**Author’s response to reviews:** We appreciate the time and effort of the reviewers to provide constructive feedback on our manuscript. We have revised the manuscript in response to the reviewers’ comments. We are happy to resubmit our revised manuscript for consideration in JoVE.

Specific responses to reviewer’s comments (non-bolded) are below.

Italicized sections indicate text that was added to the final revised manuscript.

**Editorial comments:**

**•Formatting:  
-Please include the state in the affiliation address.**

The author’s have made the requested change to the text on lines 8, 14, 21, and 27.

**-Please define all abbreviations at first occurrence (ie TCCFA).**

All abbreviations have been defined.

**-Please number steps consecutively. For instance, 4.2.6 is missing.**

All numbers has been confirmed.

**-Please remove the word “you” from all documents, including supporting documents.**

Supporting documents have been modified to remove the word “you”.

**-References – Please abbreviate all journal titles.**

We are using EndNote and have formatted the bibliography based on JoVE settings.

**•Grammar:  
-Please copyedit the manuscript for grammatical errors, which is required prior to acceptance. Some example errors are listed below.**

We have corrected the grammatical errors in the manuscript.

**-4.1.2.1 – This should be reworded so that the mice are “undergoing a necropsy.”**

The text has been edited as follows: “*After humane euthanasia by CO2 asphyxiation followed by secondary measures, mice should undergo a necropsy to obtain the cecum*.” See lines 309-310.

**-4.2.7 – “At this time enumerated”**

The text has been edited as follows: “*4.2.6) Incubate the plates anaerobically for 24 hr at 37°C. At this time enumerate C. difficile colonies to obtain CFU per gram content (feces or cecal content).***”** See lines 352-353.

**-Section 5 note – “for personnel expose”**

The text has been edited as follows: “*NOTE: Caution should be taken during this assay for personnel exposure to C. difficile and its toxin.***”**  See lines 378-379.

**-Line 557 – “Feces was”**The text has been edited as follows: “*Fecal pellets were collected prior to challenge with C. difficile spores and every 48 hours post challenge (Figure 1C).*” See lines 582-583.

**•Visualization: Protocol is discontinuous. The filmed portion should focus on the creation of the mouse model, including antibiotic treatment and gavage if possible. Alternatively, the title should be changed to reflect what will actually be filmed. In addition, a cohesive story should be highlighted rather than the few steps scattered throughout each section. For instance, the highlighting in section 2 would only require that some CFU counts are performed, which leaves out the bulk of the protocol.**

The authors have modified the highlighting to create a more synchronous visual presentation to reflect the main purpose of the protocol.  **•Additional detail is required:  
-4.1.2.1 – Please provide a citation for the dissection or describe in more detail.**

The authors have modified the text as follows: “*After humane euthanasia by CO2 asphyxiation followed by secondary measures, mice should undergo a necropsy to obtain the cecum. The cecum is the large, comma shaped section of the gastrointestinal tract.*” See lines 309-311.

**-5.1.3 – Is the flask centrifuged?**

The authors have modified the text as follows: “*Wash Vero cells with 5 ml of 1X PBS (preheated) in the tissue culture flask. Aspirate PBS and discard into waste container*.” See lines 389-390. Centrifugation is described in steps 5.1.4 to 5.1.6.

**-5.6.4 – There is no step 5.5.7.**

All step numbering has been confirmed.

**•Branding:  
-Please removing branding from supporting documents (like Milli-Q).**

The authors have removed Milli-Q and other branding from the supplemental materials.

**-Results – Jackson Laboratories; mice should appear in the materials table.**

The authors have added this to the materials excel spreadsheet.

**-Figure 4 legend - Olympus BX43F, cellSens Dimension, DP27 – all of these items should appear in the materials table.**The authors have added these items to the materials excel spreadsheet.

**•If your figures and tables are original and not published previously, please ignore this comment. For figures and tables that have been published before, please include phrases such as “Re-print with permission from (reference#)” or “Modified from..” etc. And please send a copy of the re-print permission for JoVE’s record keeping purposes.**

The figures in the manuscript are original.

**•JoVE reference format requires that DOIs are included, when available, for all references listed in the article. This is helpful for readers to locate the included references and obtain more information. Please note that often DOIs are not listed with PubMed abstracts and as such, may not be properly included when citing directly from PubMed. In these cases, please manually include DOIs in reference information.**

All references have DOIs when available. **•IMP: Please copyedit the entire manuscript for any grammatical errors you may find. The text should be in American-English only. This editing should be performed by a native English speaker (or professional copyediting services) and is essential for clarity of the protocol and the manuscript. Please thoroughly review the language and grammar prior to resubmission. Your JoVE editor will not copy-edit your manuscript and any errors in your submitted revision may be present in the published version.                                                 
  
•NOTE: Please include a line-by-line response letter to the editorial and reviewer comments along with the resubmission.**

**Reviewer comments:**

**Reviewer #1**

**Manuscript Summary:  
N/A  
  
Major Concerns:  
Fig 1c, no significant difference in C diff CFU in feces collected on postinfection days of 7, 9, 11, 13 when compared to day of infection.**

This is correct. There is no statistical significance noted in fecal CFU over the last week of the experiment. We used a more conservative statistical test (non-parametric Kruskal-Wallis one-way ANOVA test followed by Dunn’s posttest), which did not find this statistical significant.

**Fig 2a 2b, postinfection days 7 and 14 significant difference? It looks to me yes.**

Again, based on the conservative statistical test performed (non-parametric Kruskal-Wallis one-way ANOVA test followed by Dunn’s posttest) there is no statistical significance noted at these time points.

**Minor Concerns:**

**The use of cefoperazone to develop a mouse model of CDI should be further scientifically justified. Mice pretreated with antibiotic mixture (Chen et al.,) can also develop no-severe diseases depending on C diff challenging doses.**

The authors agree with the reviewer that depending on the inoculum dose administered that non-lethal illness can be observed. We have added the following sentence to the manuscript to address this comment:

“*Depending on the inoculum dose administered, a range of clinical signs and lethality can be observed using this model.”* See lines 86-87.

Additional justification for the use of the cefoperazone model to study CDI were added into the introduction section as follows: “*Since administration of third generation cephalosporins are associated with increased risk of CDI in humans, use of the cefoperazone model more accurately reflects naturally occurring disease16*.” See lines 93-95.  **Additional Comments to Authors:  
N/A  
  
Reviewer #2**

**Manuscript Summary:**

**The authors presented a mouse model of CDI with modified antibiotic regimen and a clinical relevant C difficile strain based on previous published models. Although the absolute advantage of this model version is debatable, the protocol is clearly written with great details, which will be helpful for CDI investigators. A few comments and suggestions are attached below; hopefully useful enough to make the protocol even better serve the CDI community.**

**1) The article used the term "antitoxin" multiple times based on a commercial kit. A brief description of the antitoxin or a reference would be helpful for readers to understand the principle. Is it an anti-toxin antibody derived from animals? (Polyclonal or monoclonal) Or is it *Clostridium sordellii* antitoxin?**

Based on this comment the authors have added the following text to the protocol: “*The antitoxin utilized in this assay is a neutralizing polyclonal antibody prepared in goats to C. difficile toxin26.*” See lines 614-15.

**2) Since the title of this article is "A clinically relevant platform for testing therapeutics". Therefore, an experimental example to test a therapeutic drug using this model would help validate the platform. Any data or previously published paper using this model to test a therapeutic drug would be supportive.**

The authors agree with the reviewer that based on the current title that it implies that a therapeutic has been administered in the protocol. Based on this feedback we have modified the title as follows:

“*Cefoperazone treated mouse model of clinically relevant Clostridium difficile strain R20291”*

**3) The mice were listed as male or female at 5-8 weeks old. The exact age used in the experiments of this article needs to be specified, since there are big variations in terms of size, weight and gut length in mice between 5 and 8 weeks old.**

The author’s have specified the exact age (5 weeks old) of the mice used in this protocol (see line 539).

**4) Either a reference or a brief description of the histology scoring system would be helpful for the readers to adopt the system.**

The authors included a brief description and reference for histologic scoring in the original submission. Please see text in lines 625-628 (including reference 17).

**5) Recurrent CDI model is often used by investigators to examine their drug candidates. Have the authors tried that in this model setting?**

The authors intend to use this model and another model that approximates recurrent CDI to evaluate drug candidates against this clinically relevant *C. difficile* R20291 strain. However, this is outside of the scope of the current protocol, which is to show researchers how to run the CDI mouse model.

**6) In Figure 1b, the animal weights appear to recover after day 2, and drop a second time on day 6 and 7. Is that a repeatable phenomenon in this model?**

Yes, this fluctuation in weight loss over the first week of the experiment is repeatable across multiple experiments.

**7) In Figure 1c, what control value did the authors compare and reach the significant P values for day 1, 3, 5?**

The authors compared all days to day 0 in the non-parametric Kruskal-Wallis one-way ANOVA test followed by Dunn’s posttest.  **Major Concerns:  
N/A  
  
Minor Concerns:  
N/A  
  
Additional Comments to Authors:  
N/A**