**National Cheng Kung University**

**No.1, University Road, Tainan City 701, Taiwan, Taiwan**

**Tel: +886-6-2353535 #5678**

Editor of JoVE August 20th, 2018

Dr. Alisha DSouza

Dear Dr. Alisha DSouza:

We wish to thank the reviewers and the editorial board for the excellent reviews on the manuscript entitled “**Convenient and reliable methods to evaluate the virulence and pathogenesis of *Aeromonas* infection in a *Caenorhabditis elegans* model**” (Manuscript ID: JoVE58768). We have revised the manuscript according to the valuable recommendations. The revisions we made are summarized in the following context and are marked with underlines. The draft has been edited by a native English speaker before re-submission. We hope our revised manuscript will be suitable for publication in Froniters in Immunology.

Yours Sincerely,

Yi-Wei Chen

**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.

Ans: Many thanks for the suggestion. We have re-edited the English writing of manuscript with the help from Miss Savana Moore, an English proofreading editor with 15-years experience in English editing of the language center of National Chung-Kung University.

2. Please revise the title to be more concise and clear.

Ans: Many thanks for the suggestion. We have revised the title as follow: “Convenient and reliable methods to evaluate the virulence and pathogenesis of *Aeromonas* infection in a *Caenorhabditis elegans* model”.

3. Please spell out each abbreviation the first time it is used.

Ans: Many thanks for the suggestion. We have revised to spell out each abbreviation the first time it is used. (lines 39-40; 53-54)

4. Please use centrifugal force (x g) for centrifuge speeds.

Ans: Many thanks for the suggestion. We have revised it. (lines 139)

5. JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please remove all commercial language from your manuscript and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents. For example: Prison, Nikon Eclipase, QImaging Retiga, etc.

Ans: Many thanks for the suggestion. We have removed these commercial languages. (lines 193, 219, and 238-248)

6. Please revise the protocol (1.11-1.13, 6.1-6.3, etc.) to contain only action items that direct the reader to do something (e.g., “Do this,” “Ensure that,” etc.). The actions should be described in the imperative tense in complete sentences wherever possible. Avoid usage of phrases such as “could be,” “should be,” and “would be” throughout the Protocol. Any text that cannot be written in the imperative tense may be added as a “Note.” Please include all safety procedures and use of hoods, etc. However, notes should be used sparingly and actions should be described in the imperative tense wherever possible.

Ans: Many thanks for the suggestion. We have revised the protocol to contain only action items. (lines 154-170 and 251-258)

7. Please add more details to your protocol steps. There should be enough detail in each step to supplement the actions seen in the video so that viewers can easily replicate the protocol. Please ensure you answer the “how” question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action.

Ans: Many thanks for the suggestion. We have revised and added more details and references of these protocols. (lines 75-258)

1.1: What is ddwater? What volume of ddwater is used to wash?

Ans: We have revised ddwater to deionized water and added the volume of usage. (line 136)

1.5: What eggs?

Ans: We have revised eggs to “eggs are released from the worms”. (line 143)

1.7: Please provide composition of M9 medium. What volume of M9 medium is used to wash?

Ans: Many thanks for the suggestion. The composition of M9 was described at lines 76-81. And we have revised and added the volume of usage. (lines 146-147)

1.10: How is L4 stage defined?

Ans: We have added the description of the definition of L4 stage of *C. elegans* as follow: “Note: Worms has a white dot in half-moon shape on the middle of body side at L4 stage.“ (line 165)

3.5: Please specify incubation time.

Ans: Many thanks for the suggestion. We have added the incubation time as follow “Incubate the NGM plates at 20oC until the assay is finished.” (line 188)

3.6: How many worms are transferred?

Ans: All the living worms are needed to be transferred. We have revised at lines 189-192.

4.4: Please provide composition of S medium.

Ans: Many thanks for the suggestion. The composition of S medium was described at lines 108-129.

5.7: Please describe how to paralyze the worms. For instance, for how long are the worms placed in M9 medium with 1% sodium azide?

Ans: We have revised the protocol of how to paralyze the worms at lines 238-244.

8. 2.2-2.4: Please break up into sub-steps.

Ans: Many thanks for the suggestion. We have broken up these steps into sub-steps. (lines 76-133)

9. Figure 2: Please define error bars in the figure legend.

Ans: Many thanks for the suggestion. We have revised as follow: “(Error bars: standard deviation, SD)”. (line 309)

10. As we are a methods journal, please revise the Discussion to explicitly cover the following in detail in 3-6 paragraphs with citations:  
a) Critical steps within the protocol  
b) Any modifications and troubleshooting of the technique  
c) Any limitations of the technique  
d) The significance with respect to existing methods  
e) Any future applications of the technique

Ans: Many thanks for the suggestion. We have revised the discussion at lines 336-353.

11. Please combine Finding into the Acknowledgements section.

Ans: Many thanks for the suggestion. We have revised and combined at lines 363-370.

12. References: Please do not abbreviate journal titles.

Ans: Many thanks for the suggestion. We have revised at lines 376-410.

**Reviewers' comments:**  
  
  
  
Reviewer #1:  
  
The paper entitled "Methods to study the virulence and pathogenesis of Aeromonas infection in a Caenorhabditis elegans model" describes the three approaches to evaluate virulence of Aeromonas spp. The authors have tried well to indicate that the adopted methods are convenient and useful. However it is not clear which method is more convenient and reliable to evaluate virulence? Or all three methods should be tested.  
  
It is also not indicated the need and significance of this work if methods are already exist.  
  
The authors have successfully able to generate some useful information and may be accepted for publication provided that the above points and points mentioned below should be considered.  
  
English grammar and scientific language need to be improved.  
Ans: Many thanks for the suggestion. We have re-edited the English writing of manuscript with the help from Miss Savana Moore, an English proofreading editor with 15-years experience in English editing of the language center of National Chung-Kung University.  
  
Abstract:  
All scientific names should be in italics  
\* A. dhakensis, A. hydrophila, A. veronii, and A. caviae  
Caenorhabditis elegans

Ans: Many thanks for the suggestion. We have revised to spell out each abbreviation the first time it is used. (lines 39-40; 53-54)

\* The results of the three methods determining the virulence of C. elegans were consistent.( This sentence is not clear, As you are determining the virulence of bacteria and not C elegans Pls correct it.)

Ans: Many thanks for the suggestion. We have revised at line 44.

\* Clarify change to evaluate

Ans: Many thanks for the suggestion. We have revised and changed “clarify” to “evaluate” at line 46.

\* an understanding change to Our understanding  
Ans: Many thanks for the suggestion. We have revised and changed “an” to “our” at line 47.

Protocols:  
\* References of Protocol used should be given if standard methods were adopted. If modification was done then it should be indicated that what modification done in the standard was done.

Ans: Many thanks for the suggestion. We have revised and added references at lines 76, 82, and 108.

\* Source of Bacterial cultures and C. elegans should be mentioned clearly.  
Ans: Many thanks for the suggestion. We have revised and added description at lines 76-258.

REPRESENTATIVE RESULTS:  
The methods described above offer a convenient way to differentiate virulence diversity among and within Aeromonas species. In addition, this is also a reliable model by which to study the interaction between a pathogen and host.  
  
  
  
  
  
Reviewer #2:  
  
Manuscript Summary:  
This manuscript describes the use of C. elegans as a host to assess virulence determinants for four different species of Aeromonas known to cause clinical infections. Understanding the mechanisms of virulence and toxicity that are responsible for Aeromonas pathogenicity will be helpful for developing new strategies for combatting infection. This new C. elegans - Aeromonas infection model should make it simpler to evaluate host survival and tissue pathology. I think that the procedure is appropriate for publication in JoVE.  
  
Major Concerns:  
None  
  
Minor Concerns:  
  
1) Add more information on C. elegans as a host for various kinds of bacterial and fungal infections.  
Ans: Many thanks for the suggestion. We have revised and added description at lines 64-67.

2) Define all abbreviations (e.g., ddwater). JoVE is not always read by specialists (e.g., some high school teachers use JoVE protocols for their biology labs).  
Ans: Many thanks for the suggestion. We have revised to spell out each abbreviation the first time it is used. (lines 39-40; 53-54)

3) How do you seed ENGM with OP50? What is the appropriate amount of bacteria?  
Ans: Many thanks for the suggestion. We have revised and added the description of seeding step. (lines 152-162)

4) Line 133 (step 3.1). Here and elsewhere (e.g. 4.1), what "respectively" refers to? Looks like all of the bacteria were cultured in LB.

Ans: Yes. All the bacteria are cultured separately.  
  
5) Line 134 (step 3.4). How do you transfer worms? Picking? Washing off and dropping?  
Ans: Many thanks for the suggestion. We have revised and added the description of picking to transfer worms. (lines 186-187)

6) Line 141 (step 3.6) Is the reason for the transfer to avoid progeny of non-sterile N2 worms that will interfere with the assay outcome? What happens if sterile worms (e.g. fer-15;fem-1, glp-1, glp-4, or FuDR are used?)

Ans: Yes. Transfer worms every day can avoid the effect of progeny. Besides, transfer every day can make sure the worms are fed with fresh-prepared bacteria. If using sterile worms, we still suggest to transfer worms every day. (lines 189-192)  
  
7) Line 4.10. Given that assay is run for up to 72h, there is likely some egg-laying happening. Being in liquid condition strongly enhances internal hatching, bagging and death of the mother (e.g. PMID: 10545455). Van Voorhie in his manuscript, entitled "The influence of metabolic rate on longevity in the nematode Caenorhabditis elegans" states that "In liquid as compared to solid medium, C. elegans grows more slowly, has an altered morphology, cannot mate, has reduced fertility and adult worms are subject to up to 90% mortality rates from internal egg hatching (Braeckman et al., 2000; Mitchell et al., 1979; Gandhi et al., 1980)." Was this artifact taken into account in this assay?

Ans: No. The “internal egg hatching” phenotype does not happen because of the use of FudR in the S medium in the *C. elegans* liquid toxicity assay. (lines 108-129)