

Video Article

Cefoperazone-treated Mouse Model of Clinically-relevant *Clostridium difficile* Strain R20291

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Abstract

Clostridium difficile is an anaerobic, gram-positive, spore-forming enteric pathogen that is associated with increasing morbidity and mortality and consequently poses an urgent threat to public health. Recurrence of a *C. difficile* infection (CDI) after successful treatment with antibiotics is high, occurring in 20-30% of patients, thus necessitating the discovery of novel therapeutics against this pathogen. Current animal models of CDI result in high mortality rates and thus do not approximate the chronic, insidious disease manifestations seen in humans with CDI. To evaluate therapeutics against *C. difficile*, a mouse model approximating human disease utilizing a clinically-relevant strain is needed. This protocol outlines the cefoperazone mouse model of CDI using a clinically-relevant and genetically-tractable strain, R20291. Techniques for clinical disease monitoring, *C. difficile* bacterial enumeration, toxin cytotoxicity, and histopathological changes throughout CDI in a mouse model are detailed in the protocol. Compared to other mouse models of CDI, this model is not uniformly lethal at the dose administered, allowing for the observation of a prolonged clinical course of infection concordant with the human disease. Therefore, this cefoperazone mouse model of CDI proves a valuable experimental platform to assess the effects of novel therapeutics on the amelioration of clinical disease and on the restoration of colonization resistance against *C. difficile*.

Video Link

The video component of this article can be found at <https://www.jove.com/video/54850/>

Introduction

Clostridium difficile is an anaerobic, gram-positive, spore-forming bacillus that causes life-threatening diarrhea¹. *C. difficile* infection (CDI) is associated with increased human morbidity and mortality and results in over \$ 4.8 billion in healthcare costs per year¹⁻⁴. In 2013, the Centers for Disease Control and Prevention categorized *C. difficile* as an urgent antibiotic resistance risk, indicating that it poses an urgent threat to public health¹. Currently, antibiotic treatment with vancomycin and metronidazole are considered the standard of care for CDI⁵. Unfortunately, recurrence of CDI after successful treatment with antibiotics is high, occurring in 20 - 30% of patients^{2,5-7}. Therefore, the discovery of novel therapeutics against this enteric pathogen is necessary. To evaluate therapeutics against *C. difficile*, an animal model approximating the human disease in a clinically-relevant strain is needed.

Initially, Koch's postulates were established for *C. difficile* in 1977 using a clindamycin-treated Syrian hamster model⁸. This model is still utilized today to investigate the effects of *C. difficile* toxins on pathogenesis^{9,10}. However, CDI in the hamster model results in high mortality rates and does not approximate the chronic insidious disease manifestations that can be seen in humans with CDI^{10,11}. Based on the accessibility and reagent availability of murine platforms in research, a mouse model of CDI is relevant.

In 2008, a robust mouse model of CDI was established by treating mice with an antibiotic cocktail in drinking water (kanamycin, gentamicin, colistin, metronidazole, and vancomycin) for 3 days followed by an intraperitoneal injection of clindamycin¹². This rendered mice susceptible to CDI and severe colitis. Depending on the inoculum dose administered, a range of clinical signs and lethality can be observed using this model. Since this time, various antibiotic regimens have been investigated that alter the murine gut microbiota, decreasing colonization resistance to the point where *C. difficile* can colonize the gastrointestinal tract (reviewed in Best *et al.* and Lawley & Young)^{13,14}.

More recently, a broad spectrum cephalosporin, cefoperazone, given in the drinking water for 5 or 10 days reproducibly renders mice susceptible to CDI¹⁵. Since administration of third-generation cephalosporins are associated with an increased risk of CDI in humans, use of the cefoperazone model more accurately reflects naturally-occurring disease¹⁶. Cefoperazone-treated mice susceptible to *C. difficile* have been challenged with both *C. difficile* spores and vegetative cells of a variety of strains ranging in clinical relevance and virulence¹⁷. Despite some of the original studies utilizing *C. difficile* vegetative cells as the infectious form, *C. difficile* spores are considered the major mode of transmission¹⁸.

In the last decade, *C. difficile* R20291, a NAP1/B1/027 strain, has emerged, causing epidemics of CDI^{19,20}. We sought to determine the clinical course of disease when cefoperazone-treated mice were challenged with the clinically-relevant and genetically-tractable *C. difficile* strain,

R20291. This protocol details the clinical course, including weight loss, bacterial colonization, toxin cytotoxicity, and histopathological changes in the gastrointestinal tract of mice challenged with *C. difficile* R20291 spores. Overall, this mouse model proves to be a valuable experimental platform for CDI approximating human disease. This characterized mouse model can thus be utilized to assess the effects of novel therapeutics on the amelioration of clinical disease and on the restoration of colonization resistance against *C. difficile*.

Protocol

Ethical Statement:

The Institutional Animal Care and Use Committee (IACUC) at North Carolina State University College of Veterinary Medicine (NCSU) approved this study. The NCSU Animal Care and Use policy applies standards and guidelines set forth in the Animal Welfare Act and Health Research Extension Act of 1985. Laboratory animal facilities at NCSU adhere to guidelines set forth in the *Guide for the Care and Use of Laboratory Animals*. The animals' health statuses were assessed daily, and moribund animals were humanely euthanized by CO₂ asphyxiation followed by secondary measures. Trained animal technicians or a veterinarian performed animal husbandry in an AAALAC-accredited facility during this study.

1. Administration of the Antibiotic Cefoperazone in Drinking Water to Achieve Susceptibility to *C. difficile* Colonization and Disease

NOTES: 5- to 8-week-old C57BL/6 WT mice (females and males) were purchased and quarantined for 1 week prior to starting the antibiotic water administration. Following quarantine, the mice were housed with autoclaved food, bedding, and water. Cage changes were performed weekly by laboratory staff in a laminar flow hood.

1. Prepare cefoperazone (0.5 mg/ml) in sterile distilled water 7 days prior to the targeted day of challenge (**Figure 1**).
2. Fill autoclaved water bottles with cefoperazone water (0.5 mg/ml) and place them in each mouse cage.
NOTE: Freshly-prepared cefoperazone water should be made and changed out every 2 days during a 5-day period, as denoted in steps 1.1 and 1.2. Proper disposal of cefoperazone water following institutional environmental health and safety guidelines is recommended.
3. After 5 days on cefoperazone water, replace the bottles with autoclaved water bottles filled with sterile distilled water. Give mice this drinking water for the duration of the experiment.

2. Preparation of the *C. difficile* Spore Inoculum and the Oral Gavage of the Mice

NOTE: Before beginning, ensure that the following items are placed in the anaerobic chamber for at least 24 hr: 1x phosphate-buffered saline (PBS; see Materials), taurocholate cycloserine cefoxitin fructose agar (TCCFA) plates (see Materials and the supplemental file), and a sterile L-shaped spreader.

NOTE: Mice challenged with *C. difficile* spores should be housed in a Biosafety Level 2 animal facility.

1. Obtain a *C. difficile* spore stock of the desired strain (in this case, R20291) that was prepared earlier and stored at 4 °C in sterile water^{21,22}. Determine the enumeration of this spore stock prior to use.
2. Based on the known concentration (spores/ml) of the spore stock, calculate the desired dilution to obtain 10⁵ spores per 25 µl. Ensure the inoculum volume is adequate to inoculate all mice in the experiment (25 µl per mouse), to perform the enumeration of the inoculum (approximately 30 µl), and to account for pipetting error.
3. Vortex the original *C. difficile* spore stock. Then, resuspend the calculated volume (from step 2.2) of the original *C. difficile* spore stock in sterile water within a screw-capped microcentrifuge tube. Perform this aerobically in a laminar flow hood. Identify this diluted mixture as the "spore inoculum."
4. Place the spore inoculum into a 65 °C water bath and heat it for 20 min.
NOTE: This step will ensure that only active *C. difficile* spores remain after the heat treatment.
5. Within the laminar flow hood, take 50 µl of heated spore inoculum, place it in a sterile microcentrifuge tube, and pass it into the anaerobic chamber²³. Use this sample for spore enumeration.
6. Within the laminar flow hood, load the remaining heated spore inoculum into a 1-ml syringe attached to a sterile bulb-tipped gastric gavage needle. Utilize a manual syringe stepper to ensure accurate and rapid repeat dosing between mice. Ensure that the gavage needle is filled with the heated spore inoculum prior to use.
NOTE: A new sterile gavage needle for each mouse is not required. However, if various doses of *C. difficile* spores are administered, a new gavage needle is recommended for each dose.
NOTE: *C. difficile* spores are highly resistant to traditional disinfectants. Therefore, the use of a sporicidal disinfectant, such as #62 Perisept Sporicidal Disinfectant, is necessary. The manufacturer recommends a 2-min contact time to destroy *C. difficile* spores.
7. Gavage each mouse with 25 µl of the spore inoculum. Restrain each mouse gently by grasping the animal by the loose skin of the neck and back in order to immobilize the head. Using the index finger further immobilize the animal's head and to elongate the esophagus. Ensure that the animal does not vocalize or show other signs of distress during restraint.
NOTE: The total volume given to mice by gavage should not exceed 10 ml/kg bodyweight (0.1 ml/10 g).
8. Maintain the mouse in an upright, vertical position and gently pass the gavage needle into the side of the mouth. Direct the gavage needle along the roof of the mouth and slowly advance the gavage needle into the esophagus.
NOTE: If any resistance is encountered, the gavage needle may be entering into the trachea and thus should NOT be advanced, but rather removed and repositioned immediately.
9. After the gavage needle is approximately halfway into the esophagus, inject 25 µl of the heated spore inoculum and gently withdraw the gavage needle from the esophagus.
NOTE: Forceful advancement of the gavage needle may lead to the rupture of the esophagus or stomach. Therefore, if resistance is encountered when advancing the gavage needle, it is best to immediately remove and reposition the gavage needle.

10. Return the animal to its cage and observe it for any coughing or signs of respiratory distress, as this may indicate inadvertent tracheal administration or aspiration of the heated spore inoculum.
NOTE: Mice are extremely susceptible to *C. difficile* following antibiotic treatment; therefore, precautions to prevent cross-contamination between cages is essential. This is especially important when managing antibiotic-treated but unchallenged mice as controls.
11. After inoculating all mice, place the remaining heated spore inoculum into a sterile microcentrifuge tube and pass it into the anaerobic chamber for spore enumeration.
12. For enumeration of the heated spore inoculum (from step 2.4) and the remaining gavaged heated spore inoculum, make 1:10 serial dilutions of each inoculum in 1x PBS. It is recommended to make and plate 4 - 5 dilutions (i.e., 10^{-1} through 10^{-5}).
13. Using aseptic technique, transfer 100 μ l of each serial dilution onto an individual TCCFA plate.
14. Using a sterile L-shaped spreader, evenly spread the dilution applied to the medium across the plate. Even spreading of the diluted inoculum is essential for the accurate enumeration of spores.
15. Incubate the plates anaerobically for 24 hr at 37 °C. After 24 hr, *C. difficile* colony-forming units (CFUs) can be enumerated to obtain the infective dose administered to mice in the 25 μ l (0.025 ml) volume (see the supplemental file "Example Calculations"). *C. difficile* colonies are flat and pale yellow and have irregular edges and a ground glass appearance²⁴.
NOTE: The limit of detection for bacterial enumeration is 10^2 CFU.

3. Monitoring Mouse Weight Loss and Clinical Signs of Disease throughout *C. difficile* Infection

1. Weigh animals before and after the 5-day cefoperazone antibiotic treatment, after the 2-day recovery off of antibiotics (day 0), and then for the duration of the experiment.
NOTE: Obtain weights at a consistent time each day. Weights obtained the day of challenge (day 0) should be used as the initial baseline weight for comparison throughout *C. difficile* infection.
2. Place a digital scale into a biosafety cabinet. Place a sterile plastic beaker onto the scale and tare the weight of the plastic beaker. Then, gently pick up the mouse by the base of the tail and place it into the plastic beaker.
 1. Place the beaker back on the scale. Hover a hand over the top of the beaker to ensure that the mouse does not escape. It may take 2 - 3 sec to obtain a stable weight. Then, gently place the mouse back into its cage. Record this weight and repeat the process for each mouse in that cage.
NOTE: It is imperative that precautions are taken to prevent cross-contamination between cages when obtaining weights. Each cage of mice should have its own plastic beaker for weighing. The plastic beakers should be disinfected with a sporicidal agent between each use and covered with sterile aluminum foil.
3. Monitor the challenged animals twice daily (at a minimum) for signs of clinically-severe *C. difficile* infection, including lethargy, loss of appetite, diarrhea, and hunched posture.
4. Humanely euthanize animals that lose 20% of their initial baseline bodyweight and/or develop severe clinical signs of *C. difficile* infection (listed in step 3.3).
NOTE: Mice that display clinical signs of *C. difficile* infection should be weighed as needed during the experiment to ensure that they have not lost 20% of their initial baseline bodyweight. During early time points in the experiment, this might mean twice-daily measurements.

4. Bacterial Enumeration of *C. difficile* from Mouse Feces and Cecal Content

NOTE: Before beginning, ensure that the following items are placed into the anaerobic chamber for at least 24 hr: 1x PBS (see Materials), TCCFA plates (see Materials), a sterile L-shaped spreader, and sterile microcentrifuge tubes and/or PCR plates for dilutions.

1. **Obtaining Samples for *C. difficile* Enumeration**
NOTE: Prior to obtaining feces or cecal content, weigh sterile microcentrifuge tubes on an analytical scale. Record the tube weight on the side of the tube, rounding to four decimal places (e.g., 0.9864 g). Denote this weight as the "tube weight." Each sample collected should be placed in a sterile, weighed microcentrifuge tube.
 1. Sterile collection of mouse feces
 1. Gently restrain each mouse by grasping the animal by the loose skin of the neck and back in order to immobilize the head. Use the pinky finger to gently restrain the animal's tail, thus exposing the anus. Ensure that the animal does not vocalize or show other signs of distress during restraint.
 2. Hold a sterile, weighed microcentrifuge tube directly under the animal's anus. It is imperative that the tube does not come into direct contact with the mouse.
 1. As the animal defecates, collect the fecal pellet into the tube. Keep the tube at room temperature until all fecal samples are collected. It can take several minutes for a mouse to defecate, thus necessitating repeat attempts to collect feces.
 3. Weigh the tubes containing feces on the analytical scale. Record the tube weight, rounding to four decimal places (e.g., 1.0021 g). Denote the obtained weight as the "final tube weight."
 4. Pass the fecal samples into the anaerobic chamber for bacterial enumeration directly after stool collection.
 2. Sterile collection of mouse cecal content at the time of necropsy
 1. After humane euthanasia by CO₂ asphyxiation followed by secondary measures, perform a necropsy to obtain the cecum²⁵. The cecum is the large, comma-shaped section of the gastrointestinal tract.
 2. Place the cecum onto a sterile plastic petri dish. Use a disposable, sterile no. 10 scalpel blade to cut the cecum open from the blind end of the pouch to expose the cecal contents.
 1. Gently extract the cecal contents by applying light pressure with the scalpel blade and sweeping the contents toward the incised portion of the cecum.

NOTE: Care should be taken to not disrupt the cecal tissue, which will be submitted for histopathological examination.

3. Place the cecal contents into a sterile, weighed microcentrifuge tube immediately following collection. Only about 10 mg of cecal content is required for bacterial enumeration.
4. Weigh the tubes containing cecal content on the analytical scale. Record the tube weight, rounding to four decimal places (e.g., 1.0021 g). Denote the obtained weight as the "final tube weight."
5. Pass the cecal content samples into the anaerobic chamber for bacterial enumeration.

2. Bacterial Enumeration of *C. difficile*

1. Calculate the final weight of the contents (feces or cecal) that will be enumerated for *C. difficile*. Performed this by subtracting the "final tube weight" from the "tube weight."
2. Calculate a 1:10 dilution of the contents into 1X PBS and resuspend accordingly. For fecal pellets, use the tip of the pipette to gently disrupt the pellet into solution (see the supplemental file "Example Calculations").
3. Anaerobically incubate these resuspended samples at room temperature for 30 min, allowing the contents to settle.
4. Make serial dilutions for each sample in 1x PBS for enumeration of the CFU per g of content (feces or cecal). Perform this anaerobically.
5. Using aseptic technique, transfer 100 μ l of each serial dilution to TCCFA plates. Using a sterile L-shaped spreader, spread the dilution applied to the media to evenly spread it across the plate. Even spreading of the dilution is essential for accurate enumeration of colonies.
6. Incubate the plates anaerobically for 24 hr at 37 °C. At this time, enumerate *C. difficile* colonies to obtain the CFU per g of content (feces or cecal). *C. difficile* colonies are flat and pale yellow and have irregular edges and a ground glass appearance²⁴.

NOTE: This protocol can be used to enumerate *C. difficile* from other murine GI content, including ileal and colonic content. PCR plates can be used for making serial dilutions.

5. Vero Cell Cytotoxicity Assay to Quantify *C. difficile* Toxin Cytotoxicity

NOTE: It is recommended that this assay be performed after completion of the mouse model on samples collected at necropsy and stored at -80 °C. Aseptic cell culture techniques are essential for preventing contamination of Vero cells during this assay. This protocol takes 2 days to perform. All feces and intestinal content utilized in this assay must be stored in a weighed, sterile microcentrifuge tube (denoted with the "tube weight," see section above). The final tube weight (including the contents) is measured via an analytical scale to the nearest four decimal places (see section above). Use of a multi-channel pipette is recommended for this assay.

NOTE: Before beginning, ensure that the following items are available: Vero Cells, Dulbecco's modification of Eagle medium (DMEM) 1x with 10% heat-inactivated fetal bovine serum (FBS) and 1% penicillin/streptomycin media (denoted as "DMEM 1x media;" see Materials and the supplemental file), 0.25% Trypsin-EDTA, 1x PBS, 0.4% Trypan Blue, a 96-well cell culture flat-bottom plate, a 96-well filter plate, *Clostridium difficile* Toxin A (aliquot in 3 μ l at 1 μ g/ μ l in Ultra-Pure water and store at -80 °C), *Clostridium difficile* Antitoxin, a worksheet (for calculations and plate maps; see supplemental files).

NOTE: Caution should be taken during this assay for personnel exposure to *C. difficile* and its toxin.

1. Splitting the Vero Cells (Day 1)

1. Preheat the DMEM 1x media, 0.25% Trypsin-EDTA, and 1x PBS in a 37 °C water bath.
2. Obtain a flask of Vero cells from the cell culture incubator (37 °C and 5% CO₂) and place it in the sterile cell culture laminar flow hood. Use 70% ethanol to wipe down the hood and equipment prior to use. Using a serologic pipette, aspirate the media from the tissue culture flask to remove it from the Vero cells and discard it into a waste container.
3. Rinse the Vero cells with 5 ml of 1x PBS (preheated) in the tissue culture flask. Aspirate the PBS and discard it into a waste container.
4. Add 5 ml of 0.25% Trypsin-EDTA to the tissue culture flask. Allow a contact time of 2 - 3 min. During this time, observe the cells begin to come off the flask surface. Gently rock the tissue culture flask side to side to loosen the Vero cells.
5. After contact time with Trypsin-EDTA, immediately add 5 ml of DMEM 1x media into the tissue culture flask. This will neutralize the Trypsin-EDTA. Use this solution to gently wash any unattached Vero cells off the back of the flask. Then, use a serological pipette to transfer this mixture into a 15-ml conical tube.
6. Centrifuge the Vero cells in the conical tube for 5 min at 180 x g and 25 °C. Ensure that the centrifuge is properly balanced. After centrifuging, observe a visible pellet of Vero cells at the bottom of the conical tube. Be careful not to disrupt this pellet. Aspirate off the supernatant from the conical tube and discard it.
7. Resuspend the Vero cells in 3 ml of DMEM 1x media.

2. Counting the Vero cells

1. Add 100 μ l of the Vero cell suspension (made in Step 5.1.7) to 100 μ l of 0.4% Trypan Blue. Gently mix by pipetting the solution up and down.
2. Add 10 μ l of this mixture to each chamber of a hemocytometer.
3. Count the number of viable cells within the four quadrants of the hemocytometer. Dead Vero cells will take up the Trypan Blue and thus will be stained blue (these cells should not be counted). Record these numbers in the "Vero Cell Worksheet," provided.
4. Sum the total number of viable Vero cells in all four quadrants and calculate the average. Multiply by the dilution factor, which is 2 since 100 μ l of cells are in a total of 200 μ l of liquid.
5. Multiply by the correction factor to give the "number of cells/ml." When using a hemocytometer, the correction factor is always 10⁴. Multiply by the total volume to give the "total number of cells."

3. Determining the Number of Wells Required for Each Experiment

NOTE: A single sample (either feces or intestinal content) requires 24 separate wells to be performed in duplicate. A concentration of 10^5 Vero cells per well (total volume of 90 μ l) is required.

NOTE: If one desires to incubate the Vero cell 96-well plates overnight at 37 °C, a concentration of 10^3 Vero cells per well is recommended to account for the extended incubation time. Calculations within the Vero Cell Worksheet would need to be adjusted to account for this change.

1. Determine the number of wells that can be utilized based on the amount of Vero cells available (from Step 5.2; use the Vero Cell Worksheet, provided).
2. Determine the volume of Vero cell suspension needed to fill the number of desired wells (based on the number of samples being tested). Ensure that any additional volume is added in to account for pipetting error (use the provided Vero Cell Worksheet).
3. Dilute the Vero cell suspension (obtained in Step 5.1.7) in DMEM 1x media to obtain the volume required for the experiment.

4. Seeding Vero Cells into a 96-well Cell Culture Plate

1. Using the Vero cell resuspension from Step 5.3.2, use a multi-channel pipette to add 90 μ l of the Vero cell suspension to each well of a 96-well cell culture flat-bottom plate.
2. Incubate the plate with the Vero cells in a cell culture incubator at 37 °C and 5% CO₂ for a minimum of 4 hr before adding toxin (samples) to the wells. This incubation time will allow the Vero cells to adhere to the bottom of each well.

5. Creating Stock Solutions from the Samples (Feces or Intestinal Content)

NOTE: All samples should have the "tube weight" and "final tube weight" measured via an analytical scale. Use Vero Cell Worksheet for calculations.

1. Calculate the weight of the contents. Convert g to mg, then mg to μ l. Calculate the final total number of μ l of solution needed to make a 1:10 dilution in 1x PBS. Calculate the total amount of 1x PBS to add to the sample.
2. Add the total volume of 1x PBS (calculated above) to the sample. Perform this aerobically, and using aseptic technique. For fecal pellets, use the tip of the pipette to gently disrupt the pellet into the solution.
3. Vortex the samples and then centrifuge them at 3,522 x g for 5 min at room temperature. Ensure that the centrifuge is properly balanced. Be careful not to disrupt the pellet and resuspend the sample into solution.
NOTE: During Step 5.5.3, if only a few samples are being processed, contents can be sterilized by passing them through a single 0.22- μ m filter instead of using a 96-well filter plate. Some samples may require multiple filters to sterilize the entire volume content using this technique.
4. Sterilize the sample/PBS mixture by taking 150 μ l of supernatant and placing it into separate wells on a 96-well filter plate. Place the filter plate securely on top of a 96-well cell culture flat-bottom plate so that the contents will filter into this plate.
5. Centrifuge the filter plate/cell culture plate stack at 431 x g for 5 min at room temperature. Ensure that the centrifuge is properly balanced and that the filter plate/cell culture plate stack are tightly aligned and will not shift during centrifugation.
NOTE: These filtered contents are the 10^{-1} stock that will be utilized in the dilution plates.
6. Place plate with filtered samples onto ice.

6. Creating Dilution Plate #1

NOTE: Dilution Plate #1 (DP#1) will require 6 wells per sample, including positive (*C. difficile* Toxin A) and negative (1x PBS) controls (see the DP#1 layout on the Vero Cell Worksheet).

1. To prepare the positive control (*C. difficile* Toxin A) for this assay, obtain 3- μ l aliquots (1 μ g/ μ l) from -80 °C and add 297 μ l of ultrapure water, yielding a final concentration of 0.01 μ g/ μ l. Place on ice.
2. Label the wells with their dilutions (10^{-1} to 10^{-6}) for each sample and indicate the positive and negative controls (see the DP#1 layout).
3. Add appropriate amounts of 1x PBS to each well. In the 10^{-1} wells, add **NO** PBS. For the 10^{-2} to 10^{-6} wells, add 90 μ l of 1x PBS.
4. Add appropriate amounts of the samples to each well. In the 10^{-1} wells, add 100 μ l of the 10^{-1} stock for each sample (see Step 5.5.7 from the filter plate). For the 10^{-2} to 10^{-6} wells, make serial dilutions; start by taking 10 μ l from the 10^{-1} well and adding it to the 10^{-2} well. Continue the serial dilutions out to the 10^{-6} dilution.
5. Cover the DP#1 plate with a clear plastic adhesive seal and place it on ice.

7. Creating Dilution Plate #2

NOTE: Dilution Plate (DP#2) will require 2 lanes of 6 wells for each sample, including positive and negative controls (see the DP#2 layout on the Vero Cell Worksheet).

1. Label the wells with their dilutions (10^{-1} to 10^{-6}) for each sample and indicate the positive and negative controls (see the DP#2 layout). Label the wells with the sample name (denoted as "sample wells") or the sample name with antitoxin (denoted as "antitoxin wells").
2. Calculate the amount of antitoxin required for this assay. NOTE: The antitoxin is a 25x stock solution that needs to be diluted 1:25 in 1x PBS. Place the prepared antitoxin 1x solution on ice.
3. For "sample wells," add 20 μ l of 1x PBS to each well for all dilutions (10^{-1} to 10^{-6}) of that sample. For "antitoxin wells," add 20 μ l of 1x Antitoxin to each well for all dilutions (10^{-1} to 10^{-6}) of that sample.
4. Transfer 20 μ l of the diluted sample in wells from DP#1 into the designated wells on DP#2 for rows 1 - 6 and rows 7 - 12 (see the DP#2 layout on the Vero Cell Worksheet). Mix the solution gently by pipetting up and down.
5. Cover DP#2 with a clear plastic adhesive seal and allow 40 min of incubation at room temperature. This incubation is to allow the antitoxin to bind and thus inactivate and neutralize the *C. difficile* toxin.
6. After the incubation, add 10 μ l of mixture from DP#2 to the appropriate Vero cell wells (see the Vero Cell Plate layout on the Vero Cell Worksheet). Gently mix the solution by pipetting up and down, taking care to not disrupt the Vero cells that are adhered to the bottom surface of the wells.
7. Incubate the Vero cell plate overnight in a cell culture incubator at 37 °C and 5% CO₂.

8. Assess Vero Cells for Rounding using a Light Microscope (day 2)

NOTE: Recall that the dilution factor changes by a factor of 10 when adding the sample mixtures to the Vero cell plate (e.g., 10^{-3} is now 10^{-4}). Wells that have antitoxin present should have little to no Vero cell rounding noted. Vero cell rounding (cytotoxicity) will be noted in samples that contain *C. difficile* toxin. If the cytotoxic activity is neutralized in a dilution containing the sample and the antitoxin, the presence of *C. difficile* toxin resulting in Vero cell cytotoxicity is confirmed.

1. Examine for Vero cell rounding using a light microscope at 200X magnification. For sample wells (including the positive control), record the dilution of the well that is the *last* to have 80% Vero cell rounding noted.
 2. Calculate the cytotoxic titer, which is defined as the reciprocal of the highest dilution that produced 80% Vero cell rounding per g of sample. For example, if the highest dilution is 10^{-5} , then the dilution factor would be 10^5 for this sample. Thus, the log[final dilution factor]/g of sample would be $\log[10^5]$, which yields a reciprocal titer of 6.
- NOTE: Vero cells need to have undergone at least 3 - 4 passages and no more than 30 passages prior to their use in this assay²⁶.

Representative Results

During a representative study, 5-week-old C57BL/6 WT mice were pretreated with cefoperazone in their drinking water (0.5 mg/ml) for 5 days and allowed a 2-day wash out with regular drinking water. Mice were challenged with 10^5 spores of *C. difficile* R20291 via oral gavage on day 0 (**Figure 1A**). Mice were monitored for weight loss and clinical signs (lethargy, inappetence, diarrhea, and hunched posture) of CDI for 14 days. The challenge of C57BL/6 WT mice with *C. difficile* R20291 spores resulted in diarrhea within 24 hr and significant weight loss within 48 hr post-challenge (**Figure 1B**). Although not observed in this experiment, mice challenged with a higher dose of *C. difficile* R20291 can become severely ill or have greater than 20% weight loss by 48 - 72 hr post-challenge, thus necessitating humane euthanasia. Therefore, mice require a minimum of twice-daily monitoring after challenge with *C. difficile* spores.

In this representative study, a significant amount of weight loss and clinical signs of disease in mice were also observed on days 2 - 7 post-challenge (**Figure 1B**). At 7 days post-challenge, mice began to gain weight, and clinical signs of disease subsided. By the last week of the experiment, the mice appeared clinically normal, with no evidence of the clinical signs of CDI, including diarrhea.

Fecal pellets were collected prior to the challenge with *C. difficile* spores and every 48 hr post-challenge (**Figure 1C**). Prior to the antibiotic treatment, mice were not colonized with *C. difficile*. Within 24 hr post challenge with *C. difficile* spores, mice were colonized with 10^7 CFUs of *C. difficile* per g of feces (**Figure 1C**). Mice remained persistently colonized with *C. difficile* throughout the experiment, despite no evidence of weight loss or clinical signs of CDI for the last 7 days of the experiment.

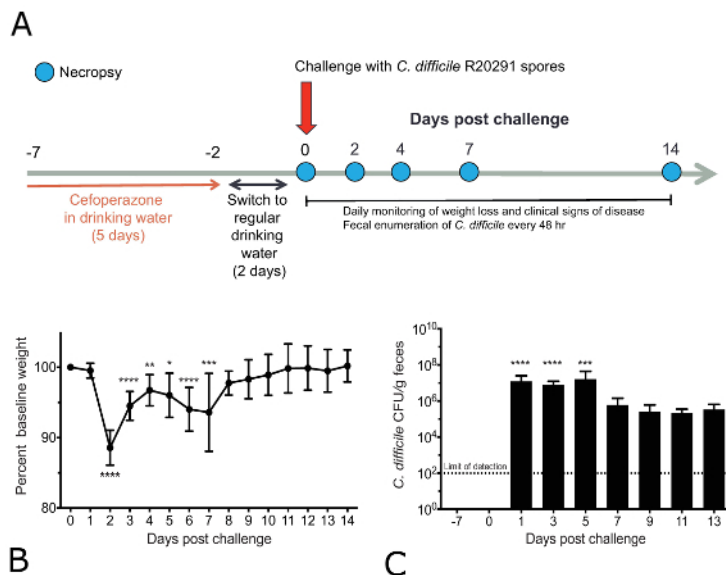


Figure 1: Cefoperazone Treated Mice Challenged with *C. difficile* R20291 Exhibit Weight Loss and are Colonized with *C. difficile*. **A)** 5-week-old C57BL/6 WT JAX mice ($n = 3M/3F$ per group) were pretreated with cefoperazone in their drinking water (0.5 mg/ml) for 5 days and allowed a 2-day wash out with regular drinking water. Mice were challenged with 10^5 spores of *C. difficile* R20291 via oral gavage on day 0. Mice were monitored for weight loss and clinical signs (lethargy, inappetence, diarrhea, and hunched posture) of CDI for 14 days. Feces was collected and used for bacterial enumeration prior to starting cefoperazone, immediately after finishing the antibiotic, and on days 0, 1, 2, 3, 4, 5, 7, 9, 11, 13, and 14 throughout infection. Necropsy was performed on days 0, 2, 4, 7, and 14. **B)** The mice lost a significant amount of their baseline weight after challenge with *C. difficile* R20291. The average bodyweight of mice was measured daily from day 0. Significant weight loss was seen on days 2 through 7 when compared to day 0, the baseline weight. **C)** *C. difficile* R20291 bacterial load in the feces after the challenge. Within 24 hr after the challenge with *C. difficile* spores, all mice were colonized with 10^7 CFUs (colony-forming units) per g of feces. No significant differences in these parameters were seen between females and males. Significance was determined by the non-parametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). Error bars represent the standard deviations from the mean. [Please click here to view a larger version of this figure.](#)

Four mice (2 males and 2 females) were humanely euthanized and prepared for necropsy on day 0, prior to infection, to serve as uninfected controls. In addition, one mouse from each cage was humanely euthanized and prepared for necropsy on days 2, 4, 7, and 14 post-challenge (**Figure 1A**). Enumeration of *C. difficile* within the cecal content was performed at each necropsy. In concordance with fecal enumeration, no *C. difficile* was detected in the cecal content of mice prior to the challenge with *C. difficile*. At 48 hr post-challenge, mice were colonized with approximately 10^8 CFUs of *C. difficile* per g of cecal content (**Figure 2A**). All mice remained persistently colonized throughout the experiment.

Cecal content was also assessed throughout the infection for the presence of *C. difficile* toxin using the Vero cell cytotoxicity assay. No evidence of *C. difficile* toxin cytotoxicity was detected in cecal contents prior to the challenge (**Figure 2B**). *C. difficile* cytotoxicity was detected 2 days after the challenge with *C. difficile* spores and persisted throughout the infection. Despite fairly uniform colonization with *C. difficile*, variation in the levels of *C. difficile* cytotoxic activity is evident in individual mice (**Figure 2B**).

The majority of mouse cecal content neutralized with *C. difficile* antitoxin during the Vero cell cytotoxicity assay and had no evidence of cytotoxicity (Vero cell rounding) in the dilutions performed. However, a few individual mice, despite retesting, had evidence of 50 - 100% cytotoxicity in the 10^{-1} dilution (dilution where the sample is most concentrated), with no evidence of Vero cell rounding in the other dilutions. The antitoxin utilized in this assay is a neutralizing polyclonal antibody to *C. difficile* toxin, prepared in goats²⁷. Therefore, this antitoxin may not neutralize the *C. difficile* binary toxin that is produced by strain R20291. However, the *C. difficile* binary toxin is not considered to be cytotoxic¹⁰. Excessive *C. difficile* toxin unable to be neutralized by the antitoxin or the presence of another cytotoxic agent other than *C. difficile* toxin could be contributing to this finding. This observation did not affect the determination of the cytotoxicity titer for these samples, since each sample's last dilution with the 80% Vero cell rounding had no evidence of cytotoxicity when combined with the antitoxin at the specified dilution.

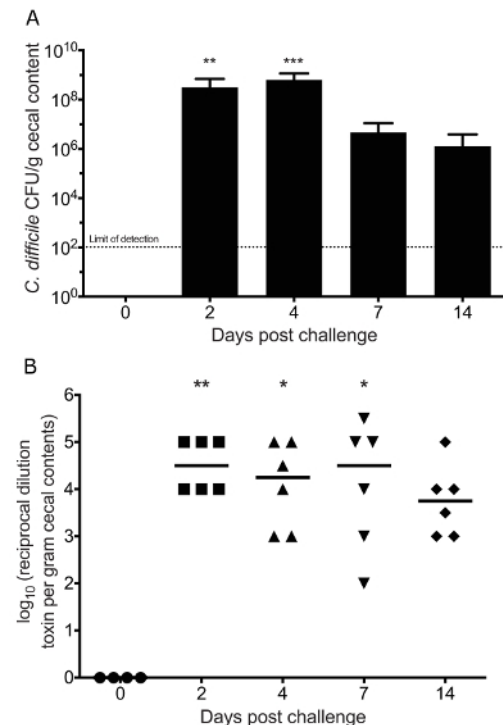


Figure 2: Colonization of the Cecum and Cytotoxicity during Infection with *C. difficile* R20291. **A)** Mice ceca remained persistently colonized with *C. difficile* throughout the experiment, despite resolution of clinical signs and observed weight gain. All mice were colonized with greater than 10^8 CFUs per g of cecal contents when assessed at 2 days post-challenge. **B)** Vero cell cytotoxicity assay from cecal content throughout infection with *C. difficile* R20291. Following the challenge with *C. difficile* spores, mice had significant levels of cytotoxicity, as measured in $\log_{10}$ reciprocal dilution of toxin per g of cecal content on days 2, 4, and 7 post-challenge compared to day 0 (medians represented by the line). Significance was determined by the non-parametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). Error bars represent the standard deviations from the mean. [Please click here to view a larger version of this figure.](#)

It is recommended that histologic evaluation is conducted by a board-certified veterinary pathologist in a blinded manner. For tissue scoring, a 0-4 numerical scoring system should be employed to separately assess edema, inflammatory cell infiltration, and epithelial cell damage in the ileum, cecum, and colon based upon a previously-published scoring scheme¹⁷.

Upon blinded histopathologic examination of the murine ileum, cecum, and colon following challenge with *C. difficile* R20291, the most significant pathologic changes were noted in the cecum (**Figure 3**). The total cecal histologic scores were significantly different between day 0 and days 2 and 4 post-challenge. No significant lesions were noted in the ileum throughout infection (**Figure 3**). Milder lesions were noted in the colon. The total colonic histological scores were significantly different between day 0 and days 2 and 7 post challenge (**Figure 3**).

Representative H&E sections of the ileum, cecum, and colon throughout infection are available in **Figure 4**. Each image has its respective total histological score and individual scores for epithelial damage, inflammation, and edema, as determined by a blinded board-certified veterinary pathologist (SAM).

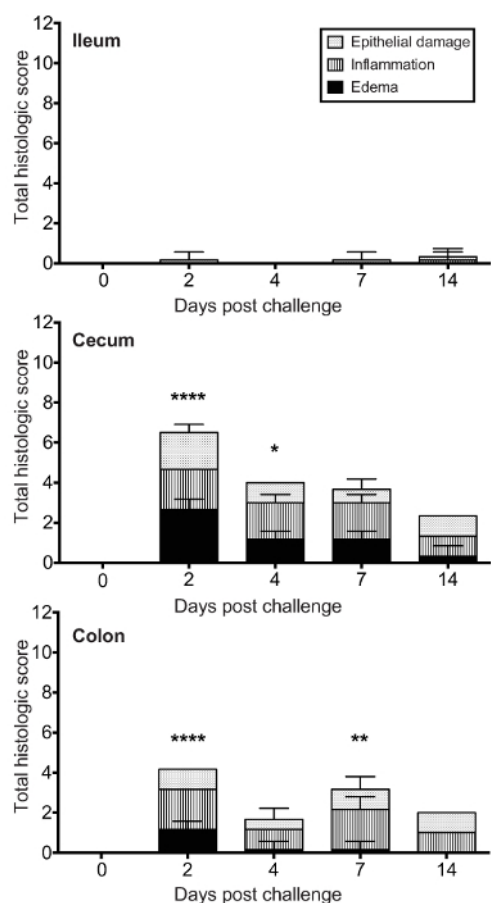


Figure 3: Histological Scoring of the Murine Ileum, Cecum, and Colon during Infection with *C. difficile* R20291. Total histological scores were calculated by adding all three scores from the parameters assessed: epithelial damage, inflammation, and edema. No significant histopathological changes were noted in the ileum throughout infection. Cecal tissue contained the most significant histological lesions during CDI. The total cecal histological scores were significantly different from day 0 on days 2 and 4 post-challenge. Milder lesions were noted in the colon. The total colonic histological scores were significantly different from day 0 on days 2 and 7 post-challenge. Significance was determined by the non-parametric Kruskal-Wallis one-way ANOVA test followed by Dunn's posttest (*, $p \leq 0.05$; **, $p \leq 0.01$; ***, $p \leq 0.001$; ****, $p \leq 0.0001$). Error bars represent the standard deviations from the mean. [Please click here to view a larger version of this figure.](#)

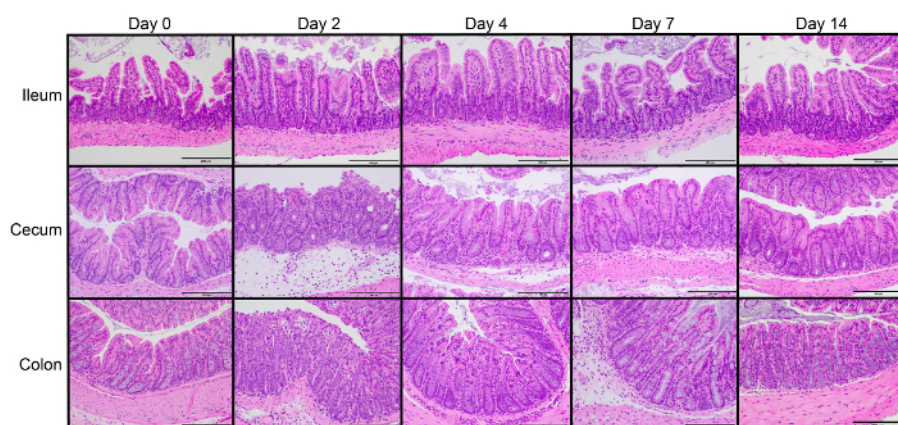


Figure 4: Histopathology During Infection with *C. difficile* R20291. This panel contains representative H&E sections of ileum, cecum, and colon throughout infection with *C. difficile* R20291. The total histological scores are in parentheses () for the corresponding day and tissue listed: for the ileum day 0 (0), day 2 (1), day 4 (0), day 7 (0), and day 14 (0); for the cecum day 0 (0), day 2 (5), day 4 (4), day 7 (3), and day 14 (3); and for the colon day 0 (0), day 2 (4), day 4 (1), day 7 (4), and day 14 (2). Photomicrographs were obtained on a light microscope with a 5 megapixel digital camera and its accompanying software (the scale bar represents 200 μ m). [Please click here to view a larger version of this figure.](#)

Discussion

This protocol characterizes the clinical course, including weight loss, bacterial colonization, toxin cytotoxicity, and histopathological changes in the gastrointestinal tract, of antibiotic-treated mice challenged with *C. difficile* R20291 spores. There are several critical steps within the protocol where attention to detail is essential. Accurate calculation of the *C. difficile* spore inoculum is critical. This calculation is based on the original *C. difficile* spore stock enumeration, which should be consistent over multiple spore enumerations prior to starting this protocol. If the inoculum calculation is erroneous, it will result in an inaccurate inoculation dose of the mice. On the same note, preparation and dilutions of *C. difficile* spores based on these calculations should be prepared with care. Any dilution errors will result in an inaccurate starting spore inoculum. Lastly, precautions should be taken to avoid contamination of antibiotic-treated control mice (not inoculated with *C. difficile* spores). Cefoperazone-treated mice are extremely susceptible to *C. difficile* colonization and thus should not be in direct contact with *C. difficile*-infected mice²⁸⁻³⁰. If the aforementioned critical steps are recognized, successful execution of the CDI mouse model is expected.

Modification of the dose or strain of *C. difficile* spores administered to the mice may alter the outcomes of this model. Increasing the spore inoculum administered to mice could increase the morbidity and mortality observed, depending on the strain of *C. difficile* administered. A dose response experiment, evaluating a range of several inoculum doses, is recommended if using other *C. difficile* strains in order to characterize the clinical course of CDI.

This protocol uses TCCFA medium for *C. difficile* enumeration of spores and vegetative cells collectively. However, if distinct phases (such as germination and outgrowth) of the *C. difficile* lifecycle are desired, differential plating is required. Non-heat-treated contents, as described in this protocol, will yield the CFU per g content of spores and vegetative cells together. If the same GI content is heat-treated (20 min at 65 °C) and then plated on TCCFA media, this will yield a CFU of spores only, since *C. difficile* vegetative cells are destroyed by heat. Comparison of *C. difficile* enumeration using these two plating techniques allows for more detailed information about the *C. difficile* lifecycle *in vivo*.

One major limitation of this mouse model is that the results may not be reproducible in mice from different strains or vendors. Recall that disruption of the gut microbiota following cefoperazone treatment renders mice susceptible to CDI²⁸. Variations in the initial composition of the gut microbiota between different murine strains from different vendors could alter the mouse model of CDI³¹. Once researchers have successfully completed this model, it is recommended that the mouse strain and vendor from where the mice are acquired stay consistent for additional studies to limit variations in the gut microbiota composition, which could alter the outcomes of the model.

Another limitation of the protocol is that the Vero cell cytotoxicity assay does not quantitatively measure *C. difficile* toxin levels *in vivo*. Instead, epithelial (Vero) cell cytotoxicity represents a qualitative approach that measures relative cytotoxicity of *C. difficile* toxins present in samples. Other techniques such as qRT-PCR and immunoblotting would be helpful to determine the amount of toxin present in the samples.

Various models of CDI are currently being used, including the Syrian hamster model, *ex vivo* models, and human intestinal organoid models^{13,14,30,32}. However, the mouse model of CDI provides the increased availability of commercially-available murine reagents and assays to further examine the pathogenesis of *C. difficile*. Other mouse models of CDI have used different strains of *C. difficile*, such as VPI 10463, which results in substantial mortality and thus does not recapitulate human CDI^{12,17,29}. In this model, *C. difficile* R20291 was selected for its genetic tractability and clinical relevance as a recent 027 epidemic strain^{33,34}. Compared to other murine models of CDI, R20291 was not uniformly lethal at the dose administered, allowing for observation of a prolonged clinical course of infection concordant with human disease. Overall, this mouse model proves to be a valuable experimental platform of CDI. This fully-characterized *C. difficile* mouse model can be utilized to assess the effects of novel therapeutics on the amelioration of clinical disease and on the restoration of colonization resistance against *C. difficile*.

Disclosures

The authors have nothing to disclose at this time.

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