The Journal of Infectious Diseases

MAJOR ARTICLE

Efficacy of Omadacycline or Vancomycin Combined With Germinants for Preventing *Clostridioides difficile* Relapse in a Murine Model

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Background. *Clostridioides difficile* infections (CDI) and recurrence (rCDI) are major health care burdens. Recurrence is likely caused by spores in the gastrointestinal tract that germinate after antibiotic therapy. This murine study explores germinant-antibiotic combinations for CDI.

Methods. Previously described murine models were evaluated using *C. difficile* VPI 10463. The severe model compared omadacycline versus vancomycin in survival, weight loss, clinical scoring, and *C. difficile* toxin production. The nonsevere model compared these antibiotics with and without germinants (solution of sodium taurocholate, taurine, sodium docusate, calcium gluconate). Additionally, colon histopathology, bile acid analysis, environmental/spore shedding, and 16S sequencing was evaluated.

Results. In the severe model, omadacycline-treated mice had 60% survival versus 13.3% with vancomycin (hazard ratio [HR], 0.327; 95% confidence interval [CI],.126–.848; $P = .015$) along with decreased weight loss, and disease severity. In the nonsevere model, all mice survived with antibiotic-germinant treatment versus 60% antibiotics alone (HR, 0.109; 95% CI, .02–.410; $P = .001$). Omadacycline resulted in less changes in bile acids and microbiota composition. Germinant-treated mice showed no signs of rCDI, spore shedding, or significant toxin production at 15 days.

Conclusions. In murine models of CDI, omadacycline improved survival versus vancomycin. Germinant-antibiotic combinations were more effective at preventing rCDI compared to antibiotics alone without inducing toxin production.

Keywords. CDI; animal; germination; recurrence; treatment.

Clostridioides difficile infections (CDI) are caused by an anaerobic, spore- and toxin-forming, gram-positive pathogen identified as 1 of 5 urgent threats by the Centers for Disease Control and Prevention [1]. It is the most commonly identified hospital-acquired infection in the United States, costing an estimated $1 billion in hospital-onset cases alone in 2017 [2–4]. Antibiotics are a major risk factor for developing CDI and recurrent CDI (rCDI) because they promote dysbiosis and reduce colonization resistance. For *C. difficile,* colonization resistance involves resource competition and bile acid profiles inhibitory to *C. difficile* growth [5]. Antibiotics that treat CDI, such as oral vancomycin (VAN), are effective at killing the vegetative form and mitigating clinical symptoms. However, they do not affect spores and can reduce colonization resistance, creating a favorable rCDI environment. Spores are impervious to antibiotics and conventional cleaning methods, and they significantly contribute to patient-to-patient transmission and rCDI [4, 6]. Even after successful treatment, up to 56% of patients asymptomatically shed spores into the environment within 4 weeks after CDI directed antibiotic therapy [6].

A major mechanism of rCDI is thought to be a gastrointestinal spore reservoir that germinates after CDI-directed antibiotic therapy prior to reestablishment of colonization resistance [7–9]. Spore germination into vegetative cells is controlled by germinants, such as taurocholate, and cogerminants, including divalent cations and amino acids [10, 11]. We hypothesized combining germinants with antibiotic therapy will germinate spores into vegetative cells, increasing antibiotic efficacy and reducing the potential reservoir for rCDI. In this study, we evaluated the efficacy of germinant administration combined with 2 antibiotics possessing in vitro and in vivo activity against *C. difficile*, omadacycline (OMC) or VAN, in rCDI murine models [12].

Received 10 April 2022; editorial decision 20 July 2022; accepted 27 July 2022; published online 29 July 2022

Presented in part. IDWeek 2021, virtual conference, 29 September to 3 October 2021, poster No. 1037.

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The Journal of Infectious Diseases® 2023;227:622–30

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https://doi.org/10.1093/infdis/jiac324

METHODS

Bacterial Strains, Antibiotics, and Chemicals

C. difficile VPI 10463 (ATCC43255) was grown and used to inoculate mice as specified below. Colistin sulfate, metronidazole, gentamicin sulfate, clindamycin hydrochloride, sodium taurocholate, and docusate sodium were purchased from Sigma-Aldrich. Taurine and kanamycin were purchased from Research Products International. Clinical powders of VAN and OMC were purchased commercially. Calcium gluconate for injection was obtained from Fresenius Kabi.

In Vitro *C. difficile* Growth Conditions and Preparation

All *C. difficile* laboratory growth took place within a type C vinyl anaerobic chamber (Coy Laboratory Products) using a gas mixture of 10% hydrogen and 10% carbon dioxide balanced with nitrogen. All media were reduced for 24 hours before use and all incubations occurred at 37°C. For the spore gavage on day 0, VPI 10463 was plated onto *C. difficile* brucella agar (CDBA) and incubated anaerobically for 24 hours [13]. A colony was then suspended into 140 mL of brain heart infusion broth (BHI; Becton, Dickinson). Incubation lasted 96 hours, being brought to 23°C for 1 hour every 24 hours to induce sporulation. After incubation, 70 mL of inoculum was heat shocked at 60°C for 20 minutes and the other 70 mL was pelleted and resuspended in a 70:30 ethanol to 1 × phosphate buffered saline (PBS) mixture for 15 minutes. Inocula were centrifuged, decanted, and all spore pellets were resuspended in 28 mL of heat-shocked supernatant. All centrifugations to pellet spores occurred at 3000*g* for 10 minutes. This spore mixture was adjusted with 1 × PBS to a concentration of 5×10^5 colony-forming units/mL, which was aliquoted and frozen at −80°C until use. Before each spore gavage, an aliquot of the mixture was diluted and plated onto CDBA to ensure no loss of spores.

Animals

All experiments were approved by the University of Wisconsin Madison's Institutional Animal Care and Use Committee. Male C57BL/6 mice aged 5–8 weeks were purchased from Jackson Laboratory (Bar Harbor, ME) and housed in groups of 5 or 3 with sterile bedding and water. Mice were fed irradiated Teklad Global 16% protein rodent diets (catalog No. 2916) throughout the experiment.

CDI Murine Models

This study evaluated antibiotics and germinants plus antibiotics in CDI models as previously described and detailed in Figure 1 [14]. Both models began with antibiotics in drinking water (kanamycin 0.4 mg/mL, gentamicin 0.035 mg/mL, colistin 850 U/mL, metronidazole 0.215 mg/mL, and VAN 0.045 mg/mL) from day −6 to day −3, 72 hours in total, and then switched to distilled water for the remainder of the experiment [14]. Mice were given a weight-based intraperitoneal (IP) clindamycin injection of 10 mg/kg on day −1 and 200 µL of the above spore mixture, totaling 1×10^5 spores, was administered via oral gavage on day 0.

In the severe model (Figure 1*A*), mice were treated with OMC (n = 10) or VAN (n = 15) starting 30 hours after spore gavage and then once every 24 hours until day 5 to assess whether OMC could treat CDI. Another group was given no CDI treatment as a positive control (infected untreated, n = 10). Lastly, a group was given antibiotic water and IP clindamycin, but no spore gavage or CDI treatment, as a negative control (uninfected untreated, n = 5).

In the nonsevere model (Figure 1*B*), all CDI treatment groups were given OMC or VAN 6 hours after spore gavage on day 0. Half the mice were then treated with either once daily OMC or VAN until day 4 (OMC, n = 13 and VAN, n = 13). The other half received antibiotics with germinants (OMC+G, n = 13 or VAN+G, n = 13) once daily on days 1–3 followed by antibiotics only on day 4. This created 4 CDI treatment groups: OMC, VAN, OMC+G, and VAN+G. In addition, 5 infected untreated mice were used to ensure the same disease course as the severe model. No uninfected untreated mice were used in the nonsevere model. Relapse was induced in the nonsevere model by administering 10 mg/kg IP clindamycin on days 10, 11, and 12. It was estimated from previous in vitro studies of antibiotic with and without germinants that a minimum of 7 mice would be needed per group to detect a statistically significant difference with an effect size of 0.8 for the mortality outcome (α level $P = .05$, power = 90%) [12].

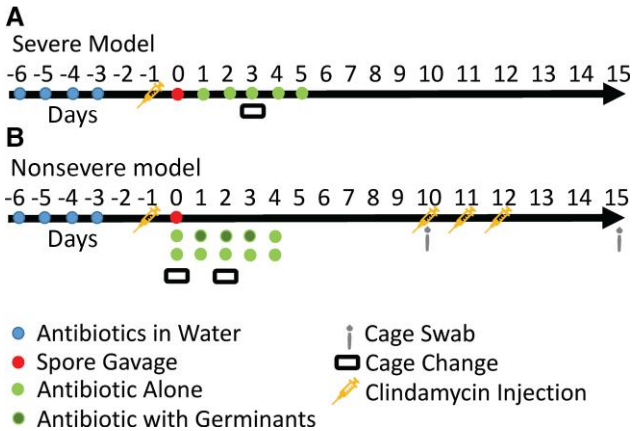

Figure 1. Model timelines. *A*, Severe model; treatments started 30 hours after spore gavage. Treatment groups were omadacycline (OMC), vancomycin (VAN), uninfected untreated, and infected untreated. *B*, Nonsevere model; treatment started 6 hours after spore gavage. Treatment groups were OMC and germinants (OMC+G), VAN+G, OMC, VAN, and infected untreated. OMC+G and VAN+G both received antibiotics only on days 0 and 5 and concomitant germinants were given on days 1, 2, and 3.

CDI Treatments

Mice in the VAN and VAN+G groups received VAN 1.5 mg while the OMC and OMC+G mice received OMC 0.25 mg daily during the CDI phase. The germinant groups also received 8 mg of sodium taurocholate, 10 mg of taurine, 0.2 mg of sodium docusate, and 1.72 mg of calcium gluconate given concomitantly with antibiotics on the specified days. All mice received 200 μL of sterile water containing antibiotics, antibiotics combined with germinants, or sterile water alone for daily treatments.

Cage Changes

Complete cage changes, including water, food, and bedding, occurred in all groups of the severe model on day 3 and nonsevere model on day 2 to remove environmental contamination and prevent coprophagy of infectious stool. Therefore, any CDI relapses are likely attributed to an internal *C. difficile* and/or spore reservoir. Complete cage changes also occurred in the nonsevere model on day 0, immediately after spore gavage.

Clinical Scoring, Weight Loss, and Survival

Clinical scoring comprised 6 categories with higher scores corresponding to more severe disease, as done previously, with an added score of 4 for mice too sick to provide fecal samples, as in Supplementary Table 1 [15]. Mice with clinical scores of ≥14 were considered to have reached a humane clinical end point and were subsequently euthanized by CO_2 asphyxiation. Mice found dead by researchers were given a score of 20 on the day of mortality. Clinical scoring and weight loss were recorded throughout treatment.

Colon Histopathology

Three mice from OMC, VAN, OMC+G, and VAN+G in the nonsevere model, and 2 uninfected untreated mice from the severe model were sacrificed on day 5 to obtain colons for histopathology. In addition, 3 mice in the infected untreated group that perished before day 5 were also harvested. The middle third of the colon was removed, flushed with 1 × PBS, and preserved. A board-certified veterinary pathologist assessed edema, cellular infiltration, and epithelial damage, as done previously [16]. The pathologist was blinded to all groups except the uninfected untreated and infected untreated groups. Scores ranged from 0–4 for all categories with higher scores representing more severe disease, as in Supplementary Table 2.

Toxin Quantification

Fecal *C. difficile* toxins A and B were quantified separately by a *C. difficile* toxin A or B Quanti kit (tgcBiomics) using sandwich enzyme-linked immunoassay according to the manufacturer's instructions. In the severe model, 3 mice in the OMC and VAN groups with individual fecal samples available on both days 1 and 2 were quantified. For the nonsevere model, individual fecal samples were chosen from 5 mice with median amounts of weight loss in OMC, VAN, OMC+G, and VAN+G on specified days. After quantification fecal pellets were washed twice in 1 × PBS and cultured for spore presence as described below.

Bile Acid Quantification in the Nonsevere Model

Individual fecal bile acids were quantified by the University of Michigan Core Facilities using negative liquid chromatography-electrospray ionization-triple quadrupole mass spectrometry. Two samples on day −6 and day 0 were evaluated. In addition, 2 samples from mice with median amounts of weight loss in OMC, VAN, OMC+G, and VAN+G on days 4 and 8 were evaluated.

Environmental Contamination and Day 15 Spore Shedding in the Nonsevere Model

On days 10 and 15 of the nonsevere model, ethanol swabs were used to collect spores from each cage wall, water bottle, and middle of the food grate for each cage, totaling 12 swabs per treatment group. Each cage wall was wiped horizontally 5 cm above the floor. Individual fecal samples were also collected in the nonsevere model on day 15. Ethanol swabs and fecal samples were transferred into a −80°C freezer until use. For evaluation, swabs were dried aerobically for 24 hours before placement in 4.5 mL of *C. difficile* brucella broth (CDBB) and incubated within the anaerobic hood [13]. Fecal samples were washed twice in 1 × PBS, resuspended in 1 mL 1 × PBS, and half the resuspended fecal sample was added to 4.5 mL of CDBB. Positive swabs and fecal samples after 1 week incubation were streaked onto CDBA. Colonies characteristic of *C. difficile* were transferred to 1 mL containers of BHI, incubated for 24 hours, heat shocked at 95°C for 10 minutes, and confirmed by polymerase chain reaction (PCR) for toxin genes *tcdA* and *tcdB*. Mice that died prior to day 15, were too sick to provide samples, or had positive stool culture were considered positive for day 15 spore shedding.

DNA Extraction and Sequencing Analysis in the Nonsevere Model

Individual fecal pellets were used to extract DNA as described previously [17, 18]. The V4 region of the 16S rRNA gene was amplified by PCR and sequenced on the Illumina MiSeq to generate 2 × 250 bp reads.

Paired-end demultiplexed sequences were checked for quality with FastQC and imported into QIIME 2 for initial processing following the moving pictures tutorial [19]. Briefly, DADA2 was used to trim reads to 230 bp and create an amplicon sequence variant table. The Silva 138 classifier was used for taxonomic classification [20]. Feature and taxonomy tables from QIIME2 were imported into R (version 4.0.5) and combined with the metadata with the Phyloseq package (version 1.34.0). The decontam package (version 1.10.0) was used to identify

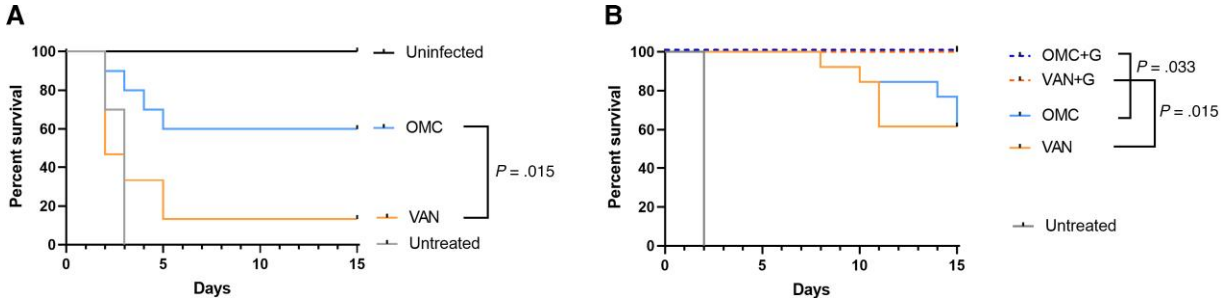

Figure 2. Survival in 2 mouse models of *Clostridioides difficile* infection (CDI): (*A*) severe CDI model; and (*B*) nonsevere CDI model. Treatment groups were omadacycline (OMC), vancomycin (VAN), OMC and germinant (OMC+G), VAN+G, uninfected untreated (uninfected), and infected untreated (untreated).

and remove contaminants based on prevalence. Phyloseq and microbiome (version 1.12.0) packages were used generate relative abundance plots.

Statistical Analysis

The data were compared between antibiotic versus antibiotic plus germinants and between individual antibiotic comparisons. CDI survival in the severe and nonsevere model compared treatment arms throughout the study duration using Kaplan-Meier survival analysis with log-rank test. Hazard ratios (HR) with 95% confidence intervals (CI) were calculated for each comparison. Parametric or nonparametric continuous variables were analyzed by Student *t* test or Wilcoxon rank-sum test, respectively. Statistical analysis was performed with GraphPad Prism with a $P < .05$ for significance.

RESULTS

Survival, Clinical Scoring, and Weight Loss

Survival is displayed in Figure 2 while clinical score and weight loss are presented in Figure 3. In both models, all the infected untreated mice perished by day 3. The uninfected untreated mice survived until the end of the experiment, showing no signs or symptoms of CDI. In the severe model, day 15 survival was significantly higher in OMC (60%) compared to VAN (13.3%) (HR, 0.327; 95% CI, .126–.848; $P = .015$; Figure 2*A*). Weight loss and clinical scoring were higher in VAN compared to OMC throughout the study, indicating more severe disease progression.

In the nonsevere model, no deaths occurred during treatment. Posttreatment survival was similar for VAN and OMC until day 11 when clindamycin-induced relapse caused an immediate survival drop in VAN but not in OMC. Notably, none of the OMC+G or VAN+G mice died from, or showed signs of, rCDI. Final survival was 100% for antibiotic/germinants (OMC+G, 9/9 and VAN+G, 8/8) versus 60% for antibiotic alone (OMC, 6/10 and VAN, 6/10) (HR, 0.109; 95% CI, .02–.410; $P = .001$). Compared to individual antibiotic survival outcomes, germinant treatment resulted in greater survival for both antibiotics (OMC+G vs OMC HR, 0.118; 95% CI, .016–.840; $P = .033$; VAN+G vs VAN HR, 0.106; 95% CI, .017–.646; $P = .015$; Figure 2*B*). Clinical scoring and weight loss for germinant-antibiotic treated mice were similar to respective antibiotic alone groups until day 8, with VAN and VAN+G showing more disease than OMC and OMC+G (Figure 3). Clindamycin-induced relapse only appeared to affect OMC and VAN, and not OMC+G or VAN+G, as noted by increased weight loss and clinical scoring in the nongerminant-treated groups.

Colon Histopathology

Day 5 colon histopathology results are summarized in Figure 4. No sizable difference in overall scores between uninfected untreated and the treatment groups occurred, suggesting that both antibiotics, if given early enough, were able to prevent damage from vegetative cells, and germinants did not cause excessive colonic deterioration. The infected untreated group displayed the most damage and was similar to previous studies [16].

Toxin Quantification

Figure 5 shows a summary of toxin quantification for both models. In the severe model, the same 3 mice were used on days 1 and 2. Toxin amounts were similar between OMC and VAN on day 1. On day 2, median toxin A and B production was significantly higher with VAN versus OMC ($P = .004$) and correlates with higher mortality in VAN at this time.

In the nonsevere model, both antibiotics, given on day 0, prevented toxin production on day 1. The first administration of germinants and antibiotics in OMC+G and VAN+G, on day 1, did not result in notable toxin production on day 2. Day 10 and 13 toxin amounts were higher in VAN compared to OMC and absent from OMC+G and VAN+G. All samples in the severe model and nonsevere model that were positive for toxin production were also positive for spore shedding (data below).

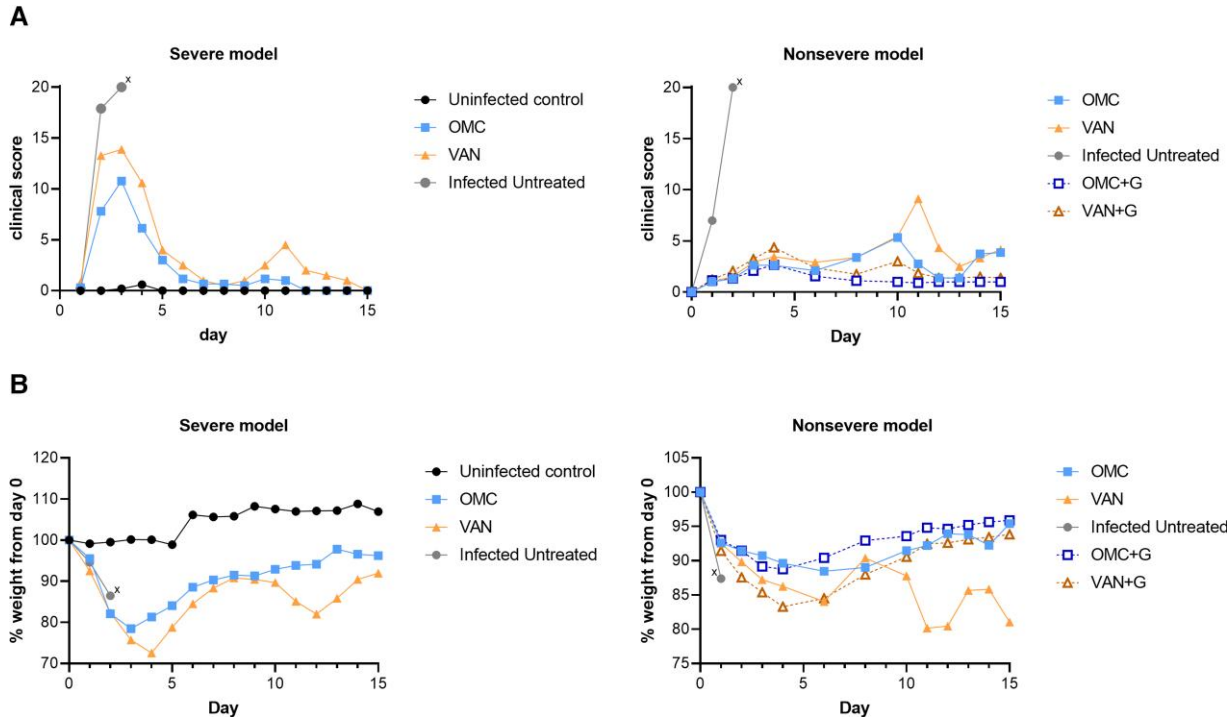

Figure 3. *A*, Clinical scoring and (*B*) weight loss. Treatment groups were omadacycline (OMC), vancomycin (VAN), OMC and germinant (OMC+G), VAN+G, uninfected untreated, and infected untreated.

Environmental Contamination and Spore Shedding in the Nonsevere Model

Both OMC and VAN had 8.3% positive cage swab rates on day 10, compared to 0% in OMC+G or VAN+G. On day 13, 25% of cage swabs from VAN-treated mice were positive. There were no positive swabs in OMC, OMC+G, or VAN+G on day 13.

For mice evaluable for spore shedding at day 15 in the nonsevere model, 100% (10/10) of VAN-treated mice were positive for *C. difficile* spores versus 60% (6/10) for OMC-treated mice ($P = .087$). Notably, none of the evaluable OMC+G (0/8) or VAN+G (0/7) mice were positive for *C. difficile* spores versus 80% (16/20) spore positivity with antibiotic alone ($P < .001$).

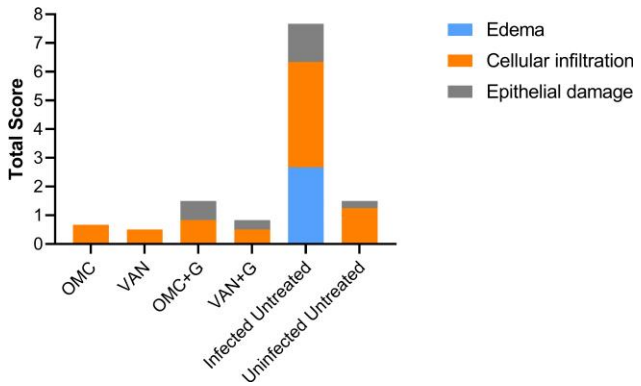

Figure 4. Day 5 colon histopathology. Treatment groups were omadacycline (OMC; n = 3), vancomycin (VAN; n = 3), OMC and germinant (OMC+G; n = 3), and VAN+G (n = 3) were taken from mice in the nonsevere model. Uninfected untreated mice (n = 2) were taken from the severe model and infected untreated mice (n = 3) were taken at time of death before day 5.

Fecal Bile Acid Quantification in the Nonsevere Model

Percentages of bile acids are summarized in Figure 6. Bile acid levels on day −6 were more diverse compared to day 0 and contained more secondary and deconjugated bile acids. By day 4, much of the bile acid diversity was gone in all treatment groups, with VAN and VAN+G showing less diversity than OMC or OMC+G. Over 95% of the bile acid pool in all groups on day 4 consisted of primary bile acids. By day 8, VAN and VAN+G still showed limited diversity, mostly unchanged from day 4, while bile acid diversity with OMC and OMC+G was more diverse. The germinant treatment did not appear to alter the bile acid pool compared to antibiotic only treatments.

Fecal 16S Sequencing in the Nonsevere Model

Microbial diversity on day −6 was greater than that on day 0, and on day 0 it was greater than on day 4 (Figure 7). On day 4, VAN+G displayed the most diversity. VAN treatments appeared to prevent much of the microbial diversity from returning compared to OMC treatments on day 8, aligning with the fecal bile acid quantification data.

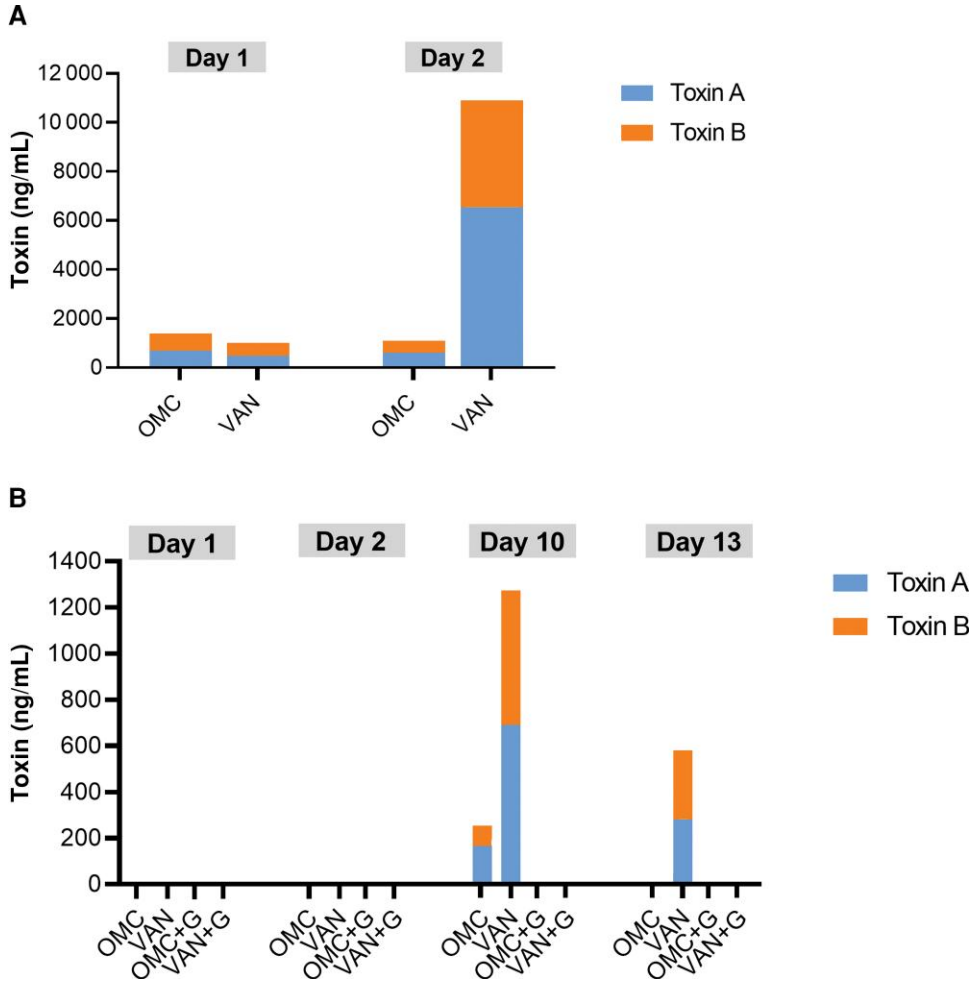

Figure 5. Toxin quantification of treatment groups omadacycline (OMC), vancomycin (VAN), OMC and germinant (OMC+G), and VAN+G. *A*, Severe model. *B*, Nonsevere model.

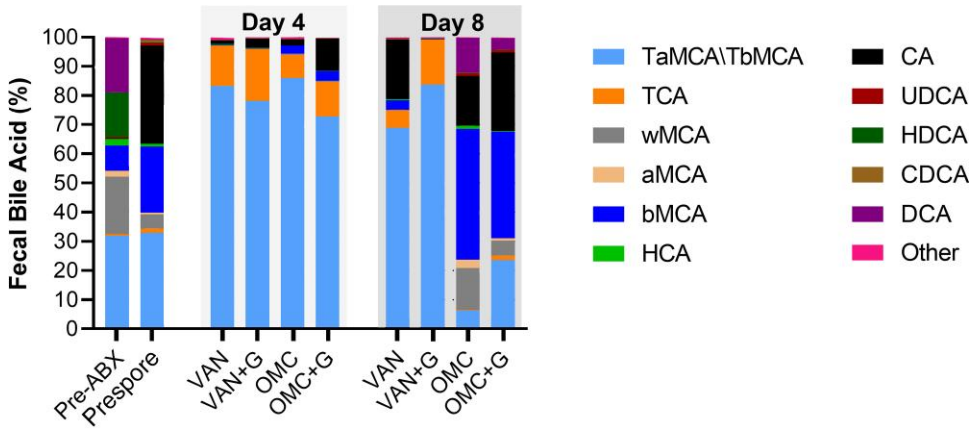

Figure 6. Fecal bile acid percentages for 2 mice in each group. Abbreviations: bMCA, β-muricholate; CA, cholate; CDCA, chenodeoxycholate; DCA, deoxycholate; HCA, hyocholic acid; OMC, omadacycline group; OMC+G, omadacycline and germinant group; Other, bile acids comprising <1%; Pre-ABX, fecal samples taken before antibiotic water; Prespore, fecal samples taken directly before inoculation; TaMCA\TbMCA, tauro-α or β-muricholate; TCA, taurocholate; UDCA, ursodeoxycholate; VAN, vancomycin group; VAN+G, vancomycin and germinant group; wMCA, ω-muricholate; αMCA, α-muricholate.

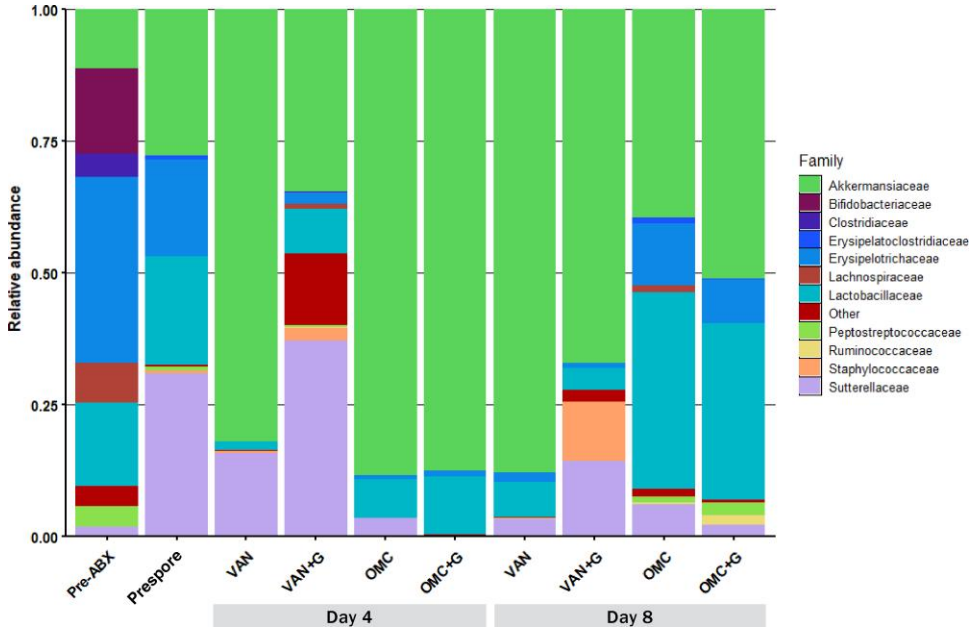

Figure 7. Fecal 16S sequencing in the nonsevere model. Fecal samples were taken before antibiotic water (Pre-ABX; n = 8); directly before inoculation (Prespore; n = 52); from vancomycin group (VAN; day 4 n = 11, day 8 n = 7); VAN and germinant group (VAN+G; day 4 n = 8, day 8 n = 6); omadacycline group (OMC; day 4 n = 11, day 8 n = 6); and OMC+G group (day 4 n = 9, day 8 n = 7).

DISCUSSION

Recurrent *C. difficile* infections are devastating for patients and current antibiotic treatments are not effective at spore removal, a major contributor to rCDI. Fecal transplants are an efficacious treatment but come with concerns of transmitting infectious organisms and have limited durability [8, 21, 22]. Another potential mechanism for treatment is antigerminant administration. This is being investigated but has had mixed results [23, 24]. Potential explanations include antigerminants preventing spores from turning into toxin-producing vegetative cells but not aiding in their removal from the gastrointestinal tract. This may preserve the spore reservoir until a time of nonadherence. Alternatively, in the current study, vegetative cell and spore decolonization produced a durable response without the need for continuing CDI-directed treatment even during subsequent clindamycin administration meant to induce recurrence.

Germinant-antibiotic combinations prevented rCDI-associated mortality, toxin production, and spore shedding compared to antibiotics alone. This suggests mice were effectively decolonized at the end of treatment. Because environmental contamination was removed via cage changes, relapses were likely due to spores attached to animal fur or gastrointestinal surfaces. Considering the similarities in bile acid profiles and 16S results, germinant-treated mice should have been equally susceptible to rCDI from externally attached spores, making an internal reservoir more likely.

Previously, we showed that antibiotics alone do not alter spore concentrations in vitro, but germinant addition, particularly with OMC, was able to remove >99% of spore colony-forming units in R027 strains without increased toxin production [12]. This initial in vitro work formed the basis of our germinant solution for this in vivo model.

Antibiotic-only treatments the day before and after germinants was to ensure the safety of the germinant coadministration regimen. This was designed to mitigate the risk of initial germination without established antibiotic presence to kill vegetative cells, which may result in increased toxin production. Continuing antibiotics 1 day after germinant was to mitigate the concern for germination on the last treatment day, promoting vegetative cell presence as antibiotic concentrations dwindle. Buffering germinant/antibiotic administration on days 1, 2, and 3, with antibiotics alone on days 0 and 4, appeared to alleviate these concerns and would be translatable to clinical infection management.

Although either germinant/antibiotic combination prevented rCDI, OMC appeared to be more efficacious for disease severity. This was especially evident in the severe model, with lower mortality, decreased toxin production, clinical scoring, and weight loss. Similar results were found in the nonsevere model. In addition, OMC groups had more microbial and bile acid diversity compared to VAN groups at day 8, which may signify faster return of colonization resistance, a critical attribute when treating CDI. This corresponds to previous literature showing OMC does not induce CDI in vitro and that tetracyclines may be protective [25, 26].

While these results are encouraging for further murine model investigations of germinant-based decolonization treatment, there are some limitations worth noting. The first is the proclivity of VPI 10463 spores to germinate in response to our treatment solution. We chose VPI for its ability to produce large amounts of toxins, which was our greatest concern. Other *C. difficile* strains, such as ATCC 1870, reached a germination plateau in our previous work [12]. Further work in animal models with germinant-resistant strains and those more associated with clinical disease is needed. A second limitation is the selection of stool for toxin analysis. Severely ill mice were often unable to produce fecal samples, creating selection bias. We attempted to lessen this by evaluating animals with median amounts of weight loss. Another limitation in the severe model was a 30-hour interval prior to treatment. This may have allowed excessive damage to occur, making comparisons between VAN and OMC difficult. Lastly, although we have many different types of encouraging data, the amount in any 1 category was limited. Specifically, evaluations of colon histopathology, toxin quantification, fecal bile acid quantification, and fecal 16S sequencing help understand the biological impact of different antibiotic treatments with and without germinants, but these were determined in only a small subset of mice.

In summary, antibiotic/germinant combinations were effective at decolonizing mice in this murine model of CDI without causing a burst of toxin production. Although both antibiotic-only treatments had similar outcomes in the nonsevere model, the addition of germinant solution to either resulted in complete survival. Lastly, in the severe model of CDI, OMC was more effective than VAN. These results indicate OMC should be further explored for treatment of CDI. The novel antibiotic-germinant treatment approach has potential to prevent rCDI and decrease spore shedding in health care environments. Future studies will focus on the efficacy of this regimen in murine models using germinant-resistant and contemporary strains found clinically.

Supplementary Data

Supplementary materials are available at *The Journal of Infectious Diseases* online. Consisting of data provided by the authors to benefit the reader, the posted materials are not copyedited and are the sole responsibility of the authors, so questions or comments should be addressed to the corresponding author.

Notes

Disclaimer. Paratek did not influence the design or reporting of this research.

Financial support. This work was supported by an investigator-initiated research grant from Paratek Pharmaceuticals.

Potential conflicts of interest. All authors: No reported conflicts of interest. All authors have submitted the ICMJE Form for Disclosure of Potential Conflicts of Interest. Conflicts that the editors consider relevant to the content of the manuscript have been disclosed. Funding to pay the Open Access publication charge for this article was provided by Paratek Pharmaceuticals.

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