Case Study

Culture-independent Profiling of the Fecal Microbiome to Identify Microbial Species Associated with a Diarrheal Outbreak in Immunocompromised Mice

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Immunocompromised mice are used frequently in biomedical research, in part because they accommodate the engraftment and study of primary human cells within a mouse model; however, these animals are susceptible to opportunistic infections and require special husbandry considerations. In 2015, an outbreak marked by high morbidity but low mortality swept through a colony of immunocompromised mice; this outbreak rapidly affected 75% of the colony and ultimately required complete depopulation of the barrier suite. Conventional microbiologic and molecular diagnostics were unsuccessful in determining the cause; therefore, we explored culture-independent methods to broadly profile the microbial community in the feces of affected animals. This approach identified 4 bacterial taxa—*Candidatus Arthromitus*, *Clostridium celatum*, Clostridiales bacterium VE202-01, and *Bifidobacterium pseudolongum* strain PV8-2—that were significantly enriched in the affected mice. Based on these results, specific changes were made to the animal husbandry procedures for immunocompromised mice. This case report highlights the utility of culture-independent methods in laboratory animal diagnostics.

Abbreviations: ANOSIM, analysis of similarities; NSG mice, NOD-SCID γ mice; SFB, segmented filamentous bacteria

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Immunodeficient mice are ideal recipients of xenografts, including primary tissue from human patients, and thus provide a valuable platform for assessing cancer immunotherapeutics. Two commonly used mouse models for engraftment of human tissues are NOD-SCID γ mice (NSG; NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}/SzJ) and triple-transgenic NSG mice, which express human IL3, granulocyte-macrophage colony stimulating factor, and stem cell factor (NSGS; NOD.Cg-Prkdc^{scid}Il2rg^{tm1Wjl}Tg(CMV-IL3, CSF2, KITLG)1Eav/MloySzJ). These mice completely lack an adaptive immune system, including T, B, and natural killer cells, and exhibit a severe deficiency in cytokine signaling. Innate immune cells, including neutrophils, monocytes, macrophages, and dendritic cells, are present but are functionally impaired.³⁷ Although this level of immunodeficiency accommodates the engraftment of a wide range of primary human cells and enables modeling of human biology and disease, it also poses a major challenge for disease prevention in a laboratory animal setting. NSG and NSGS mice require special husbandry practices to limit their exposure to opportunistic pathogens,²⁵ including the use of autoclaved microisolation

or pressurized IVC, weekly cage changes, acidification of sterile water to pH 2.5 to 3.0 (to restrict *Pseudomonas* species),²⁴ and irradiated or autoclaved food. Despite these precautions, infections can still occur. The susceptibility of immunocompromised animals to a broad range of microbes that are not normally pathogenic to immunocompetent animals complicates surveillance and diagnostic methods. In this case study, we describe an infectious outbreak of diarrheal disease of unknown origin in an NSG–NSGS core facility that resulted in the euthanasia of more than 2000 mice and the complete shutdown and decontamination of a barrier suite. Conventional microbiologic and molecular diagnostic methods were unsuccessful in identifying potential causes of the outbreak, thereby limiting proactive measures to reduce chances of future outbreaks.

Advances in high-throughput sequencing technology, together with the development of multiplex protocols for large-scale marker-gene-based studies,^{7,20} have revolutionized microbiology, allowing scientists to complement culture-based approaches with culture-independent profiling of complex microbial communities (that is, microbiomes). We hypothesized that profiling the fecal microbiomes of diseased and control mice would provide insight into microbes associated with this costly outbreak. 16S rRNA gene sequencing and shotgun metagenomics were used to identify suspect bacteria. This report is the first description of microbiome sequencing used to identify organisms associated with an outbreak in a laboratory animal facility,

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and the results helped to guide the decontamination protocol and subsequent husbandry practices.

Case Report

Approximately 2000 NSG and NSGS mice were housed in a suite within a barrier facility. The suite consisted of 6 rooms, with the NSG and NSGS mice occupying 4 of the 6 rooms. One room was a breeding room, where mice were housed in semi-rigid flex-front isolators (Park Bioservices, Groveland, MA). The suite included an 'engraftment room,' where mice initially were transferred after weaning but before study and engraftment. In addition, the suite contained 2 multiuser housing rooms, one of which contained an in vivo imaging system (IVIS Spectrum, PerkinElmer, Akron, OH) dedicated to immunodeficient mice. The suite also had a shared procedure room and an ABSL2 room that housed immunocompetent mice. Any immunodeficient mouse that went to the procedure room could not return to housing. The breeding room and engraftment rooms were security-restricted to animal care staff and 3 core employees, whereas a maximum of 45 users had free access to the housing rooms. The entry order for animal care personnel was the NSG breeding room, then the engraftment room, followed by the multiuser rooms, and finally the ABSL2 room. Required personal protective equipment comprised shoe covers, gown, bonnet, and gloves, which were donned prior to entering the suite. Once personnel were inside the suite, Tyvek sleeves (VWR, Radnor, PA) and an additional pair of gloves were required. Clidox-S (Pharmaceutical, Naugatuck, CT), prepared at a 1:18:1 concentration, was used as the disinfectant between mouse cages, on gloves, and for cleaning of imaging equipment and isoflurane boxes.

In July 2015, an animal care technician noticed diarrhea in several cages in one of the multiuser rooms. Over the course of the next few weeks, diarrhea spread throughout the NSG and NSGS rooms in the suite. The diarrhea was first noted in 2 cages of mice in a multiuser housing room. Although these mice were euthanized, animals in several other cages in the same room started having diarrhea. Within 2 wk of the first cases, dozens of new cages were affected daily in both of the multiuser housing rooms. Initially, we planned to cull mice under study and preserve the breeder rooms (who were under higher barrier protection); however, despite a strict entry order, restricted personnel access, and the use of individual semirigid isolator units, diarrhea was noted in the breeder room on 22 July 2015. The outbreak was marked by high morbidity, but low mortality, with more than 75% of the colony affected but fewer than 12 adult mice succumbing to disease. Due to the spread of diarrhea and the likely confounding effects on research, the entire suite, consisting of approximately 2000 NSG and NSGS mice in total, was depopulated by 4 September 2015. Mice in the ABSL2 room, which housed immunocompetent animals, did not show signs of disease and were relocated to another vivarium. Attention was focused on decontaminating the suite and identifying organisms associated with the outbreak, to ensure that appropriate preventative measures were in place when the suite was repopulated. Traditional diagnostic methods, including histopathology, PCR analysis of infectious agents, and culturing, were unsuccessful in identifying the cause of the outbreak. Positive identification of the bacteria through microbiome analysis was sought after these other methods were exhausted with no pathogen identified. No mice remained in the suite after depopulation, but intestinal tissue and fecal samples recovered at necropsy were used for histology and microbiome analysis, respectively, to identify a potential cause of the outbreak.

Materials and Methods

Animal care and infection control. The NSG and NSGS mice initially were obtained from Jackson Laboratories (Bar Harbor, ME). The outbreak encompassed breeder mice from Jackson, as well as mice bred inhouse for experiments (1 or 2 generations of breeding). Clidox-S (Pharmaceutical, Naugatuck, CT) prepared at a 1:18:1 concentration was used to disinfect equipment. All mice in housing rooms were kept in autoclaved IVC (Allentown Caging, Allentown, NJ) and fed an irradiated diet (LabDiet 5058, Purina, St Louis, MO), with unrestricted access to autoclaved water acidified to pH 2.5 to 3.0. IACUC guidelines allow as many as 5 adult mice per cage. Mice were housed on autoclaved 1/4-in. corn cob bedding (Animal Specialties and Provisions, Quakertown, PA). All singly housed mice received either an autoclaved cotton square (Animal Specialties and Provisions) or other autoclaved forms of enrichment unless an exemption was granted by the IACUC. Cages were checked daily and typically were changed once weekly. Facility temperatures were maintained at 22.2 ± 1.1 °C (72 ± 2 °F); the humidity was between 30% and 70%, with 10 to 15 air changes hourly. Each facility had 24-h environmental monitoring, and notifications of deviations were sent automatically to facility managers or onsite staff.

During each of 3 quarters, sentinel mice were examined on-site for fur mites and pinworms, and serology (ELISA) was performed by using antigens for mouse hepatitis virus, epizootic diarrhea of infant mice virus, Theiler virus, mouse minute virus, and mouse parvovirus. Antigen-coated plates were purchased from Charles River Laboratories (Wilmington, MA). Confirmatory testing, when required, was performed by indirect immunofluorescence using antigens prepared internally or provided by Dr. Susan Compton (Yale University, New Haven, CT). Annually, live sentinel mice were shipped to Charles River Laboratories for more comprehensive monitoring. All sentinel mice were tested by using the HM Plus profile (serology for 23 viral and bacterial agents; upper respiratory and gastrointestinal tract cultures; endo- and ectoparasites; and gross necropsy), and lymph nodes from sentinel mice housed in barrier facilities were tested by PCR assay for mouse parvoviral DNA. The NSG breeding room did not contain any sentinels. Instead, randomly chosen NSG breeding colony mice were collected on a quarterly basis, and heart blood, swabs from ears, eyes and peritoneal cavities, as well as liver and kidney samples were cultured for aerobic bacteria. The University of Pennsylvania does not exclude murine norovirus or *Helicobacter* spp. from its mouse housing facilities; in fact, both organisms are present enzootically. All mice were housed and maintained in accordance with the guidelines of the University of Pennsylvania IACUC.

Histopathology. Mice were euthanized by CO₂ inhalation. A complete necropsy was performed, and lungs, heart, spleen, liver, gastrointestinal tract, kidneys, lymph nodes, adrenal glands, reproductive organs, and brain were obtained. All tissues for histopathology were fixed in 10% neutral buffered formalin (Thermo Fisher Scientific, Waltham, MA) for at least 72 h prior to paraffin embedding, and sections were subsequently stained with hematoxylin and eosin and Gram stain by the Histology Laboratory of the Veterinary Hospital of the University of Pennsylvania (Philadelphia, PA). All slides were evaluated by a board-certified veterinary pathologist (AKB).

Conventional diagnostics. Aerobic, anaerobic, *Salmonella*, and *Campylobacter* cultures were performed in the clinical microbiology laboratory at PennVet by using prepared media (Remel, Lenexa, KS). All cultures were performed under appropriate atmospheric conditions (Mitsubishi Gas Chemical, New York, NY) and temperatures. Briefly, feces were first cultured in

thioglycollate, anaerobic thioglycollate, GN broth, and *Campylobacter*–thioglycollate broth for approximately 18 h. Samples were streaked on blood agar, MacConkey agar, and Columbia CNA agar for aerobic culture; *Brucella* and CDC PEA agar for anaerobic culture; XLD and MacConkey agar for *Salmonella* culture; and *Campylobacter* blood agar for that organism. Isolated organisms were identified by manufacturer protocols by using MALDI-TOF analysis (Bruker, Billerica, MA). Molecular detection of pathogens was performed by using the manufacturer's protocol for a multiplex gastrointestinal pathogen panel (Luminex, Austin, TX). DNA was extracted by using the QIAmp Powerfecal DNA Kit (Qiagen, Hilden, Germany), and PCR assays for markers for enteroaggregative and enteropathogenic strains of *E. coli* were performed at the *E. coli* Reference Laboratory at Pennsylvania State University. Fecal samples were sent to Charles River Research Animal Diagnostic Services (Wilmington, MA) to undergo testing in an infectious disease PCR panel that included lymphocytic choriomeningitis, mouse adenovirus (types 1 and 2), mouse hepatitis virus, murine norovirus, mousepox virus, mouse parvovirus, murine roseolovirus, reovirus, Theiler murine encephalomyelitis strain GDVII, β *Streptococcus* spp. (groups B, C, and G), *Bordetella hinzii*, *Campylobacter*, *Citrobacter rodentium*, *Clostridium piliforme*, *Corynebacterium bovis*, *Corynebacterium kutscheri*, *Helicobacter* spp., *Klebsiella oxytoca*, *K. pneumoniae*, *Pasteurella pneumotropica* Heyl, *P. pneumotropica* Jawetz, *Proteus mirabilis*, *Salmonella* spp., *Staphylococcus aureus*, *Moniliformis moniliformis*, *Streptococcus pneumoniae*, *Cryptosporidium* spp., *Entamoeba* spp., *Giardia* spp., and *Spironucleus muris*.

16S rRNA gene and metagenomic profiling. Fecal samples from 8 affected NSG mice from separate cages in an affected user room (6 male, 2 female; age, 3 to 6 mo) and 10 healthy NSG control mice (obtained after the outbreak from separate cages) were stored at -80°C . The affected mice were progeny from NSG mice obtained from Jackson Laboratories, whereas the healthy mice were new breeding stock (5 male, and 5 female; age, 3 mo) obtained from Jackson Laboratories and acclimated to the facility. Genomic DNA was extracted from feces by using the PowerSoil DNA Isolation Kit (MO BIO Laboratories, Carlsbad, CA) according to the manufacturer's recommendations. A mock community pool containing purified genomic DNA from 12 known bacterial isolates was amplified and sequenced as a quality control. Additional controls included extraction of blank-processed samples (in which the DNA extraction process was followed without the addition of input material), and water only, to determine background microbial signal.³⁴ A dual-index amplicon sequencing method was used for PCR amplification of the V4 region of the 16S rRNA gene.²⁰ Amplicons were sequenced on a MiSeq platform (Illumina, San Diego, CA) using 250 base-pair paired-end chemistry at the University of Pennsylvania Next Generation Sequencing Core; this process generated 508,747 sequences after quality filtering and trimming. Sequencing depth ranged from 11,456 to 58,163 (median, 24,061) reads per sample. Reads were filtered to remove sequences with a Q-score lower than 30. Homopolymers longer than 10 bp and sequences shorter than 248 bp or longer than 255 bp were removed by using mothur.³⁵ Data were rarefied to 11,000 reads per sample. Quantitative Insights into Microbial Ecology (QIIME, version 1.8)⁶ was used to process the sequence data and open-reference operational taxonomic units were picked using UCLUST.⁹ The reads were aligned to the Greengenes database (release 13_8)²³ by using PyNast.⁵ α - and β -diversity metrics were calculated for the fecal microbiome in affected and control mice.

For metagenomic profiling, 1 ng of fecal DNA from the same extracts used for 16S rRNA sequencing, along with the technical controls described earlier, was processed by using the NexteraXT Library Preparation Kit (Illumina) according to the manufacturer's instructions. Libraries were sequenced by using 150 base-pair single-end chemistry on an Illumina NextSeq 500 (Illumina) to generate 13,880,440 to 103,818,258 reads (median, 17,187,034 reads). Reads were trimmed and filtered by using Trimmomatic⁴ and subsequently analyzed by using the CosmosID cloud computing resource (CosmosID, Rockville, MD) described elsewhere^{14,21,29,31} to achieve rapid identification at the species, subspecies, or strain level. The Galaxy implementation¹ of LefSe³⁶ was used to identify differentially abundant taxa between affected and control samples.

Data Accessibility. The 16S rRNA sequencing data and meta-data generated in this study are available through the NCBI Sequence Read Archive (SRA; <http://www.ncbi.nlm.nih.gov/sra/>) under the SRA study accession number SRP118081 and BioProject accession PRJNA407772.

Statistics. *P* values for the 16S rRNA gene sequencing were determined by using the Wilcoxon rank-sum test. The QIIME implementation of Analysis of Similarities (ANOSIM) was used to measure determine differences in microbial community diversity between groups. The R package MetagenomeSeq was used to normalize the metagenomics data.³⁰ *P* values for normalized metagenomics data were determined using the Student *t* test. All *P* values were adjusted for multiple comparisons by using the Benjamini–Hochberg false-discovery rate. All statistical tests were run in R (version 3.3.1).³²

Results

Intestinal pathology and gram-positive bacteria in affected mice. The first 3 mice to present with diarrhea were euthanized, and necropsy showed a poor body condition score (2 on a scale of 5), ruffled fur, severely dilated ceca, and intestines with liquid feces. Hepatomegaly and splenomegaly were present in 2 of the 3 mice. Histology revealed long segmented, filamentous bacteria (Figure 1 A) attached to the mucosa of the small intestine (Figure 1 B, arrow), with mild to moderate enterocyte necrosis and sloughing. In addition, abundant bacterial rods were attached to the mucosal surface of the cecum and colon (Figure 1 C, arrow). Gram staining of fecal smears from affected mice showed gram-positive rods with a large centralized spore (Figure 1 D, arrows).

Results of conventional diagnostics. Despite histologic evidence of a bacterial infection (top differentials being a *Clostridia* species or *Escherichia coli*), aerobic and anaerobic microbiologic cultures were inconclusive. Standard aerobic and anaerobic culture of fecal material from multiple affected mice yielded only growth of *Lactobacillus johnsonii*, *Enterococcus* spp., and a coagulase-negative *Staphylococcus*, none of which were suspected to be the causative bacteria. There was no growth of aerobic gram-negative rods, and specific cultures for *Salmonella* and *Campylobacter* spp. were negative as well. In addition, targeted molecular assays were performed, including a gastrointestinal panel that was negative for *Salmonella* spp., *E. coli* (O157:H7), *Campylobacter* spp., *Giardia* spp., *Cryptosporidium* spp., *Clostridium difficile* toxin gene, and *C. perfringens* toxin gene. In light of the histologic evidence of microbial adherence to the epithelium of affected mice (Figure 1 A through C), DNA extracted from feces was PCR-tested for enteroaggregative and enteropathogenic *E. coli* but yielded negative results. Finally, feces from affected mice was submitted to Charles River Research Animal

Figure 1. Intestinal pathology and gram-positive bacteria evident in affected mice. (A through C) Formalin-fixed, hematoxylin-and-eosin-stained sections from affected mice. (A and B) Villi of the small intestine with long, segmented bacteria attached to the epithelium (B, arrow). (C) Abundant bacterial rods (arrow) attached to the mucosal surface of the large intestine. (D) Gram stain of fecal smear from affected mice showing gram-positive bacteria with large, centralized spore (arrows). Bar: 20 μ M (A through C), 200 μ M (D).

Diagnostic Services for an infectious disease PCR panel and was negative for all agents (see Methods).

Gut microbiome of outbreak-affected mice. Given the lack of conclusive results from culture- and PCR-based diagnostics, we pursued culture-independent profiling of the fecal microbiome. Diversity and composition of the fecal microbiome was determined by targeted sequencing of the V4 region of the 16S rRNA gene from control and affected animals. α -diversity was calculated by using the Shannon index (Figure 2 A), which showed that control and affected mice had similar community diversity in the feces ($P = 0.4598$). In contrast, the 2 groups showed a clear separation by Weighted UniFrac analysis²² (Figure 2 B), indicating that the outbreak resulted in a marked shift in the composition of the gut community (ANOSIM, $P = 0.001$). Compared with control samples, feces from affected mice showed an increase in the relative abundance of *Candidatus Arthromitus* (segmented filamentous bacteria, SFB), unclassified *Clostridiaceae*, *Turicibacter*, and *Bifidobacterium* (Table 1 and Figure 2 C) and a decrease in *Lactobacillus* relative abundance. In control mice, *Candidatus Arthromitus* and *Turicibacter* were detected in feces at very low relative abundance; in contrast, the median relative abundance of these taxa were 8.5% ($P < 0.001$) and 7.6% ($P = 0.01$), respectively, in affected mice (Table 1 and Figure 2 C). Similarly, *Bifidobacterium* increased from undetectable levels in

control animals to 5.3% median relative abundance in affected mice ($P = 0.001$). Unclassified members of the *Clostridiaceae* family increased from 0.06% in controls to 3.7% in the affected mice ($P = 0.001$). Median *Lactobacillus* levels decreased from 11.2% in controls to 4.5% in affected animals ($P = 0.03$). Due to the short length of the V4 region, taxonomy assignment beyond the genus level was not possible. Taken together, these data show that disease was associated with marked changes in the relative abundance of specific taxa, rather than a more general loss of diversity, as has been reported to occur during chronic intestinal inflammatory diseases.²⁸

Metagenomic sequencing of outbreak-associated taxa at strain-level resolution. We next sought to obtain a higher-resolution view of the bacterial species and strains associated with this outbreak and to ask whether viral or protozoal pathogens were associated with this outbreak. The same fecal samples as used for 16S analysis were used for ‘shotgun’ metagenomic sequencing. After sequencing, data were trimmed and annotated with CosmosID, and the sequence read counts were normalized by using MetagenomeSeq.³⁰ Control and affected animals showed no differences with regard to protists, viruses, fungi, and bacterial virulence factors (data not shown). Similar to our 16S rRNA gene sequencing results (Figure 2), metagenomics analysis identified *Candidatus Arthromitus* SFB and *Bifidobacterium*

Figure 2. 16S rRNA gene sequencing reveals dysbiosis in outbreak-affected mice. (A) Box-and-whisker plot showing the Shannon α diversity index for control (blue triangles) and affected (red circles) mice. (B) β -diversity analysis using weighted UniFrac distance to compare control (blue triangles) and affected (red circles) mice. Data were analyzed by using ANOSIM ($R = 0.51$, $P = 0.001$). (C) Stacked bar charts showing the relative abundance of the top 10 most-abundant taxa in the feces of control and affected mice. Taxa significantly enriched in affected and control mice are indicated with + and x, respectively.

Table 1. Bacterial taxa enriched in feces from control and affected mice, as assessed by 16S V4 rRNA sequencing

	Control mice		Affected mice		<i>P</i> ^a
	Range	Median	Range	Median	
Enriched in affected mice					
<i>Candidatus Arthromitus</i>	0% to 0.0009%	0%	3.6% to 20.0%	8.5%	0.0009
Unclassified <i>Clostridiaceae</i> spp.	0.009% to 0.9%	0.06%	0.65% to 17.5%	3.7%	0.001
<i>Turicibacter</i> spp.	0% to 10.3%	0.02%	0.45% to 10.2%	7.6%	0.01
<i>Bifidobacterium</i> spp.	0% to 0%	0%	0% to 12.7%	5.3%	0.001
Enriched in control mice					
<i>Lactobacillus</i>	1.2% to 15.6%	11.2%	0.1% to 9.9%	4.5%	0.03

^aAdjusted for false-discovery rate

pseudolongum strain PV8-2 as the most prevalent organisms in affected mice, compared with controls ($P < 0.01$; Figure 3 A and B, respectively). In addition, *Clostridium celatum* DSM 1785 and *Clostridiales bacterium* VE202-01, although present only at very low relative abundance, were significantly enriched in affected mice ($P < 0.01$, Figure 3 C and D, respectively).

Discussion

In this case study, profiling of the fecal microbiome was used to investigate putative causes of a costly diarrheal outbreak in immunocompromised mice. This approach identified several species that were enriched in affected animals compared with healthy controls including: *Candidatus Arthromitus* (SFB),

Bifidobacterium pseudolongum strain PV8-2, *Turicibacter* spp., and *Clostridium celatum*. Members of the *Bifidobacterium* genus are well-known commensals and among the first organisms to colonize the neonatal gut.⁴² The use of *B. pseudolongum* in probiotics and its ability to limit gut inflammation in a mouse model of colitis¹³ argue against this organism being a disease-promoting agent in this outbreak. Similarly, SFB dominate the terminal ileum of recently weaned mice but recede to low levels in adults.^{3,10} Consistent with our histology results (Figure 1 A and B), SFB are known to attach directly to the intestinal epithelium, which induces the development of Th17 cells,¹⁶ production of IgA,^{19,41} and modulation of gut immunity.¹¹ The striking increase in the relative abundance of SFB in the feces of affected mice,

Figure 3. Metagenomic sequencing identifies bacterial strains associated with the outbreak. Relative abundance (% abund.) of (A) *Candidatus Arthromitus*/SFB, (B) *Bifidobacterium pseudolongum* PV8-2, (C) *Clostridium celatum* DSM 1785, and (D) *Clostridiales* bacterium VE202-01 in feces from affected and control mice. †, Significant ($P < 0.01$) difference between control and affected mice as determined by Student *t* test after adjusting for multiple testing.

compared with controls, might be explained by either a bloom of this organism in the intestine or by increased sloughing of the intestinal mucosa during diarrheal disease. Although SFB are regarded as commensal in mice,¹⁰ these organisms induce colitis in a SCID mouse model when SFB are administered as part of a consortium of bacteria, whereas the same consortium without SFB, or SFB on its own, failed to induce colitis.³⁹ Taken together, these data raise that possibility that SFB acted in concert with at least one other bacterial species or product to induce inflammation in NSG/NSGS mice.

Both 16S rRNA gene sequencing and metagenomic sequencing identified an increase in the relative abundance of *Clostridia* in affected mice, although levels were extremely low relative to SFB and *B. pseudolongum*. This result may reflect genuinely low relative levels of *Clostridia*, or it could be a consequence of incomplete disruption of clostridial spores (Figure 1 D, arrows) during DNA extraction. The *Clostridium* genus exhibits extensive genetic diversity,¹⁸ and its members are fastidious anaerobes⁴⁰ that are challenging to identify by culture-based methods. *C. celatum* and *Clostridiales* bacterium VE202-01 are poorly studied clostridial species and therefore would not have been detected on the initial clinical diagnostics. *Clostridiales* bacterium VE202-01 was one of 17 bacterial strains isolated from healthy human feces and was shown to elicit CD4⁺FOXP3⁺ regulatory T cells and attenuate disease in a mouse model of colitis, providing no evidence for a pathogenic role of this organism. *C.*

celatum was originally isolated from human feces¹⁵ and recently was implicated in 2 serious human postoperative infections.² Perhaps even in low abundance, *C. celatum* may have triggered diarrheal disease in immunocompromised mice.

Several factors inherent in microbiome studies necessitate caution when interpreting our results. First, although dysbiosis was apparent in all affected mice, and changes in community membership were dominated by a few bacterial taxa, additional mechanistic studies would be required before a causal role could be determined. Second, sequence-based identification methods do not distinguish live and dead organisms. Third, it is also possible that no single organism was responsible for the outbreak, but that multiple changes in the microbial community resulted in disease—a concept that has prompted several groups to suggest a reinterpretation of Koch's postulates for microbiome studies.^{26,38} Finally, since control animals were newly acquired and acclimated to the facility after the outbreak, we cannot rule out the possibility that organisms observed in affected mice were present in the facility prior to the outbreak and therefore not associated with disease.

Given the observation of increased numbers of bacteria with spore-forming potential in the fecal smears and microbiome from affected animals, several husbandry changes were made to prevent future outbreaks of this nature. The entire suite was decontaminated with chlorine dioxide gas to help eliminate bacterial spores, and a custom chlorine-dioxide chamber (DRS

Laboratories, Lehigh Valley, PA) was designed that sterilizes all nonautoclavable items entering the suite. No outside materials are permitted to enter the immunodeficient mouse suite unless they have undergone sterilization in this chamber (live cells being the exception, in a sanitizable container and disinfected on entry). The surface disinfectant was changed from Clidox-S to Oxivir-Tb (Diversey, Charlotte, NC), a H₂O₂-based cleaner with sporicidal efficacy. Food was changed from irradiated food that was distributed from a common container by using a scoop, to a high-fat, autoclavable diet (LabDiet 5V0G, Purina) portioned out directly to the cage prior to autoclaving, which then avoided a potential fomite source. The decision to change food was based upon the concern that there is some evidence that irradiated food can carry pathogens.³³ A Virkon sticky mat (VWR) was placed at the entrance to the suite, rooms were limited to specific personnel, and the entire suite was converted to housing immunocompromised mice exclusively. Since implementing these changes, no diarrheal disease or opportunistic bacterial infections have affected the immunodeficient colony.

Microbiologic and serologic assays of small numbers of sentinel mice have long been a field standard for monitoring the overall health of a mouse room and to identify specific pathogens in mouse facilities.²⁷ In the current study, we showed that profiling the microbiome can be used in the face of an outbreak to identify potential pathogens. Future directions should include investigating this tool as a culture-independent assay to complement sentinel programs. Banking and storing fecal samples on a routine basis may be advantageous to facilitate tracking the microbiome status of immunocompromised mice in the event of gastrointestinal symptoms. The scalability and relative low cost of 16S rRNA gene-based surveys makes this method broadly applicable, even for large laboratory animal operations. The recent discovery of inexpensive, rapid, and scalable DNA extraction and 'direct PCR' methods for microbiome studies will help to make large surveillance studies more feasible.^{43,44} Moreover, portable sequencing technologies¹⁷ together with cloud-based analytics,¹² such as CosmosID which we used in this study, would provide near real-time monitoring of infectious disease, potentially curtailing infections before they spread throughout a facility.

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