Rebuttal letter: addressing reviewer’s comments, and concerns regarding JOVE manuscript titled: “Neonatal polymicrobial sepsis: A guide for a controlled mouse model”

Reviewer 1’s concerns:

We thank the reviewer for their careful critique and excellent suggestions to edit the manuscript and video. We have addressed each of the critiques as outlined below.

1. The first major concern regarding the relatability of using adult mouse cecal slurry injected into neonatal mice:

* in the first paragraph of the introduction we discuss some of the limitations regarding studying sepsis in neonatal mice. One major limitation is that the gold-standard used in adult mice, cecal ligation puncture, is not possible in neonatal mice as the mice do not survive the mock-surgery. The point that was brought up where neonatal mice could be exposed to bacteria that they had not previously been exposed to is a legitimate concern, and has been included as a limitation in the discussion section. We have also cited 10 articles that have used the cecal slurry method to study neonatal sepsis (references 4 to 14). It is not the goal of this manuscript to biologically validate the cecal slurry method, but only to standardize its use and improve humane endpoint definitions.

1. The second major concern regarding bacterial viability in frozen cecal slurry stored in dextrose water, in absence of glycerol:

* The article was revised to include more rational behind this decision. The use of frozen stocks was used instead of fresh donors to be both a more cost-friendly method than using weekly shipments of donor mice, and also to utilize excess male mice from in-house breeding that were otherwise being euthanized without any purpose/function that would have led to mouse wastage. This method was adapted from its previous use by other researchers (references 13-15). We have reviewed Pubmed, searching for “Steele 2017 PLOS one” to see if there was a new 2017 reference that was available but of the 9 search results none of the 2017 papers contained the term glycerol. We also searched Pubmed for “2017 PLOS one glycerol 10%” and were not able to find any papers that utilized 10% glycerol to freeze bacteria. We suspect that the 2017 year was in error, as Steele et al from PLOS one in 2014 used 10% glycerol, but if the reviewer could provide a specific reference (title/PMID) we will update the manuscript.
* Furthermore we have revised the article to present data in Figure 2 that showed consistent bacterial viability of the cecal slurry stock that was frozen without glycerol over 6 months of storage at -80 oC which suggests that the lack of glycerol in the storage media is inconsequential.

1. Regarding the third concern about the glycerol and filter size within the introduction:

* Following Reviewer 1’s suggestion about expanding on the range of glycerol used we have reviewed our references and found that 10% glycerol was used by Steele et al in a 2014 publication (already cited). A direct quote from the Steele et al 2014 PLOS one publication is “Similar results were obtained when CS was stored in 5% and 10% glycerol buffer with the colony forming ability maintaining 100% of original capability after cryopreservation at 280 oC for 6 weeks (data not shown).” The text in our manuscript was reformatted to talk about the larger range of Glycerol that has been used.
* The description of the filtration steps was also re-worded to include the accurate two-stage filtration steps that were cited, as recommended.
* Note that these points were brought up simply to acknowledge that there is heterogeneity in published methods used in the study of neonatal mouse sepsis.

1. Regarding the minor concern of the glycerol-induced kidney injury:

* The previously provided references were, as pointed out, only in rats. We have appended another citation that utilized glycerol as an inducer of acute renal failure in mice (Zager, et al, 2006) which is a species-relevant example of the *potential* damage caused by glycerol. This was provided to explain the reasoning behind what could potentially cause additional damage, and was something that was avoided so to not introduce more variables to the challenge model.
* The added Figure 2 displays that storage without glycerol resulted in consistent bacteria viability, and that since the viability is not affected then the removal of a potential confounder is acceptable.

1. Regarding the minor concern about sterile weight paper:

* We have edited the manuscript and the audio to describe the sterilization technique (70% ethanol spray), or the alternate use of sterile petri dishes

Reviewer 2’s concerns:

We thank the reviewer for their comments, recommendations, and positive feedback. Concerns raised by this reviewer were addressed as outlined below.

1. Regarding the first major concern of cross-study applicability of endpoints in the abstract:

* as recommended a note about the requirement of a pilot study was added to the abstract

1. Regarding the second major concern of filtering cecal content stock solutions to remove large debris.

* The dose that we administer to mice is not adjusted for the weight removed in the cell strainer, as the dose is in reference to the original weight of cecal slurry that was resuspended with D5W. When producing each batch of cecal slurry the filtrate is bubbled through the cell strainer until there are no more droplets coming from the cell strainer. From each of these experiments there is a similar consistency of filtered material. From our personal experience there has not been great variability of the challenge dose resulting in different mortality between different cecal slurry preparations which suggests that if there is noise added by not adjusting by filtrate-weight then it is either stable and accounted for proportionally with each preparation, or is a small enough change that does not drastically impact the results.
* never-the-less we recommend throughout the protocol (and ourselves do) a dose-titration of every new cecal slurry batch as a control to determine whether the expected dose of cecal slurry results in the desired lethal dose, so that the experiment is standardized and comparable to previous results, based on the important biological readout of mortality.

1. Regarding the major concern of dead mice and location in the nest

* as suggested we have removed the location of dead mice, as it detracted from the point which was that the sick mice were not being identified early enough to be able to consistently euthanize them at a suitable humane endpoint and that there was excess suffering that could be reduced with an earlier humane endpoint that does not sacrifice accuracy of assigning outcome.

1. Regarding the major concern of hip movement demos and data consistency

* a new video was added to each of the hip-movement sections to provide another example to increase clarity.
* As recommended to address the consistency of scoring we took 60 videos of mice that had been recorded after being placed on their back and gave them to 7 individuals who had only received this manuscript, figures, and video for training (no in person training) and found that the proper assignment of humane endpoints was assigned 97% of the time. This is presented as Figure 4 in the manuscript.

1. Regarding the minor concern of data used as a singular:

* we have gone through the manuscript and corrected these