Figure 1) and single-investigator (Figure 2) rooms, respectively, after which cases decreased in frequency until the last case presented at approximately 7 wk after cessation of antibiotic therapy. Throughout the outbreak, which lasted 24 d in the multiple-investigator rooms and 12 d in the single-investigator room, mice that deteriorated were euthanized or found dead, recovered, or were submitted for diagnostic evaluation (Table 1). Clinically affected and apparently healthy mice were often cohoused. Of the mice in both the multiple-investigator and single-investigator rooms (total $n = 182$), 168 had been engrafted with a xenograft, whereas the remaining 14 were experimentally naïve. Affected mice were adults and were female ($n = 11$), male ($n = 67$), or of unknown sex ($n = 104$). Affected mice in the 2 multiple-investigator rooms were found on 3 of the 10 racks total; these 3 racks held cages from 3 different investigative labs only.

Diagnostic testing of the 60 submitted mice (58 sick mice, 2 clinically unaffected cage mates) included both gross necropsy and histopathology ($n = 21$), histopathology of formalin-fixed gastrointestinal tracts only ($n = 5$), and gross necropsy only ($n = 4$; Table 2). Intestinal contents or feces ($n = 54$) and blood ($n = 2$) were cultured aerobically and anaerobically (Table 3). Antimicrobial sensitivity assays ($n = 3$) were conducted under anaerobic growth conditions by using antimicrobial-containing strips (Table 4). *C. difficile*-specific antigen and toxin assays were performed on feces ($n = 3$) or cecal contents ($n = 4$; Table 3). Whole-genome sequencing was performed on representative isolates identified as *C. difficile* ($n = 3$; Table 3).

Outbreak 2. A few months after the end of outbreak 1, a total of 3 adult NOD-*scid* mice used for breeding in 2 cages (housing a total of 9 mice) belonging to a single investigator (investigator E) were found dead. In contrast to those in outbreak 1, these mice were otherwise experimentally naïve and had never been exposed to antibiotic treatment. One cage contained female mice only ($n = 4$), and the other had only male mice ($n = 5$). These mice were housed in a different vivarium than those in outbreak 1 and were bred inhouse. Five of the 6 remaining cage mates presented hunched and lethargic with piloerection. These 5 mice were euthanized, and 3 (female, $n = 1$; male, $n = 2$) were submitted for complete necropsy (Table 5). All 3 of the mice necropsied showed neutrophilic typhlitis or typhlocolitis, both of which are highly suggestive of *C. difficile* enterotoxemia.

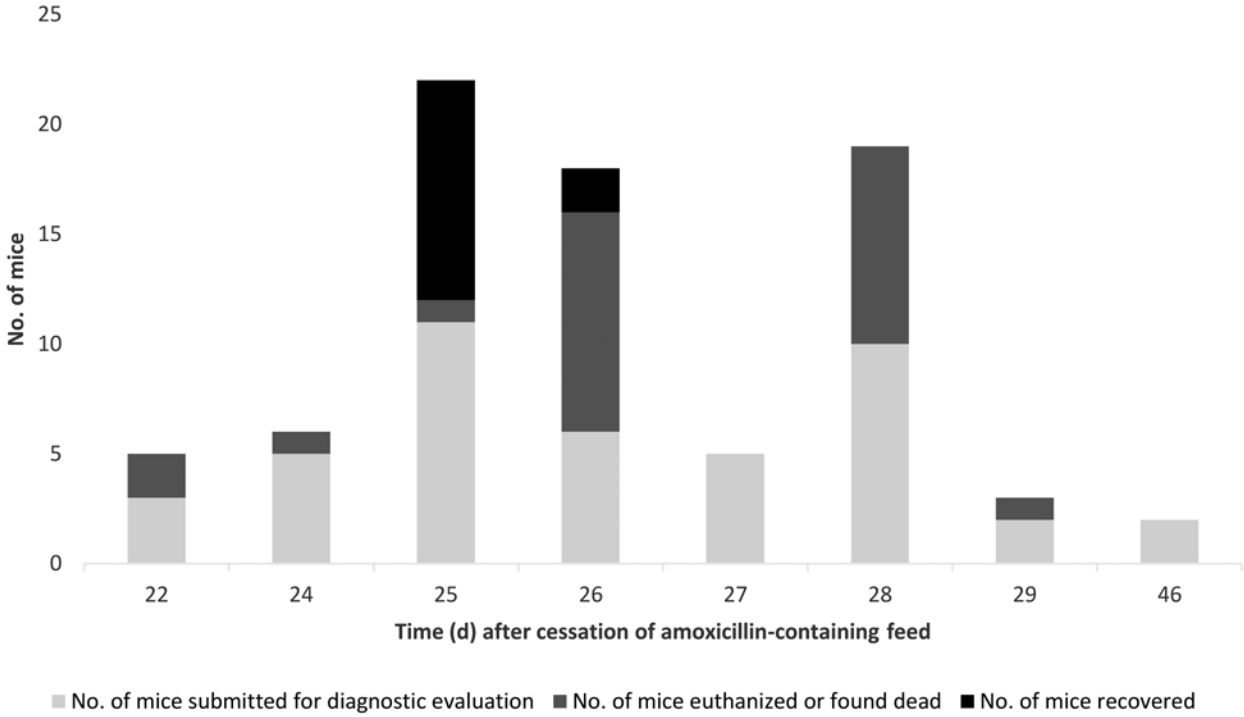

Figure 1. Number of mice affected by *C. difficile* in outbreak 1, including the proportions found dead or that required euthanasia, recovered, or were submitted for diagnostic evaluation in 2 multiple-investigator rooms after gradual cessation of amoxicillin administration. The total estimated population in both rooms at the time of the outbreak was approximately 1677 mice.

These results prompted us to collect feces from another 2 separate NOD-*scid* breeding colonies (belonging to 2 different investigators) housed on the same rack; we pooled the samples by investigator and tested for *C. difficile* antigen and toxins A and B. One of 2 pooled fecal samples was positive for *C. difficile* antigen and one or both toxins; this sample was from the colony that experienced the aforementioned morbidity and mortality. Neither *C. difficile* nor its toxins were detected in feces from the other pooled fecal sample.

No additional unexpected morbidities or deaths were detected in this colony until 3 mo later when a NOD-*scid* breeding dam of a breeding pair was found hunched and lethargic, with perineal fecal staining; these clinical signs are consistent with *C. difficile* enterotoxemia. The female mouse was housed with a clinically normal, male cage mate. These 2 mice, and a clinically normal NOD-*scid* mouse from a different cage, underwent gross necropsy and histopathology ($n = 3$), aerobic and anaerobic culture of intestinal contents ($n = 3$), antimicrobial sensitivity assays ($n = 1$), and *C. difficile* toxin immunoassays of cecal and colonic contents ($n = 3$; Tables 4 through 6). Whole-genome sequencing was performed on an isolate identified as *C. difficile* from the clinically affected female ($n = 1$; Table 6).

Materials and Methods

Animal housing. Mice were maintained in individually ventilated polysulfone cages with stainless-steel wire-bar lids and filter tops (no. 19, Thoren Caging Systems, Hazelton, PA). They were housed on autoclaved aspen chip bedding (PWI Industries, Quebec, Canada), with each cage provided 2 compressed cellulose squares (Nestlets, Ancare, Bellmore, NY) for enrichment. Mice were fed a closed-formula, natural-ingredient, γ-irradiated diet (no. 5053, PicoLab Rodent Diet 20, PMI Nutrition International, St. Louis, MO), which was surface-decontaminated by using 'flash' sterilization (100 °C for 1 min).[79] All animals in outbreak 1 were also fed γ-irradiated, 0.12% amoxicillin-impregnated feed (TestDiet, Richmond, VA). Mice were provided reverse-osmosis–purified acidified (pH 2.5 to 2.8, with hydrochloric acid) water in polyphenylsulfone bottles with stainless-steel caps and sipper tubes (outbreak 1; Techniplast, West Chester, PA) or drilled polysulfone bottles with neoprene stoppers (outbreak 2; Thoren Caging Systems). Cage bottoms were changed weekly, whereas wire-bar lids, filter tops, and water bottles were changed biweekly within a class II, type A2 biologic safety cabinet (LabGard S602-500, Nuaire, Plymouth, MN). The rooms were maintained on a 12:12-h light:dark cycle, relative humidity of 30% to 70%, and room temperature of 72 ± 2 °F (22.2 ± 1.1 °C). The animal care and use program at Memorial Sloan Kettering Cancer Center (MSK) is accredited by AAALAC, and all animals are maintained in accordance to the recommendations provided in the *Guide for the Use and Care of Laboratory Animals 8th Edition*.[31] All animal use described in this investigation was approved by Memorial Sloan Kettering Cancer Center's IACUC.

Colony health monitoring. The soiled bedding sentinel program has been previously described in detail.[47] Briefly, 4 to 6 wk old female Tac:SW mice (Taconic Biosciences, Germantown, NY) are obtained for use as soiled bedding sentinels. On arrival, animals are free of antibodies to mouse hepatitis virus, mouse rotavirus, lymphocytic choriomeningitis virus, ectromelia virus, mouse parvovirus, minute virus of mice, murine norovirus, pneumonia virus of mice, Reovirus, Sendai virus, mouse rotavirus, Theiler meningoencephalitis virus, mouse adenovirus, K virus, murine polyoma virus, mouse cytomegalovirus, mouse T-lymphotropic virus, hantavirus, and lactate dehydrogenase-elevating virus and do not have *Filobacterium rodentium*, *Mycoplasma pulmonis, Helicobacter* spp., *Salmonella* spp., *Clostridium piliforme*, *Corynebacterium kutscheri*, *Citrobacter rodentium*, endoparasites, and ectoparasites. Each sentinel cage

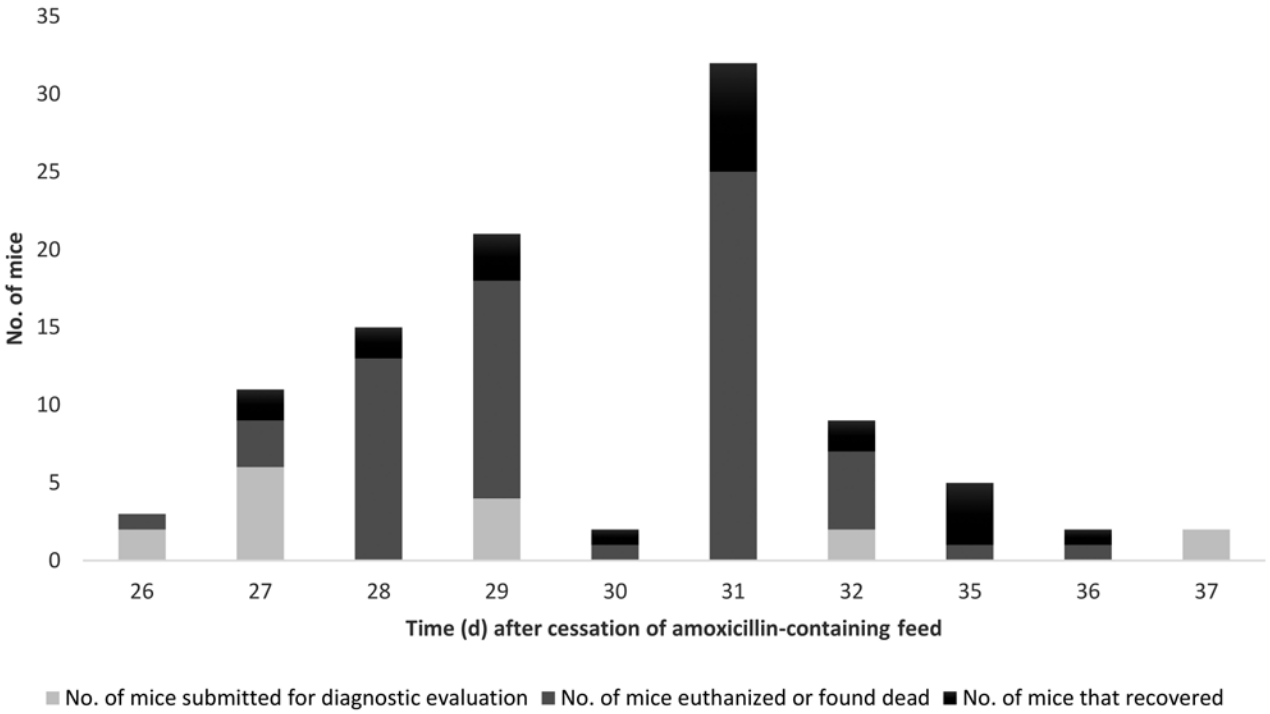

Figure 2. Number of mice affected by *C. difficile* in outbreak 1, including the proportions found dead or required euthanasia, recovered, or submitted for diagnostic evaluation in a single-investigator room after abrupt cessation of amoxicillin administration. The total estimated population at the time of the outbreak was 724 mice.

Table 1. Total number of mice affected by *C. difficile* in outbreak 1 in the multiple- and single-investigator rooms, including the proportions of mice found dead or that required euthanasia, that recovered, or that were submitted for diagnostic evaluation

	Multiple-investigator rooms (total no. of mice, 1677)	Single-investigator room (total no. of mice, 724)
Euthanized or found dead	24 (1.4%)	64 (8.4%)
Recovered	22 (1.3%)	12 (1.7%)
Submitted for diagnostic evaluation	44 (2.6%)	16 (2.2%)
Total no. of affected animals	90 (5.4%)	92 (12.7%)
	182 (total no. of mice overall, 2401; 7.6% affected overall)	

serves a maximum of 4, single-sided, 70-cage racks and receives approximately 15 mL of dirty bedding from a maximum of 40 different colony cages (1 column per rack) weekly. One sentinel mouse from each cage is identified every 8 wk and its blood collected for serologic testing and fecal samples and pelt swabs collected for PCR testing. At 6 and 12 mo after placement, one sentinel mouse from each cage is euthanized for blood collection, pelt and large intestinal content examination for ecto- and endoparasites, and gross necropsy, with histologic examination when gross lesions are found and when necessary to confirm positive parasitology or serologic tests. Survival blood collection (approximately 20 µL) is performed through tail vein nicking using a sterile 25-gauge needle and collecting blood in a microsampler (HemaTop, CRL, Wilmington, MA) or by cardiocentesis after euthanasia by CO_2 asphyxiation.

Gross necropsy and histopathologic analysis. Live mice were euthanized by CO_2 asphyxiation. A gross necropsy was performed and macroscopic lesions recorded. Samples for aerobic and anaerobic culture were collected from the small and large intestines or feces. Select tissues were collected (heart, lungs, thymus, mandibular, mesenteric and mediastinal lymph nodes, kidneys, liver, spleen, stomach, salivary glands, uterus, cervix, skin, urinary bladder, adrenals, ovaries, oviducts, uterus, bulbourethral glands, epididymis, prostate, seminal vesicle, testes, thyroid, trachea, esophagus, hindlimb [femur, tibia, stifle joint, skeletal muscle, and peripheral nerves], vertebral column with spinal cord, sternum [for bone marrow evaluation], head, and coronal sections [including brain, eyes, ears, nasal and oral cavities, teeth]). Only small and large intestines were submitted and processed for a subset of mice. Tissues were preserved in 10% buffered formalin and, when deemed necessary, processed into paraffin blocks, cut into 5-µm–thick sections, and underwent hematoxylin and eosin and Gram (select tissues) staining for microscopic evaluation by a board-certified veterinary pathologist (AP).

Bacterial isolates, growth conditions, and species identification. Samples were plated directly on solid agars as well as inoculated into a nutrient broth supplemented with sodium thioglycolate, hemin, and vitamin K (BBL Thioglycolate Medium, Becton Dickinson, Franklin Lakes, NJ). Samples were

Table 2. Macroscopic and microscopic pathologic findings from the mice evaluated in outbreak 1

Investigator (room type)	Accession ID (no. of mice)	Macroscopic findings (no. mice w/lesion/no. total)				Microscopic findings (no. mice w/lesion/no. total)			
		Perineal staining	GI mucoid or no content	LI edema	SI serosal congestion	Typhlocolitis	Typhlitis only	Ileitis	LI edema
A (multiple-investigator room 1)	18-1108 (4)	2/4	2/4	1/4	2/4	2/4	2/4	1/4	4/4
	18-1066 (3)	0/3	3/3	2/3	1/3	2/3	1/3	0/3	3/3
	18-1434 (6)	NP	NP	NP	NP	NP	NP	NP	NP
	18-1163 (3)	2/3	2/3	3/3	0/3	3/3	0/3	0/3	3/3
B (multiple-investigator room 1)	18-1146 (2)	0/2	2/2	2/2	0/2	2/2	0/2	0/2	2/2
	18-1139 (5)	NP	NP	NP	NP	5/5	0/5	0/5	5/5
	18-1162 (1)	0/1	1/1	1/1	0/1	1/1	0/1	0/1	1/1
D (single-investigator room)	18-1346 (8)	6/8	8/8	4/8	1/8	4/4	0/4	1/4	4/4
	18-1422 (2)	0/2	1/2	0/2	0/2	1/2	0/2	0/2	2/2
	18-1410 (2)	1/2	2/2	2/2	0/2	1/2	1/2	0/2	2/2
Total	60	11/25	22/25	15/25	4/25	21/26	4/26	2/26	26/26

GI: gastrointestinal tract (stomach, small intestine, large intestine, colon, cecum); LI, cecum–colon; NP, not performed; SI, small intestine

incubated aerobically and anaerobically at 37 °C for a maximum of 96 h. For aerobic culture, samples were plated on trypticase soy agar with sheep blood (BBL Prepared Plated Media TSA II, Becton Dickinson. For anaerobic culture, samples were plated on *Brucella* agar with 5% sheep blood supplemented with hemin and vitamin K1 (BBL Prepared Media, Becton Dickinson) or *C. difficile* selective-media plates (AnaeroGro Cycloserine–Cefoxitin Fructose Agar, Hardy Diagnostics, Santa Maria, CA). The plates were placed promptly into an anaerobic incubation sachet (BD GasPak EZ Container Systems, Becton Dickinson). On anaerobically incubated solid media, the detection of flat, circular to irregular, gray to white, nonhemolytic colonies on nonselective media, with gram-positive rods and large, oval, subterminal endospores under light microscopy and yellow colonies on selective media plates, warranted suspicion of clostridioidal species. Identification at species level was determined by using an anaerobe biochemical test panel (RapID ANA II System, ThermoFisher Scientific, Waltham, MA), L-proline aminopeptidase activity (PRO Disks, ThermoFisher Scientific, Waltham, MA), and finally MALDI-TOF mass spectrometry (conducted on representative clinical isolates [n = 4]; Weil Cornell Medicine's Genomics and Epigenomics Core, New York, NY).

Other bacterial colonies found during routine culture were identified to the genus level by using microbial identification kits (API NE, 20S, and 20E, BioMérieux, Durham, NC). In addition, we received a fecal sample from a vendor whose mouse colony had a history of *C. difficile* enterocolitis and from which we periodically receive mice.[1] This fecal sample was subject to the same anaerobic culture technique described earlier.

Antimicrobial sensitivity testing. *Brucella* blood agar (Becton Dickinson), which was reduced overnight in anaerobic incubation sachets, was used for all susceptibility testing. Bacterial isolates were resuspended in thioglycolate medium to a density of 1.0 McFarland. A sterile cotton swab was dampened in the suspension. The swab was then spread evenly over the entire surface of the agar in 3 different directions. After strips containing antimicrobials were applied carefully, plates were incubated anaerobically at 37 °C. Zones of inhibition were read after 48 h. Minimal inhibitory concentration (MIC) determination was performed by using gradient strips (E-Test, BioMérieux, Durham, NC) containing ampicillin, amoxicillin–clavulanic acid, clindamycin, metronidazole, enrofloxacin, sulfamethoxazole–trimethoprim, and vancomycin. Susceptibility was categorized as susceptible, intermediate, or resistant when interpretation guidelines were available. Zones due to ampicillin, amoxicillin–clavulanic acid, clindamycin, and metronidazole were interpreted according to Clinical Laboratory Standards Institute M100 ED29:2019 guidelines implemented for the agar dilution method.[15] For enrofloxacin, interpretation was performed by using the moxifloxacin MIC breakpoint available in Clinical Laboratory Standards Institute M100 ED29:2019.[15] Zones due to vancomycin were interpreted by using MIC breakpoints or epidemiologic cut-off values according to the European Committee on Antimicrobial Susceptibility Testing clinical breakpoints version 9 (http://www.eucast.org/).

***C. difficile*-specific antigen and toxin assay.** Feces or cecal contents were collected and tested immediately, or frozen at -80 °C and tested using an assay that detects *C. difficile*-specific glutamate dehydrogenase antigen (confirms presence of the bacterium only) as well as toxins A and/or B (C. DIFF QUICK CHEK COMPLETE, TECHLAB, Blacksburg, VA) according to the manufacturer's recommendation.

Whole-genome sequencing, assembly, annotation, and in silico analysis. Four representative *C. difficile* isolates collected from clinically affected mice belonging to 3 distinct investigative teams (investigators A, C, and D) from outbreak 1, a single isolate from outbreak 2 (investigator E), and an isolate from the vendor described earlier underwent whole-genome sequencing and subsequent computational analyses (Memorial Sloan Kettering Bioinformatics Core, New York, NY). Single colonies of each *C. difficile* isolate were grown anaerobically overnight at 37 °C in brain–heart infusion medium supplemented with yeast extract and cysteine. DNA underwent phenol–chloroform extraction with bead beating and purified by using a DNA purification kit (QiAmp DNA mini kit, Qiagen Sciences, Germantown, MD). Purified DNA was sheared by using a focused ultrasonicator (Covaris, Woburn, MA) and prepared for next-generation sequencing (LTP Library Preparation Kit for Illumina Platforms, KAPA Biosystems, Wilmington, MA; TruSeq Adaptors, Illumina, San Diego, CA) to create 300- by 300-bp nonoverlapping paired-end reads, as previously described.[7]

C. difficile genomes were assembled and annotated by using PATRIC 3.5.27.[52] Genome feature tables for VPI 10463, CD 196, Lem1, vendor strains, and 33 previously reported clinical

Table 3. Microbiologic results from mice evaluated in outbreak 1

Investigator (room type)	Accession ID (no. of mice)	*C. difficile* culture results (no. positive/no. plated [sample source])	Confirmation results (test type; no. positive/no. tested)	*C. difficile* antigen and toxin testing (no. positive/no. tested)	Whole-genome sequencing
A (multiple-investigator room 1)	18–1138 (18)	11/18 (feces)	RapID ANA; 11/11	1/1	No
	18–1108 (4)	2/4 (SI)	RapID ANA; 2/2	1/1	No
		4/4 (LI)	RapID ANA; 4/4		
	18–1066 (3)	3/3 (SI, LI, feces)	MALDI-TOF; 2/2 RapID ANA; 3/3	NP	Yes
	18–1434 (6)	6/6 (LI)	RapID ANA; 6/6	1/1	No
	18–1163 (3)	NG (LI)		NP	No
B (multiple-investigator room 1)	18–1146 (2)	NG (LI)		NP	No
	18–1139 (5)	1/5 (feces)	RapID ANA; 1/1	1/1	No
	18–1162 (1)	NG (LI)		NP	No
C (multiple-investigator room 2)	18–1421 (2)	2/2 (LI)	MALDI-TOF; 1/1 RapID ANA; 2/2	1/1	Yes
D (single-investigator room)	18–1502 (2)	2/2 (LI)	MALDI-TOF; 1/1 RapID ANA; 2/2	NP	Yes
	18–1346 (8)	2/4 (LI)	RapID ANA; 2/2	2/2	No
	18–1422 (2)	2/2 (LI)	RapID ANA; 2/2	NP	No
	18-1410 (2)	2/2 (LI)	RapID ANA; 2/2	NP	No
	18–1426 (2)	NG (blood)		NP	No
Total	60	35/56		7/7	3

LI, cecum–colon; NG, no growth; NP, not performed; SI, small intestine

isolates were downloaded by using PATRIC.[45] *C. difficile* protein annotations were hierarchically clustered according to the presence or absence of PATRIC Local Families.[82] A UMAP plot was generated by setting n_neighbors and visualized by using ggplot2 in R version 3.5.1.[78] The PATRIC Similar Genome Finder Service, which contains approximately 1617 publicly available *C. difficile* genomes, was used to find the most closely related genomes. Multilocus sequence typing (MLST) predictions were made by using BLASTn to compare genome assemblies against the PubMLST database for *C. difficile*.[4,27]

Results

Histologic lesions consistent with *C. difficile*-associated disease. Table 2 provides an overview of the macroscopic and microscopic lesions found in mice evaluated during outbreak 1. Of the 25 mice that underwent gross necropsy, 20 had a normal body condition score (score, 3 [maximum, 5]); the remaining mice were underconditioned (score, 2).[80] Macroscopic findings included perineal fecal staining (11 of 25 mice), depleted intestinal contents (22 of 25 mice), translucent and turgid cecum and colon due to mural edema (15 of 25 mice), diffuse reddish discoloration of the intestinal serosa (interpreted as congestion) from the duodenum to the colon (4 of 25 mice), and white pinpoint foci on the hepatic surface (1 mouse). The most frequent microscopic lesion in clinically affected mice was a necrotizing and neutrophilic typhlocolitis (21 of 26 mice) of variable severity with the presence of rod-shaped, gram-positive bacteria with endospores within the lumen, and prominent submucosal edema (Figure 3 A through C) in clinically affected mice (24 of 24 mice). The large intestinal mucosa was characterized by multifocal areas of epithelial necrosis and neutrophilic exudation through necrotic mucosa (so-called 'volcano lesions'; Figure 3 C). Fibrin exudation leading to pseudomembrane formation was observed occasionally. Typhlitis, in the absence of colitis, was present in a few mice (4 of 26). In 2 mice, the mucosal lesions extended into the ileum and corresponded to the cases with marked serosal congestion (Figure 3 D). Additional microscopic changes included bilateral acute renal tubular necrosis

Table 4. Minimal inhibitory concentrations (mg/L) and susceptibility interpretation of various antimicrobials for 4 *Clostridioides difficile* isolates from outbreaks 1 and 2

Investigator (room type)	Accession ID	Ampicillin	Amoxicillin–clavulanic acid	Clindamycin	Metronidazole	Enrofloxacin	Trimethoprim–sulfamethoxazole	Vancomycin
A (multiple-investigator room 1)	18–1066	0.75 / I	0.19 / S	1.5 / S	0.047 / S	> 32 / R	0.38 / NA	0.5 / S
C (multiple-investigator room 2)	18–1421	1 / I	0.5 / S	4 / I	0.047 / S	> 32 / R	> 32 / NA	0.75 / S
D (single-investigator room)	18–1502	1 / I	0.5 / S	1.5 / S	0.064 / S	> 32 / R	> 32 / NA	0.5 / S
E	19–0880	NP	0.38 / S	NP	NP	> 32 / R	> 32 / NA	NP

I, intermediate; NA, interpretation not available; NP, not performed; R, resistant; S, sensitive

Table 5. Macroscopic and microscopic pathologic findings from mice evaluated in outbreak 2

		Macroscopic findings (no. mice w/lesion/no. total)				Microscopic findings (no. mice w/lesion/no. total)			
Investigator	Accession ID (no. of mice)	Perineal staining	GI mucoid or no content	LI edema	SI serosal congestion	Typhlocolitis	Typhlitis only	Colitis only	LI edema
E	18–4848 (3)	0/3	2/3	0/3	0/3	1/3	2/3	0/3	3/3
	19–0880 (3)	1/3	1/3	1/3	0/3	1/3	0/3	1/3	1/3
Total	6	1/6	3/6	1/6	0/6	2/6	2/6	1/6	4/6

GI, gastrointestinal tract (stomach, small intestine, large intestine, colon, cecum); LI, cecum–colon; SI, small intestine

(6 of 26 mice) and myeloid hyperplasia in the bone marrow (6 of 26 mice). No histologic lesions were associated with the macroscopic lesions in the liver in 1 mouse. In the subclinically affected mice, lesions were limited to prominent submucosal edema in the cecum (2 of 2 mice).

Table 5 provides an overview of the macroscopic and microscopic lesions found in mice evaluated during outbreak 2. Of the 6 mice, 5 had a normal body condition score and 1 was underconditioned (score, 2). Perineal fecal staining was seen in one of the 6 mice. The small intestines were fluid-filled, and the colon and cecum were devoid of contents (3 of 6 mice) and the colon was edematous (1 mouse). There were no macroscopic findings in the remaining 2 mice. Microscopic findings included a diffuse, neutrophilic, and hyperplastic typhlocolitis (2 of 6 mice), colitis only (1 of 6 mice), typhlitis only (2 of 6 mice), and mild, multifocal, neutrophilic enteritis (2 of 6 mice). Orthokeratotic hyperkeratosis of the nonglandular portion of the stomach, consistent with inanition, was present in 3 of the 6 mice.

Isolation of *C. difficile* from clinically affected mice. Table 3 summarizes the microbiologic findings from Outbreak 1. *C. difficile* was isolated from 35 of the 56 mice as confirmed by anaerobic biochemical testing or MALDI-TOF mass spectrometry (or both). Some mice had gross and histopathologic lesions compatible with *C. difficile* infection but were culture-negative (12 of 56 mice). In 9 of the 56 samples (cecal contents, 7; blood, 2), no bacterial growth was seen on solid media despite growth of a bacterium of clostridioidal cell morphology in broth confirmed by Gram staining and were thus considered negative; these samples were submitted for bacterial culture only. In addition to isolating *C. difficile*, we cultured only *Enterococcus* and *Staphylococcus* species from a large number of mice during routine culturing of samples. Table 6 summarizes the microbiologic findings from outbreak 2. *C. difficile* was isolated from 2 of 3 cecum-content samples. The culture-positive animals were a breeding pair from the same cage. Mixed enteric bacteria were identified in all mice evaluated.

Toxigenic *C. difficile* isolates. In further support of *C. difficile* as the etiologic agent, intestinal contents were tested for the presence of a *C. difficile*-specific antigen (glutamate dehydrogenase) and toxins A and B. All 7 samples tested ($n = 4$ feces, $n = 3$ cecal contents) from outbreak 1 were positive for *C. difficile* antigen and toxin A or B or both (Table 3). In addition, 2 of the 3 cecum-content samples from outbreak 2 were positive for *C. difficile* antigen and toxin A or B or both (Table 6). The toxin-positive animals were a breeding pair from the same cage.

Sensitivity of *C. difficile* isolates to ampicillin or amoxicillin–clavulanic acid. To better understand the clinical impact of amoxicillin feed, we assessed in vitro antibiotic sensitivity. Table 4 summarizes antibiotic sensitivity results and the MIC for the antibiotics tested for both outbreaks. The 3 submitted isolates from outbreak 1 were sensitive to amoxicillin–clavulanic acid, metronidazole, and vancomycin and had at least intermediate sensitivity to clindamycin and ampicillin, the surrogate for amoxicillin. In outbreak 2, the single isolate evaluated was sensitive to amoxicillin–clavulanic acid and resistant to enrofloxacin. Although the MIC was large (greater than 32 mg/L) for sulfamethoxazole–trimethoprim in isolates from both outbreaks, we are unable to comment regarding their susceptibility due to a lack of universal guidelines regarding the interpretation of results from *C. difficile* for this antibiotic. Among the 4 isolates evaluated, slight differences in MIC were found for the antibiotics tested.

Inclusion of isolates in a hypervirulent (ST1) MLST group. Whole-genome sequencing and comparative genomic analysis were performed to compare sequence similarity of our *C. difficile* isolates with other strains (VPI 10463, CD 196, Lem1, and vendor-supplied) and to determine MLST types. We chose VPI 10463 and CD 196 for comparison because they are common human clinical strains used to induce experimental *C. difficile* disease in mice.[2,22,35] Lem1 is a toxin-producing but nonpathogenic strain, genetically similar to VPI 10463, that has recently been identified in mice from both The

Table 6. Microbiologic results from mice evaluated in outbreak 2

Investigator	Accession ID (no. of mice)	*C. difficile* culture results (no. positive/no. plated [sample source])	Confirmation results (test type; no. positive/ no. tested)	*C. difficile* antigen and toxin testing (no. positive/no. tested)	Whole-genome sequencing
E	18–4848 (3)	NP		NP	No
	19–0880 (3)	2/3 (LI)	RapID ANA; 2/2	2/3	Yes
Total	6	2/3		2/3	1

LI, cecum–colon; NP, not performed

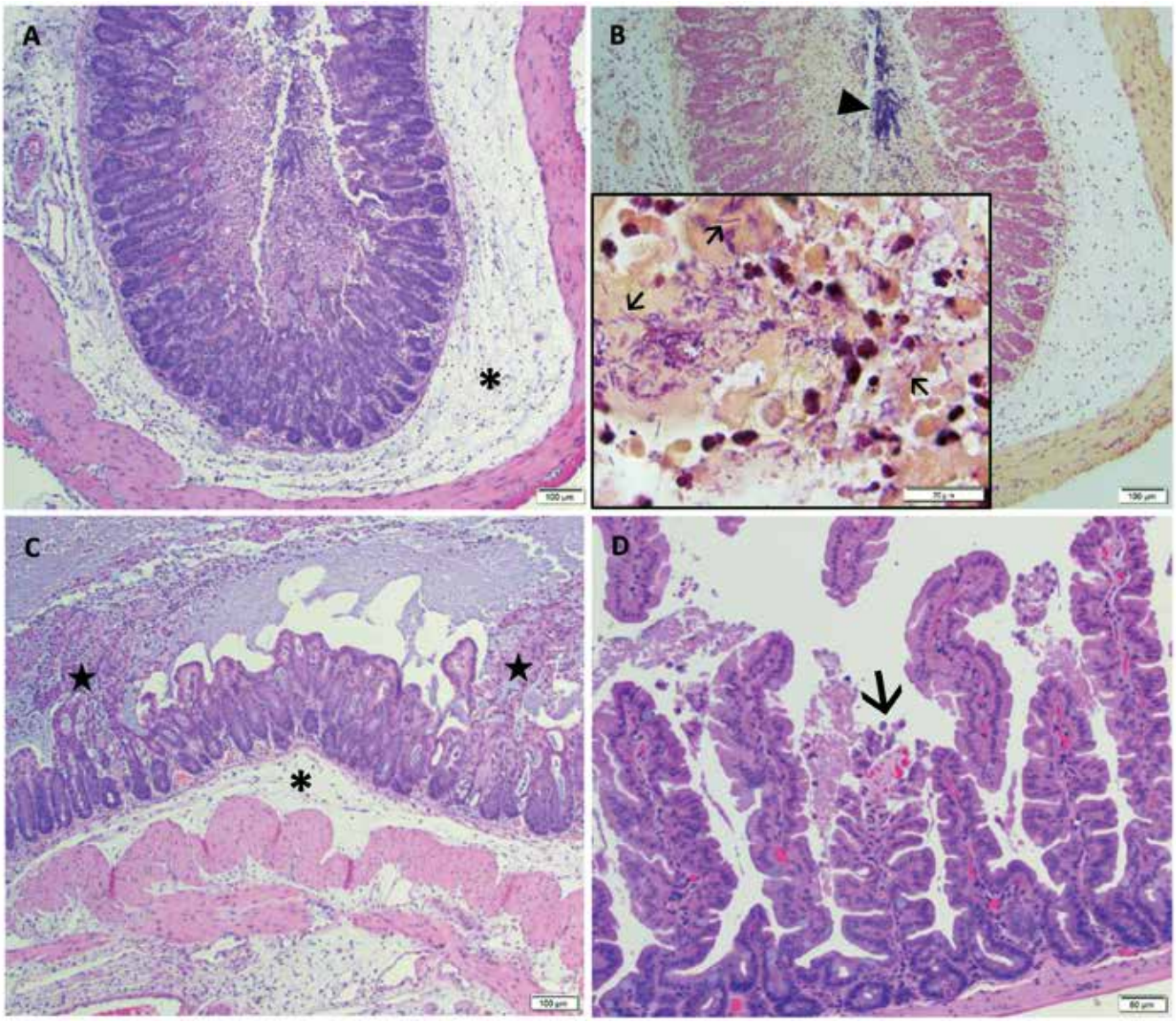

Figure 3. Cecum. Loss of continuity of the intestinal mucosa, with efflux of neutrophils and luminal accumulation of necrotic cellular debris. Numerous rod-shaped bacteria are present within the exudate. Prominent submucosal edema (asterisk) is shown. (A) Hematoxylin and eosin stain. (B) Gram stain. High-power magnification of luminal content containing gram-positive, rod-shaped bacteria (arrowhead; inset) with endospores (arrows). (C) Colon. Multifocal 'volcano lesions' (star) characterized by vertical neutrophilic exudation and sloughed off necrotic enterocytes through the damaged mucosa. Submucosal edema (asterisk) was consistently present. Hematoxylin and eosin stain. (D) Small intestine. Congestion of the villi and degeneration of the enterocyte lining (arrow). Hematoxylin and eosin stain.

Jackson Laboratories and Charles River Laboratories.[22] We also compared sequences from isolates from both outbreaks with the vendor-supplied strain to determine whether this vendor could have been the source of the offending isolate(s). Isolates from both outbreaks (1: A, C, and D; 2: E) and CD 196 belong to the ST1 group.[27] In contrast, VPI 10463 and the vendor-supplied strain belong to ST46 and ST55, respectively. Figure 4 reveals the percent presence or absence of proteome match for *C. difficile* strains CD 196, VPI 10463, Lem1, the vendor-supplied sample, and *C. difficile* isolates from both outbreaks (1: A, C, and D; 2: E). Isolates A, C, D, and E are genetically most similar (although not identical) to strain CD

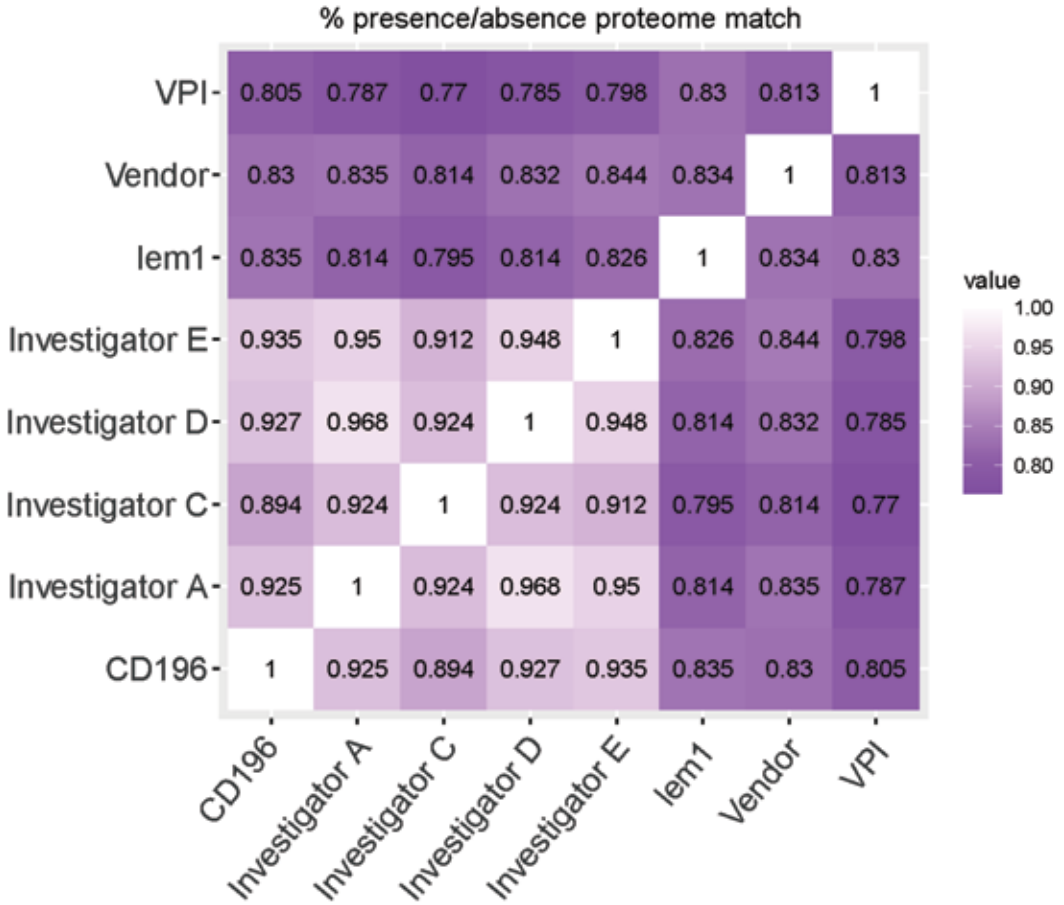

Figure 4. Analysis of percent presence or absence of proteome match for *C. difficile* strains CD 196, VPI 10463, Lem1, vendor-supplied sample, and *C. difficile* isolates from outbreaks 1 and 2 (investigators A, C, D, and E).

196, with at least 89.4% proteome match. In addition, isolates A, C, D, and E and CD 196 cluster closely, along with 5 human clinical isolates belonging to ST1, indicating similarities among the genomes (Figure 5).

Discussion

C. difficile enterotoxemia is a significant clinical problem in humans and select animal species that receive broad-spectrum antibiotics, which alter the intestinal microbiota, leading to overgrowth of toxigenic *C. difficile* and toxin-induced disease.[30] Although mice have been used experimentally for many years to study *C. difficile*, they typically are refractory to clinical disease, requiring the administration of multiple antibiotics or the use of axenic models followed by administration of bacterial spores.[30] Here, we report 2 outbreaks of *C. difficile* enterotoxemia in mouse strains with significant immune deficiencies. The earlier outbreak was associated with amoxicillin administration, which had been used in our program without adverse effects for many years. The second outbreak, which was not antibiotic-associated, involved far fewer mice, and the causative factor(s) remain unknown. We speculate that immunodeficiency of the murine host plays an important role in determining whether clinical disease results from colonization with toxigenic *C. difficile*.

Immunocompetent mice colonized with *C. difficile* are relatively resistant to *C. difficile*-induced disease.[13,30] This characteristic makes mice a desirable model for CDI, given that the more traditional hamster model invariably results in fulminant disease in this highly susceptible species.[30] Mice are now used extensively to study the pathogenesis of primary CDI, recurrence, microbiota alterations resulting from disease, and therapies aiming to restore a healthy microbiota.[13,16,18,71,77] Protective commensal bacteria likely inhibit *C. difficile* growth.[22,42,84] The microbiota of the mouse contains a myriad of species, such as *Lachnospiracea* and *Clostridium scindens*, that confer protection against *C. difficile*.[3,18,42,64,70] Antimicrobial treatment decreases 'colonization resistance' and induces dysbiosis through decreased competition and increased metabolites supporting growth of opportunistic bacteria that normally exist in low quantities. Amoxicillin, a β-lactam antibiotic, is known to reduce the protective microbiota due to its 'broad-spectrum' action against gram-positive and anaerobic bacteria; long-term use of oral amoxicillin also is associated with *C. difficile* enterotoxemia in humans.[6,19,60] The first clinical cases with diarrhea in outbreak 1 occurred 22 d (multiple-investigator rooms) and 26 d (single-investigator room) after the cessation of providing amoxicillin feed. The variation in the onset of clinical disease may reflect the gradual withdrawal of amoxicillin-containing feed in the multiple-investigator rooms compared with the abrupt replacement with the standard diet in the single-investigator room. The protracted exposure to amoxicillin in the multiple-investigator rooms may have suppressed recolonization of normal, protective bacteria, thus allowing more rapid *C. difficile* colonization, growth, and disease. Even though our isolates had at least intermediate sensitivity to ampicillin, this antibiotic may have been more detrimental to the protective microbiota in these cases.

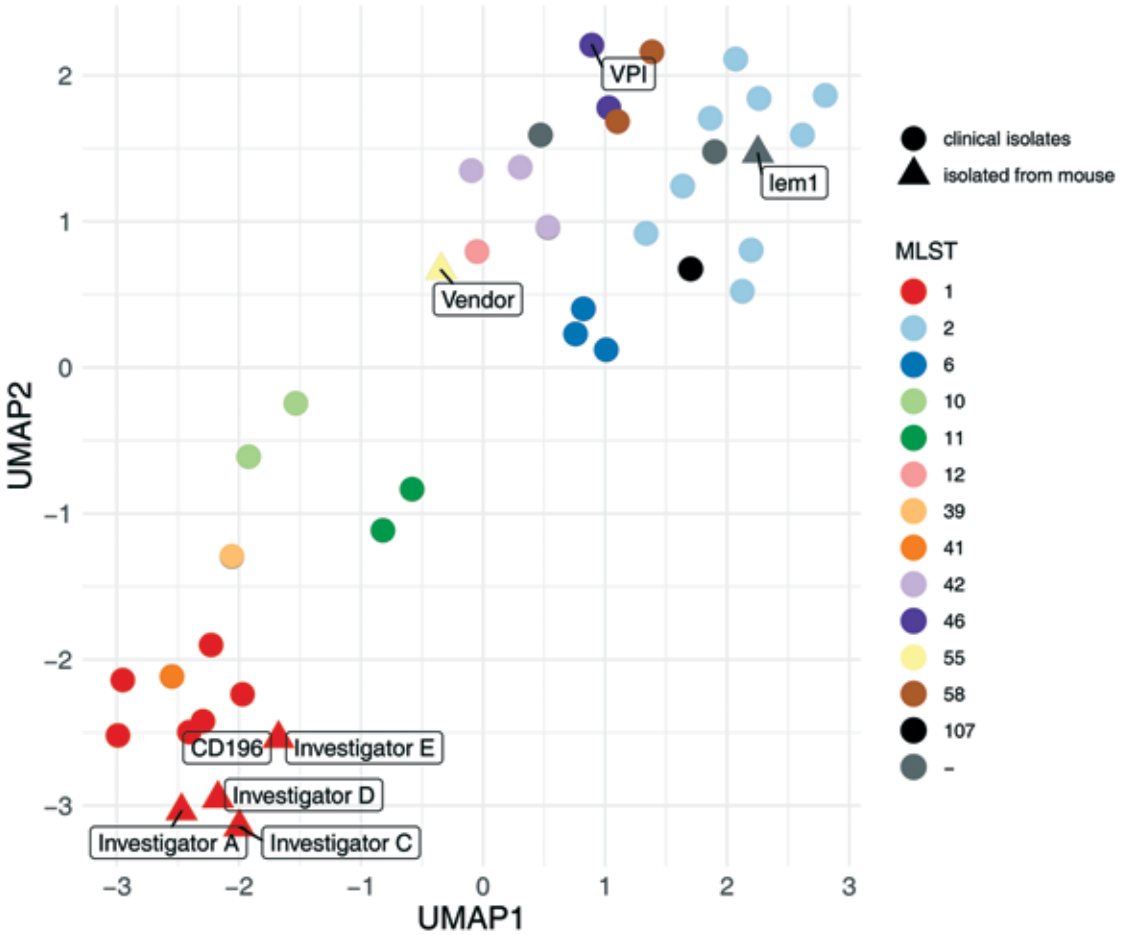

Figure 5. Analysis of clustering genomes by using UMAP according to the presence or absence of PATRIC Local Families for *C. difficile* strains CD196, VPI 10463, Lem1, Vendor sample, 33 clinical isolates from Lewis and colleagues (2017) and *C. difficile* isolates from outbreaks 1 and 2 (investigators A, C, D, and E). Isolates are characterized by shape (mouse compared with human clinical isolates) and color (MLST grouping). Isolates are characterized by shape (triangle: mouse compared with circle: human) and color (MLST grouping). Proximity of isolates indicates greater genome similarity.

Further evidence of dysbiosis included the overgrowth during outbreak 1 of *Enterococccus* spp., a feature that has also been reported in humans with CDI.[9,29] However, we have never observed clinical signs of *C. difficile* in mice placed prophylactically on sulfamethoxazole–trimethoprim-supplemented feed, which is commonly used to prevent opportunistic infections and was being administered to select mice in the colonies affected in outbreak 1. The offending isolates demonstrated a large MIC against sulfamethoxazole–trimethoprim and thus may be resistant. The potential mechanism(s) of protection provided by sulfamethoxazole–trimethoprim feed needs further elucidation.

Functional adaptive and innate immune responses (e.g., complement, macrophage, dendritic cell and natural killer cell functions) are critical in protecting mice from C. *difficile* enterotoxemia. C57BL/6 mice treated with immunosuppressive doses of dexamethasone are more susceptible to severe CDI and relapse.[77] The innate immune system is critical in initial defense and recovery.[2,3,35,42] Antibiotic-treated *MyD88*$^{-/-}$ or TLR5$^{-/-}$ mice, with deficiencies in innate signaling at the mucosal surface, develop severe and fatal infections when challenged with *C. difficile* spores.[34,42] MyD88 signaling is essential for recruiting neutrophils to prevent systemic dissemination of bystander bacteria.[35] Furthermore, type 1 and 3 innate lymphoid cells prevent fatal infection and aid in recovery from acute infection.[2] At the lamina propria, IFNγ production by type 1 innate lymphoid cells recruits neutrophils to destroy invading bacteria and provide support for epithelial cell repair, whereas type 3 innate lymphoid cells produce IL22, thus activating the C3 complement pathway to clear translocated pathogenic bacteria.[2] Antitoxin IgG and mucosal IgA provide important protection against initial and recurrent *C. difficile* disease but not colonization or recovery.[2,36,77] Highly immunodeficient mouse strains such as NSG or NOD-*scid* are deficient in both innate and adaptive immune responses. Therefore, these mice likely cannot mount effective immune responses to control *C. difficile* expansion leading to excessive toxin production and disease. This situation was evident in outbreak 1, when C57BL/6 and athymic nude mice in the affected rooms lacked clinical disease. These mice have intact innate immune responses and normal or impaired (i.e., athymic nude mice) adaptive immune responses. Immunodeficiency is a risk factor for primary CDI caused by ribotype 027 strains in humans.[57]

Affected animals exhibited 3 distinct clinical syndromes: 1) peracute death (very few animals); 2) severe diarrhea leading to euthanasia or death; and 3) mild to moderate diarrhea followed by recovery. Mice with different syndromes were often housed in the same cage as clinically unaffected but genetically identical cage mates; however, the clinically unaffected cage mates had

mild intestinal pathology (e.g., mural edema in the cecum). In outbreak 2, even though the NOD-*scid* breeding pair had detectable *C. difficile* toxin, only the female mouse developed clinical signs. In humans, women are overrepresented in both hospital- and community-acquired CDI.[44] In particular, pregnant women undergo a Th2 shift in immune response, resulting in decreased antitoxin A and B IgG, increasing their susceptibility to severe infection.[67] Cages of male mice also had multiple clinical presentations. Stress associated with social dynamics might explain the disparity in clinical presentation. Male mice subjected to chronic psychosocial stress have dysbiosis and altered immunoregulatory profiles, the combination of which may increase disease susceptibility.[8] Differences in the microbiome due to vendor, experimental manipulations, mouse strain, and other internal or external factors also could account for the variation in susceptibility and clinical presentations within affected rooms.[14,20]

Whole-genome sequencing revealed that strains from both outbreaks were similar to each other and belonged to ST1, the 'hypervirulent' MLST group, which is associated with increased lethality in antibiotic-treated mouse models.[27,45] ST1 contains PCR ribotype 027 *C. difficile* strains, which are known for hypervirulence and the ability to cause outbreaks with severe infections, high mortality, and recurrence in humans.[45,76] In addition, members of this ribotype are resistant to fluoroquinolones, which we noted in our isolates.[74,76] Our isolates were most similar to CD 196, a strain responsible for an outbreak in Canadian men and women.[76] Our isolates exhibited some differences in proteome and antibiotic sensitivity. These differences could have resulted from microevolutionary changes in the genome over time, as is known to occur in ribotype 027 strains.[75,76] Most of our isolates evaluated were positive for toxin A or B (or both). The typical PaLoc encodes for toxins A and B, and 3 proteins that appear to regulate toxin production and secretion (toxins R, E, and C). Toxins A and B both stimulate cytokine release from local cells and glucosylate enterocyte rho GTPases, resulting in cytoskeleton breakdown and loss of tight junctions.[72,73] These effects recruit neutrophils to the site, leading to further destruction of the intestinal barrier.[72,73] Although strains encoding toxin B alone appear to result in severe disease, the role of toxin A alone remains uncertain.[40,41,50,51] However, independent of PaLoc composition or the isolate's ability to produce toxin A or B, virulence within the MLST ST1 group in mice is heterogeneous, suggesting that additional unknown genetically encoded products affect virulence.[45] Binary toxin (i.e., *C. difficile* transferase) is encoded on a different locus, and in strains missing both toxin A and B, binary toxin is insufficient to cause disease in hamsters.[25] Although its exact role in disease remains unclear, binary toxin may be associated with an increased risk of mortality in the less than 10% of clinical isolates in which it is found.[7,24,26] The presence of other virulence genes does not necessarily equate to increased pathogenicity; Lem1 produces relatively high levels of toxins, including toxins A and B and binary toxin but is considered nonpathogenic in mice.[22] Nonetheless, all of our isolates (A, C, D, and E) contain the gene that encodes binary toxin (data not shown).

Outbreak 2 resembled community-acquired infections in humans in that the classic risk factor of antibiotic exposure was absent. These mice were used only for breeding. Proponents that consider *C. difficile* a zoonotic pathogen propose that long-term exposure to the bacterium leads to eventual disease in the absence of a known inciting cause.[68] In humans, this continuous exposure can come from retail meats, grass lawns, and even pet dogs.[61,62,65,66] Even though the mice in this outbreak were housed in a different facility, the isolated *C. difficile* strain had at least a 91.2% proteome match with the strains from outbreak 1. We cannot rule out possible spread and maintenance of these strains between facilities, either by animal transfer or personnel. Because of its production of environmentally resilient spores, *C. difficile* can be difficult to eradicate from a facility.

We cannot ascertain the original source(s) of *C. difficile* associated with these outbreaks, because we do not survey for *C. difficile*. Although its presence in the microbiome of some or all of our colonies is unknown, the fact that no previous outbreaks have occurred suggests that if the organism has been present, it may have been a less virulent strain. Mice colonized with very low levels of the nonpathogenic Lem1 strain escaped detection by quantitative PCR analysis but could be detected by a more sensitive nested PCR assay.[22] This finding suggests that the bacteria could potentially be introduced through the importation of colonized mice, even if *C. difficile* screening with quantitative PCR testing was performed. The *C. difficile* strain responsible for the outbreak of diarrhea at the vendor's facility was from a distant MLST group as compared with our isolates and therefore was not the source of the bacteria causing our outbreaks.

Fecal–oral transmission of environmentally resilient spores, rather than of the more fragile vegetative state, is the primary mode of transmission of *C. difficile*. Shoes (worn by animal care or investigative staff), biofilms, human carriers (whether by serving as fomites or who are themselves infected carriers), and food or water are potential sources of the offending agent.[32,63] Although our food is γ-irradiated and even though we use reverse-osmosis–purified water, bacterial spores can still survive irradiation and pass through membranes in reverse-osmosis systems.[48,55] In addition, introduction of the bacterium through human xenografts cannot be ruled out. The building housing our vivarium is interconnected with a hospital, and staff with clinical responsibilities access the vivarium. The prevalence of ST1 in human clinical isolates from the associated hospital remained unchanged, as compared with prior years, nor were any hospital-acquired ST1 outbreaks detected during this period (data not shown). At least 6 y prior to the described outbreaks, an investigator had experimentally inoculated mice with CD 196, the strain most similar to our clinical isolates, in the same vivarium.[35] The limited genetic differences observed within and between our isolates and CD 196 could be explained by horizontal gene transfer via bacteriophages or homologous recombination through acquisition of DNA via a conjugation-like mechanism.[10,38] Although the studies with CD 196 were conducted at ABSL 2+, it is possible that spores from this strain contaminated the environment, were disseminated, and colonized mice within the facility. This lab has since moved to a different institution.

Treatment of CDI in humans depends on severity of infection and number of recurrences.[54,73] Physicians recommend vancomycin, fidaxomicin, or metronidazole for the treatment of moderate diarrhea, severe fulminant infections with or without ileus and megacolon, and first-time recurrences.[54] The focus of treatment is eradication of vegetative, toxin-producing forms of *C. difficile* rather than of highly resistant spores;[59] consequently recurrent CDI in humans after antimicrobial intervention is relatively high (20% to 30%).[17] Bezlotoxumab, a monoclonal antibody against toxin B that received FDA approval in 2016, can be used as an adjunct therapy in patients with a high risk of recurrence.[38] Surgical interventions (subtotal colectomy, diverting loop ileostomy) may be necessary for severely ill patients with megacolon.[54] After the outbreaks that we describe here, 10 NSG mice with first-time (nonrecurrent) CDI in which the *C. difficile* isolate was confirmed to be sensitive to vancomycin

were treated with vancomycin (50 mg/kg daily) in the drinking water indefinitely.[21] The dose was derived from earlier work describing vancomycin's protective effects against CDI-induced mortality in the C57BL/6 CDI mouse model, although relapse and death occurred after vancomycin was discontinued.[21] In our mice, vancomycin treatment reversed clinical signs within a few days of implementation and suppressed *C. difficile* shedding within 2 wk of initiating treatment; clinical recurrence was not noted prior to euthanasia at the experimental endpoint, which was approximately 40 d after therapy was initiated (data not shown). Further investigation is required to determine any consequences of vancomycin treatment and to confirm the observed therapeutic effectiveness of vancomycin in NSG mice with CDI-associated disease. FMT is now strongly recommended as a rescue treatment for patients experiencing more than 2 recurrences and are deemed refractory to traditional antimicrobial therapies.[5,12,38,54] FMT provides the patient with microbiota from a healthy individual to reestablish colonization resistance in both humans and mice.[12,23,52,71] We attempted to treat a few mice from outbreak 1 by using FMT but without success; however, we implemented treatment at a late stage when animals already exhibited severe clinical signs. The benefit of early FMT and its use after initial vancomycin therapy in mice with moderate to severe disease should be explored further in highly immunocompromised strains of mice with *C. difficile* enterotoxemia.

In human healthcare settings, a multifaceted approach is essential to control further infections during outbreaks.[49] Sporicidal products, such as sodium hypochlorite dilutions, and various phenol- or peroxide-based agents reduce—but not eliminate—the environmental load of viable spores.[49,54,63] Washing hands, wearing single-use personal protective equipment, and thorough wiping of surfaces can also physically remove spores.[54] Near the end of outbreak 1, we introduced the use hypochlorous acid (Clorox Healthcare Fuzion, Clorox Company, Oakland, CA) as a disinfectant in affected rooms. The effectiveness of such sporicidal agents on decreasing or eliminating the spore burden in a vivarium setting should be evaluated in the future. Although individual cases of *C. difficile* enterotoxemia still arise occasionally in our facilities, the number of CDI cases has decreased considerably since the implementation of oral vancomycin treatment, the use of sulfamethoxazole–trimethoprim-supplemented feed, and application of hypochlorous acid disinfectant in the affected rooms.

In conclusion, the administration of amoxicillin-containing feed to immunocompromised mice led to the development of diarrhea and a significant number of fatalities associated with toxigenic *C. difficile*; we also observed morbidity and mortality in experimentally naïve NOD-*scid* mice in association with the same organism. Whole-genome analysis of the offending isolates identified a hypervirulent strain. *C. difficile* disease may become more problematic in mouse colonies in association with the increased use of mouse strains with both severe innate and adaptive immune deficiencies. The use of these strains may dictate the need to exclude this agent from immunocompromised mouse colonies.

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