Author's response to reviews

Title: Virulence difference between the prototypic Schu S4 strain (A1a) and Francisella tularensis A1a, A1b, A2 and type B strains in a murine model of infection

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Author's response to reviews: see over
Dear Dr. Chen,

Thank you for your recent correspondence regarding our manuscript entitled “Virulence difference between the prototypic Schu S4 strain (A1a) and *Francisella tularensis* A1a, A1b, A2 and type B strains in a murine model of infection” for publication in BMC Infectious Diseases. We thank the reviewer’s for a careful evaluation of our manuscript and have made changes to improve the manuscript by incorporating and addressing all suggested changes. We are submitting this revised manuscript along with our response to the reviewer’s comments (see below).

**Editor’s comments:**
(1) Limited amount of new data

-Please see comments below.

(2) The reproducibility of the results and the robustness of the conclusion since the conclusion appears to be derived from a single experiment.

-Please see comments below.

**Reviewer #1 Qingmei Jia:**

**Major Comments:**

-**Comment 1:** Reviewer states that 1) the study is limited to small numbers of one mouse strain and 2) a similar study comparing differences between Schu S4 and other A1a isolates using a different mouse strain would enhance the strength of the conclusion.

1) For this study, seven mice were used per infection. A power calculation to ensure sufficient power to detect differences was performed and is stated in the methods section on page 12. Additionally, there is only one time point (the drop point that is a surrogate end point that was previously evaluated, tested and published by Molins et al. 2012) that
is being used to generate survival curves and to perform bacterial burden comparisons. The use of seven mice for one time point is actually quite high as compared to what other papers in the field describe. For example, in Twine et al. 2005, only 3 mice per strain were used on day 3 comparisons and only 5 mice were used to generate survival curves. In a separate study comparing type A and type B infection (Conlan et al. 2003) only 3 mice were used per time point for comparisons. We feel that the use of seven mice is sufficient given that we were able to obtain statistical differences. The two commonly used mouse strains for F. tularensis research are BALB/c and C57BL/6. The drop point model used here was developed in the C57BL/6 mouse strain. To do this again using a BALB/c model would require additional time-to-death experiments which are not justifiable given that Twine et al. 2005 performed a similar study (although not as extensive as ours and without the use of as well-characterized F. tularensis strains) using BALB/c mice that gave similar results as those found here. A discussion of Twine et al. study is included in the manuscript.

2) We agree that expanding the study with additional A1a strains would be interesting and would further support our findings; however, the focus of the work here was not just to compare Schu S4 and A1a infections but to compare Schu S4 infection to infections by all the F. tularensis subpopulations, A1a, A1b, A2 and type B. Our main objective with this manuscript is to communicate that Schu S4 is not fully representative of type A strains regardless if it is A1a or A1b, but instead causes infection in mice that is more similar to type B strains. Our study nicely corroborates the work previously performed by Twine et al. 2005 where a different mouse strain (BALB/c) was used and gave similar results. Performing a study that focuses on comparisons of Schu S4 to a large number of A1a clinical isolates would be justified to address whether A1a clinical isolates differ in virulence. However, this is beyond the scope of the objectives of this paper. Statements about future studies are included in the discussion.

-Comment 2: Reviewer states that organ bacterial burden comparisons are missing between Schu S4 and A1a isolates and that these comparisons will further clarify our conclusion that differences exist between Schu S4 and A1a isolates.
- We agree that bacterial burden comparisons between Schu S4 and A1a isolates would be interesting to investigate; however, for this study the objective was to determine if Schu S4 is an appropriate surrogate for type A infections, particularly for studies evaluating therapeutics and vaccines. To minimize the number of animals used, we infected mice with an A1b strain, which is reported in the literature as being the most virulent in mice and causing the highest mortality in humans, and showed that mice infected with Schu S4 resulted in a different survival curve and in different bacterial burdens within the blood and spleen as compared to mice infected with A1b.

Minor Comments:

-Comment 1: Reviewer asked that we correct the typo in “and LD100” on page 3, line 8.
-We corrected this typo by changing the word “and” to “an” as suggested by the reviewer.

Reviewer #2 Anders Sjostedt:

Major Comments:

-Comment 1: The reviewer suggests that we rephrase sentences where the word “virulence” is used because our study measures time to death and not “the degree of pathogenicity of a microorganism as indicated by case fatality rates and/or its ability to invade the host tissue”. He also states that we did not measure any symptoms and that it is unclear if onset differed between Schu S4 and MA00-2987 infected mice.

- We agree with the reviewer that the term virulence can be misused. In writing this manuscript we considered this point and in the end we used the definition that is used to instruct microbiology students at Colorado State University which is “the degree or intensity of pathogenicity of an organism as indicated by case fatality rates and/or ability to invade the host tissues and cause disease” (Microbiology, Prescott, Harley and Klein, 7th edition, 2007). The measurement of the rate at which an animal succumbs to an infection is a measure of the “intensity or degree of pathogenicity”. It should be noted that A1b strains have been defined as more virulent because they correlate with higher rates of mortality in humans (Kugler et al. 2009). This F. tularensis subpopulation was also shown to result in more rapid death in mice (Molins et al. 2010). Thus, we argue that a comparison of drop point (surrogate of time-to-death) is a measure of the intensity
and degree of virulence. Additionally, we did measure fever as a symptom and to
highlight this point, we have added data on pages 6 and 7 that address differences in the
length of temperature phases that were observed between Schu S4 and A1a infected mice
and Schu S4 and A1b infected mice.

-Comment 2: Reviewer suggests that we 1) reproduce the experiments because there is confusion
as to what experiment was done when and 2) there was some variability observed in previous
published experiments between Round 1 and Round 2 infections.

1) In the methods section we have better clarified when each experiment was done. This
information was also added to the table created as Additional file 1. Additionally, we
previously showed (Molins et al. 2012) that we can reproduce similar results using this
temperature-based mouse model by infecting mice with strain MA00-2987 in two
different rounds and showing that there was no statistical difference between the two
survival curves created using drop point. It is difficult to justify repeating an experiment
using animals when the model being used is validated, all controls were in place and
there were no discrepancies or inconsistencies observed. Additionally, seven mice were
used per time point to generate the survival curves and for bacterial burden comparisons.
This replicate number of mice is higher than what most investigators use. Based on these
reasons, we do not feel that repeating the study would be justifiable.

2) The slight variability that was observed in the previous published study was due to the
use of multiple strains of each *F. tularensis* subpopulation for the infections. Two strains
each of A1a, A1b, A2, and type B were used with one strain from each subpopulation
used in Round 1 and the other used in Round 2. The survival curves for the two strains
representing each subpopulation were found to not differ statistically. This allowed us to
combine the A1a, A1b, A2 and type B data from both rounds to create one representative
survival curve for each subpopulation (Molins et al. 2010). As also noted above we did
perform repeat experiments with strain MA00-2987 (A1b) (Molins et al. 2011) and found
no differences in the infection results which served as validation for this mouse model.
Furthermore, Figure 1 nicely demonstrates how similar the two resulting survival curves
are when two different A1a strains were used and tested in different rounds of infection.
Comment 3: Reviewer suggests that we add information regarding if bacterial numbers in the blood of MA00-2987 and Schu S₄ correlate with time to death.
- To address this comment, we performed calculations to determine if there is a correlation between the bacterial burden in the blood and/or spleen with drop point for mice infected with Schu S₄ and MA00-2987. Results for this show a moderate correlation between the bacterial burden within the spleen and drop point for Schu S₄ infected mice. We have added this information to the results section on page 8 and have also incorporated the methods used to perform these calculations in the methods section on page 15.

Minor Comments:
Comment 1: Reviewer asks what the passage history is for the strains used in the study.
- We have addressed this comment by adding the following on page 10 in the discussion: “A few of the strains (recent clinical isolates) used in this study are also single colony picks. However, we tested whether this resulted in a selected phenotype by doing a side-by-side comparison of survival curves generated from mice infected with the single colony pick MA00-2987 strain and the original MA00-2987 isolate. There were no statistical differences between the two survival curves generated for these two infections (data not shown)”.

Comment 2: Reviewer suggests we add a supplemental table with the strains used in this study to simplify the reading.
- We created Additional file 1, which is a table that lists the strains used in this study.

Comment 3: Reviewer suggests that we add the route of infection to the abstract.
- We have added the route of infection to the abstract.

Comment 4: Reviewer finds the symbols of the curves in Figure 1 and 2 to be too small and difficult to differentiate.
- To address this comment, we colored each curve a different color and used different symbols for each curve to facilitate the differentiation of each curve. This was done for both Figures 1 and 2 and colors and symbols are defined in the corresponding Figure legend.

Comment 5: Reviewer states that the sentence “Bacterial load was consistently lower in mice infected with Schu S₄ as compared to mice infected with MA00-2987” reiterates what was written in the previous sentence.
-To address this comment, we have removed this sentence from the discussion.

Reviewer #3 Barbara Mann:

Minor Comments:

-Comment 1: Reviewer suggests that we define drop point when it is first used in the paper.

-We have added the definition of drop point to the Results section on page 5.

-Comment 2: Reviewer points out that a study of Schu S4 infection in rabbits has been conducted.

-The study referred to by the reviewer describes pneumonic tularemia in rabbits but it is not an LD50 or LD100 study. Therefore, all of the statements that we make in the manuscript regarding rabbit studies hold true. No changes were made to the manuscript in response to this comment.

We look forward to hearing from you regarding this manuscript.

Sincerely,

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