September 14th, 2018

Vineeta Bajaj, PhD
Review Editor
JoVE

Dear Dr. Bajaj,

We thank you and the reviewers for the thorough review of our manuscript, JoVE58930 "*Murine model to test host-pathogen responses and vaccine efficacy*". Our responses to the comments are below:

Editorial comments:
Changes to be made by the Author(s) regarding the written manuscript:
1. Please take this opportunity to thoroughly proofread the manuscript to ensure that there are no spelling or grammar issues.

- As requested.

2. Please print and sign the attached Author License Agreement (ALA). Please then scan and upload the signed ALA with the manuscript files to your Editorial Manager account.

- As requested.

3. Please rephrase the Introduction to include a clear statement of the overall goal of this method.

- As requested, a more explicit explanation was included in the first paragraph.

4. Please move the ethics statement before your numbered protocol steps, indicating that the protocol follows the animal care guidelines of your institution.

- As requested.

5. 1.2: Please specify the vaccines and buffer used in this step, and the ratio of vaccine/buffer to mix.

- As requested. This is detailed in step 1.1.1-1.1.3

6. 3.3: Please break into two steps.

- As requested.

7. 4.1: Please refrain from using bullets, dashes, or indentations. Sub-steps of 4.1 should be 4.1.1, 4.1.2, 4.1.3, etc.

- As requested, these have been changed to follow the appropriate format.

8. Line 190: Please remove commercial language (Kontes™).

- As requested.

9. 6.8: Please mention how to calculate CRUs/total organ.

- As requested, a description of CFU calculations was included in step 6.5.

10. Please combine some of the shorter Protocol steps so that individual steps contain 2-3 actions and maximum of 4 sentences per step.

- As requested.

11. After you have made all the recommended changes to your protocol (listed above), please highlight 2.75 pages or less of the Protocol (including headings and spacing) that identifies the essential steps of the protocol for the video, i.e., the steps that should be visualized to tell the most cohesive story of the Protocol.

- As requested.

12. Please highlight complete sentences (not parts of sentences). Please ensure that the highlighted part of the step includes at least one action that is written in imperative tense. Please do not highlight any steps describing anesthetization and euthanasia.

- As requested.

13. Please include all relevant details that are required to perform the step in the highlighting. For example: If step 2.5 is highlighted for filming and the details of how to perform the step are given in steps 2.5.1 and 2.5.2, then the sub-steps where the details are provided must be highlighted.

- As requested.

14. Please upload each Figure individually to your Editorial Manager account as a .png, .tiff, .pdf, .svg, .eps, .psd, or .ai file.

- As requested.

15. Figures 2 and 3: Please include a space between numbers and their units (1 mL, 0.9 mL, 0.1 mL, 50 µL, etc.). Please make 600 a subscript in Figure 2. Please consider converting Figure 2 to a table. Please use exponential notation (instead of E notation) for numbers in Figure 3.

- As requested.

16. Figures 4 and 5: Are these figures reprinted from a previous publication? If yes, please obtain explicit copyright permission to reuse any figures from a previous publication. Explicit permission can be expressed in the form of a letter from the editor or a link to the editorial policy that allows re-prints. Please upload this information as a .doc or .docx file to your Editorial Manager account. The Figure must be cited appropriately in the Figure Legend, i.e. "This figure has been modified from [citation]."

- Yes, the figure come from reference #10: Jennings-Gee, J. *et al.* The adjuvant Bordetella Colonization Factor A attenuates alum-induced Th2 responses and enhances Bordetella pertussis clearance from mouse lungs. *Infection and Immunity.* 10.1128/IAI.00935-17, (2018). The manuscript is cited in the figure legend. Explicit permission to republish/adapt parts of articles are given in the following statement from their editorial policy:

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the first page of the PDF version, or "Copyright © American Society for Microbiology, [insert journal name, volume number, year, page numbers and DOI]" of the HTML version.

17. Figure 5: Please label the y-axis. Please define error bars.

- The figure is republished from reference #10 and the y-axis has been labeled as "pg/mL" in that article. A sentence was added to the figure legend to define error bars as standard deviations.

18. Figures 3 and 6: Please use the capitalized (A, B, C) letters for panel labels.

- As requested.

19. Discussion: As we are a methods journal, please also discuss critical steps within the protocol and any limitations of the technique.

- As requested. We have included expanded sections to include a more thorough discussion about immunizations, infections, and tissue harvest and limitations of each method.

20. Please revise the Acknowledgements section to include any acknowledgments and all funding sources for this work.

- As requested.

21. References: Please do not abbreviate journal titles.

- Corrected as requested.

22. Please remove trademark (™) and registered (®) symbols from the Table of Equipment and Materials. Please use SI abbreviations for all units (µm) and include a space between all numerical values and their corresponding units (15 mL, 60 mm, etc.).

- As requested.

Reviewers' comments:

Please note that novelty is not a requirement for publication and reviewer comments questioning the novelty of the article can be disregarded.

Reviewer #1:

Manuscript Summary:

The manuscript entitled "Murine model to test host-pathogen responses and vaccine efficacy" provides a clear and concise protocol for the immunization and intranasal infection of mice, in addition to instruction on the harvesting and processing of tissue for the purpose of immune profiling. The manuscript is well written, suitably titled and describes appropriate controls. The protocol is easily followed and the expected results are reasonable given the information provided. The figures provided are beneficial and easily interpreted.

Major Concerns:

*Step 4.11, a description of blood collection is provided but the blood processing steps stop at "Place tube on ice". A description of how the blood is processed should be added to the manuscript

- As requested. This was included and is Step 8.

*The manuscript would benefit from a description of the timeline used e.g immunization day, infection day and harvest day

- As requested. This was included and is Figure 1.

Minor Concerns:
*Line 114, step 1.8, please describe the gauge and size of needle, and depth of insertion

- As requested.

*The authors should detail controls used for the vaccine immunization procedure

- As requested. This is detailed in step 1.1.1-1.1.3.

*The abbreviation "10% BG + antibiotic" is used throughout the text without prior description, add abbreviation to description in section 2.1

- As requested.

*Step 2.6 & 3.5, describe the buffer in which bacteria should be diluted

- As requested.

*Line 173/ 174, typo: "as negative control" is repeated twice in the sentence

- As requested.

*Line 210, add a description of the type of tubes used for blood collection

- As requested.

*Line 225, typo: "Make sure lay to skin to the side" correct

- As requested.

*Step 4.16, this step could benefit from further description, for example a description of where the first incision should be made and what direction etc.

- The description was expanded to include these details.

*Line 324/ 325, assuming both antigens are not mixed in the same sample, line should read "Therefore, mix 2ug/ml Prn or 1ug/ml FHA in 0.5 ml" not "Prn and 1ug/ml FHA"

- As requested.

*Step 6.8, the authors describe the earlier removal of the right superior lobe for histopathology, but describe the calculation for bacterial burden as CFUs/total organ, how is the missing lobe accounted for?

- We thank the reviewer for the comment. For the lung CFU enumeration, it is technically correct that we are not enumerating CFUs for the total organ as the superior lung is taken for histopathology. Therefore, we have changed the text to read "CFU/lung" instead of whole organ. Since the superior lung is taken for histology from all animals, the CFUs are enumerated from the same amount of lung tissue for each animal.

*Line 404, Figure 5 legend, a more accurate description would highlight that this is cytokine production by immunized spleen cells following stimulation with ... e.g. vaccine components/ antigens/ adjuvants

- Thank you for the suggestion, we have changed the legend accordingly.

*Line 420-425, references should be added to support each statement

- We thank the reviewer for the comment and have added references #21-24.

*Table of materials, the table should include information on the type/ source of 48 well plate, needle information and details on the source of SS media

- As requested.

Reviewer #2:

Manuscript Summary:

In this manuscript "Murine model to test host-pathogen responses and vaccine efficacy" by Caution et al., the authors describe the in vivo evaluation of vaccine effectiveness and host immune responses using a Bordetella pertussis inactivated vaccine.

Major concerns:

There are several major and minor (not included in this review) concerns that the authors should contemplate to provide a more focus and overall novel manuscript.

1) The authors claim that the protocol can be adapted for vaccine models that study viral, bacterial or other parasitic pathogens. However, there are several parameters that would need to be adapted for a particular pathogen. These include, among many others, vaccine dose and route of administration, dose and route of administration of the challenged pathogen, animal anesthesia, evaluation of adaptive B and T cell responses, etc....

- Thank you for the comment. We acknowledge that an investigator would need to modify the protocol to fit the individual experimental design.

2) There is several key information missing in the manuscript. This include, for instance, how many mice would be needed to provide with sufficient statistical power analysis to conclude from the described experiments. Likewise, experiments to assess morbidity (e.g. mouse body weight and/or clinical signs of disease) as well as mortality (e.g. percentage of animal survival) have not been included in the manuscript. Moreover, information regarding the sex of the animals should be provided since it has been shown how sex is an important biological variable for different pathogens. Finally, the authors did not include information regarding the use of appropriated controls to validate their results, etc....

- We thank the reviewer for the comment and have added information to clarify these points. We have an animal ethics statement at the beginning of the protocol to detail number of mice per group for determination of statistical power, and a statement regarding the gender of experimental animals. In addition, vaccine treatment groups and necessary control groups are detailed in steps 1.1.1-1.1.3. A statement regarding monitoring and animals post vaccination and infection for clinical signs of disease has been included at step 1.10.1 and 3.4.1, respectively.

3) The authors have not provided details on the preparation and source of the B. pertussis vaccine used in this study.

- We thank the reviewer for the comment and have added information about the acellular vaccine (contents and ratios used) and appropriate controls in steps 1.1.1-1.1.3.

4) There are several sections in the manuscript that can be obtained from basic microbiology books. This include, for instance, the preparation/growth of the bacteria for the challenge experiments. There are also several figures in the manuscript that can be found in basic microbiology and/or biology books. This include, for instance, Figure 2 (Preparation of 1 OD600 bacterial suspension), Figures 3A (Schematic representation of serial bacterial dilutions) and 3B (Calculation of the bacterial dilutions for mice infections), Figure 4 (Schematic representation of mouse lungs), and Figure 6 (B. pertussis colony formation and calculation of bacterial titters).

- We agree that some procedures are established in the field. For completeness, and smooth protocol flow, descriptions for those procedures have also been included..

5) Some of the experimental approaches described in the manuscript have been described in other Jove manuscripts (e.g. intranasal infection of mice, harvest of animal lung and nasal mucosa tissues, etc....)

- See response to #4 above

6) The authors should include a schematic representation of the overall vaccination/challenge protocol for the reader (and reviewer) to understand the overall experimental approach.

- As requested. This was included and is Figure 1.

Reviewer #3:

Manuscript Summary:

The manuscript describes a clear protocol to vaccinate mice followed by intranasal challenge with B. pertussis. The authors also discuss how tissues can be processed to determine bacterial load and/or immune responses, or a combination thereof.

Minor Concerns:

There are a couple of questions/minor concerns that should be addressed:

-1.3 It is not clear to me why the mice would need to be sedated for intramuscular immunization. In our facility we only knock mice out if the procedure cannot be performed without significant discomfort to the mice and even though mice recover quickly from isoflurane treatment it also causes stress. I.m. vaccination can easily be performed without anesthesia by two qualified technicians with one fixating the mouse and the other administering the vaccine.

- Step 1.4 now includes this option. -1.5 To minimize variation between experiments we typically sedate mice one at a time in the isoflurane box, leaving them in for a set amount of time. This is particularly useful in the challenge studies to minimize spread in challenge dose.
- Step 1.4 has been modified to address this option.

-2.4 The authors use freshly grown bacteria to prepare the challenge inoculum. There are both advantages and disadvantages to this approach. One of the disadvantage compared to using frozen challenge stocks is that variation may be introduced in the challenge dose during culture that can only be identified when the inoculum is counted retrospectively. This may become important when experiments become too big to challenge all mice on the same day or when comparing multiple experiments.

- This option has been addressed in step 2.6.1.

-3.3 50 ul is a very high volume for Balb/c mice, which are quite small, and it may be difficult to deliver the full inoculum into the lungs. In our experience, a volume of 40 ul is also sufficient to induce infection of the lower respiratory tract.

- We modified step 3.3 to reflect the range of 40-50 µl. -3.5 I suggest that the authors also plate out the inoculum prior to challenge as the viable count may go down when many mice have to be challenged over a longer period of time.
- A note after step 2.6 includes this control. -5.13 I suggest to store the supernatants in multiple aliquots as repeated freeze-thaw cycles will negatively affect detection of many cytokines.
- We thank for the reviewer for the suggestion and have included this modification to step 6.2.

-Figure 3: From the calculations that are shown I understand that the average CFU is taken of 3 different serial dilutions. It may be useful to provide a minimum and maximum number of CFU per plate that is used for counting as it will be difficult to count colonies when there are very dense plates. This may lead to inaccurate counts as bacteria may clump together and form one colony, resulting in lower CFUs.

- Step 6.5 & 6.5.1 are modified as per the suggestion.

-Figure 6: I suggest that undiluted tissue samples are always plated as the bacterial counts on this plate will determine the lower limit of detection.

- Step 6.5.1 & 7.4 now includes this control.

-In the discussion the authors mention that infection of mice with B. pertussis has many similarities with human infection. Some more references are warranted in this section.

- References #21-24 have been added to address this point.

-Mice are challenged by intranasal inoculation. This is a common method but is certainly not the only one and there are also groups that infect mice using aerosols, which is likely closer to human infection. Some discussion of these two methods may be useful.

- We thank the reviewer for the comment and have included this point in the Discussion.

We hope this revised manuscript is suitable for publication in JoVE

Sincerely,

Purnima Dubey, PhD