Journal of Visualized Experiments

A Controlled Mouse Model for Neonatal Polymicrobial Sepsis

--Manuscript Draft--

Article Type:	Invited Methods Article - Author Produced Video
Manuscript Number:	JoVE58574R3
Full Title:	A Controlled Mouse Model for Neonatal Polymicrobial Sepsis
Keywords:	Neonatal mouse; Cecal slurry; Sepsis; Polymicrobial sepsis; Humane endpoint; Behavior monitoring; Health score; Health outcome
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Additional Information:	
Question	**Response**
Please indicate whether this article will be Standard Access or Open Access.	Open Access (US$3000)

Friday, June 1, 2018

Jaydev Upponi, Ph.D.
Science Editor of Immunology and Infection, *JOVE*
Alewife Center, Suite 200
Cambridge, MA, USA, 02140

Dear Dr. Upponi,

As per our email discussions we hereby submit to *JOVE* our manuscript titled, "**Neonatal polymicrobial sepsis: A guide for a controlled mouse model**."

Human newborns are notably susceptible to infections in early life, and sepsis is one of the leading causes of death in this vulnerable population. Understanding disease in this group, and development of effective therapeutics has not been as impactful as work in other age groups, partly because neonatal sepsis is difficult to study in human newborns due to major limitation of sampling small volumes of blood as well as the rapid changes that occur in early life. As an adjunct strategy to investigate the mechanisms and test possible interventions, several animal models have been proposed, including a mouse model of neonatal sepsis (all references are provided in our manuscript). While neonatal mouse sepsis models, similar to the human, also are subject to inter-individual variability, they offer the distinct advantage of allowing standardization of the model to be implemented.

We here report our rigorous approach to standardize methods used to induce sepsis in neonatal mice. This guide will serve not only the larger community working on neonatal sepsis, but all who work with neonatal mouse models, as they provide a detailed range of assessments of the health/illness of newborn mice undergoing experimentation. Specifically, we provide the data supporting the development of an objective a humane endpoint that accurately identifies mice that would not otherwise recover from disease. We also present data showing that the traditional parameters used, such as weight change are not sufficient to separate mice that would recover from those that would not recover. This data-driven humane endpoint will greatly reduce animal suffering, without impacting the ability to experimentally study early life events.

This manuscript has not been published before, has only being submitted to JOVE, and will not be submitted elsewhere during the review process. If the manuscript is published then we will not publish it elsewhere in either similar form or verbatim without express written permission from the editors. The listed authors were involved with the conceptual development, design, interpretation, recording, drafting or revising of the manuscript and video, and have approved the manuscript.

If there is anything else we could to do help in the submission process, please do not hesitate to let us know.

Sincerely,

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TITLE:

A Controlled Mouse Model for Neonatal Polymicrobial Sepsis

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KEYWORDS:

Neonatal mouse, cecal slurry, sepsis, polymicrobial sepsis, humane endpoint, behavior monitoring, health score, health outcome

SUMMARY:

This protocol provides the necessary steps to establish and evaluate neonatal sepsis in 7-day-old mice.

ABSTRACT:

Neonatal sepsis remains a global burden. A preclinical model to screen effective prophylactic or therapeutic interventions is needed. Neonatal mouse polymicrobial sepsis can be induced by injecting cecal slurry intraperitoneally into day of life 7 mice and monitoring them for the following week. Presented here are the detailed steps necessary for the implementation of this neonatal sepsis model. This includes making a homogeneous cecal slurry stock, diluting it to a

weight- and litter-adjusted dose, an outline of the monitoring schedule, and a definition of observed health categories used to define humane endpoints. The generation of a homogeneous cecal slurry stock from pooled donors allows for the administration into many litters over time, reducing the variation between donors, and preventing the use of potentially toxic glycerol. The monitoring strategy used allows for the anticipation of survival outcome and the identification of mice that would later progress to death, allowing for an earlier identification of the humane endpoint. Two main behavioral features are used to define the health scores, namely, the ability of the neonatal mice to right themselves when placed on their back and their level of mobility. These criteria could potentially be applied to address humane endpoints in other studies of neonatal disease in mice, as long as a pilot study is performed to confirm accuracy. In conclusion, this approach provides a standardized method to model newborn sepsis in mice, while providing resources to assess animal welfare used to define early humane endpoints for challenged animals.

INTRODUCTION:

Sepsis is a leading cause of human newborn infectious deaths[1]. Because newborn sepsis is poorly understood, little progress has been made in both the identification of at-risk newborns early during the disease and the development of efficacious treatments or prophylaxes. This necessitates the use of animal models of sepsis to better understand the process and test possible interventions. Furthermore, adult rodents respond differently to sepsis, with statistically significant differences in the number of bacteria to administer to obtain the same lethal dose (LD) and differences in the resulting host response as compared to newborns[2]. Thus, neonatal sepsis has to be studied in neonates. Several adult sepsis models have been used in sepsis research. These include an intravenous challenge with specific organisms implicated in adult human sepsis or cecal ligation and puncture (CLP). CLP is an endogenous challenge model where the cecum is surgically isolated, ligated, and punctured to allow leakage of intestinal contents into the peritoneum, eventually leading to the systemic dissemination of microbes and their products[3]. However, the surgical procedure required to establish CLP is lethal to newborn animals; therefore, an alternate method is necessary to mimic the polymicrobial challenge of CLP to induce neonatal sepsis. The cecal slurry model for neonatal polymicrobial sepsis was developed to address this need, whereby the cecal contents of animals are harvested, suspended in sterile dextrose 5% in water (D5W), and intraperitoneally injected into newborn mice[2]. This has, since, become an increasingly popular model to study sepsis in both newborn and adult animals and has substantially advanced mechanistic insights in the disease's process[4–15].

Given the increasing use of this model and desire of researchers to directly compare results across publications, there is a need for the technical aspects to be well described and standardized across studies. Standardization applies to three aspects of the model, namely, i) the preparation of the cecal slurry stock, ii) the preparation of the challenge aliquots for injection into the experimental animals, and iii) the definition of the humane endpoint whereby animals are deemed nonsurvivors in challenge experiments. Specifically, methods to prepare the cecal slurry stock are often referenced to the original article introducing the model[2]. A brief summary of that model is that cecal contents from adult mice were harvested, suspended in sterile D5W to a concentration of 80 mg/mL, and used within 2 h to inject the experimental animals. This

original model used mice of the same age, from the same vendor location, which were housed in their respective research facilities for less than 2 weeks prior to harvesting cecal contents. The use of in-house bred mice, although reducing the cost from regular vendor delivery and allowing for the use of excess mice of a broader range of sex and age, also substantially increased donor-to-donor variability. This motivated the development of an alternative technique, whereby cecal contents from multiple mice were pooled together to prepare a large stock, which was then aliquoted and stored at -80 °C[13]. This alternate method was adapted by multiple groups[14,15]. However, that adaptation resulted in some technical variations, both in the storage media used (10% or 15% glycerol, or D5W alone) and in the strategy of filtration to remove particulate (multistage filtration through a 860 µm and, then, a 190 µm filter, or individual filtrations through 100 µm or 70 µm filters)[13–15]. The injection of glycerol alone could potentially cause harm, given that 25%–50% glycerol injections have been used as a rodent model of renal injury[16–20]. To avoid unintended side effects of glycerol, the cecal slurry stock preparation for mice in this study is frozen in D5W without glycerol, and tests of bacterial viability from storage at -80 °C are performed. The filtration strategy used in this study is one pass through a 70 µm filter, which has not been directly compared to the other filtration strategies listed.

Lethal weight-adjusted doses of injected cecal slurry may vary from facility to facility and should be titered out to the desired lethality for individual groups. With different challenge doses, the accompanying challenge volumes change by necessity. However, this methodological detail has not been reported before. Furthermore, strategies for standard procedures, such as intraperitoneal injection, are rarely elaborated on within the literature, but individual techniques may affect whether newborn mice leak when injected and impact their final outcomes.

Animal welfare, including a definition of humane endpoint, is a central aspect of this model and in any model of infection and inflammation in rodents[21]. In 1998, the Canadian Council on Animal Care (CCAC) published extensive guidelines for humane endpoint selection, defining the humane endpoint as "any actual or potential pain, distress, or discomfort should be minimized or alleviated by choosing the earliest endpoint that is compatible with the scientific objectives of the research"[22]. Others also caution that humane endpoints must be established based on scientific justification rather than on a subjective interpretation of the animal's state alone[21]. While there is a wealth of resources for clinical, behavioral, and body-condition sign-based criteria for humane endpoint, even in the context of infection and inflammation specifically[21,23,24], none of these, including the CCAC guidelines for humane endpoint[22], mention newborn mice. Thus, objectively and scientifically justified humane endpoints are much more difficult to establish for newborn animals, given both their limited behavioral capabilities and the lack of evidence from criteria like weight loss, which is commonly used for adult mice. Currently, the criteria for the humane endpoint used for 5- to 12-day-old neonatal mice in the cecal slurry literature all reference back to the original manuscript that introduced the model[2]. In this original paper, the definition of humane endpoint for newborn animals was based on two criteria; namely, the location of a mouse outside of the nest (scattering) and the lack of milk spots had been seen to result in death within hours. A complicating matter in assigning a humane endpoint is that milk spots become difficult to see in mouse strains with dark fur, such as the commonly employed C57BL/6J strain, after the first week of life, while sick animals are monitored until the

14th day of life (DOL). Further, dead animals can be found postchallenge when applying these criteria (own observation; unpublished); thus, a more rigorous definition of humane endpoint is necessary to alleviate suffering to experimental animals and avoid mortality in situations where the outcome could be accurately discerned earlier.

All three methodological aspects of the cecal slurry model are presented in a standard operating procedure detailing the preparation of cecal slurry stock, a method for injecting experimental animals that keeps the injection volume constant between doses and reduces the risk of leaks, and a definition of humane endpoint for 7- to 12-day-old mice based on a system of behavioral modeling. Behavioral information of mouse health scores from over 240 experimental animals was collected and grouped by final survival outcome, demonstrating an evidence-driven definition of humane endpoint. The suffering of experimental animals is reduced by identifying moribund neonatal mice at the earliest possible time point, while biologically significant survival outcomes can be inferred by observing key variables. The visual representation of both cecal slurry preparation and neonatal mouse behaviors will serve as an excellent resource to any group studying sepsis or newborn challenge model animals.

PROTOCOL:

All experiments in this protocol have been approved by the University of British Columbia Animal Care Committee under protocol number A17-0110.

1. Tool sterilization

1.1. In a biological safety cabinet (BSC), turn on and preheat the hot bead sterilizer to 250 °C, at least 30 min before use.

1.2. Dip the tools in 70% ethanol.

1.3. Submerge the tools into the preheated hot bead sterilizer for a minimum of 1 min.

NOTE: The handles of the tools will get hot and may burn if left in the hot bead sterilizer for over 1.5 min.

1.4. Spray a mat of paper towels with 70% ethanol to sterilize it.

1.5. Remove the tools from the hot bead sterilizer without touching the sterilized part of the tool to the nonsterile handles of other submerged tools and place them on the ethanol-sprayed paper towels.

1.6. Wait for 30 s to 2 min for the tools to cool down before using them for dissection.

2. Cecal slurry preparation

2.1. Preweigh 15 mL centrifuge tubes (one tube for every five mice being euthanized).

2.2. Euthanize cecal slurry donors according to local animal care guidelines or use the protocol below.

NOTE: Up to 40 C57BL/6J mice between 6 and 12 weeks old were used for cecal slurry preparation, with up to five mice being euthanized at a time.

2.2.1. Transfer the mice to the euthanasia chamber and set the isoflurane anesthesia machine to 5% with oxygen perfusion.

2.2.2. Monitor the mice to observe the loss of the ability to move and to see them entering the surgical plane of anesthesia and finally, stop breathing.

2.2.3. Remove a mouse from the euthanasia chamber, pinch its paw and observe any leg retraction or inhalation. If either is present, return the mouse to the euthanasia chamber; otherwise, continue.

2.2.4. Terminally euthanize the mice by sharp cervical dislocation.

2.3. Perform cecum dissection, using presterilized and cooled tools (see section 1) in a BSC.

2.3.1. Pin the legs of the mouse to an extruded polystyrene foam board using 23 G needles so that the mouse has its abdomen up. Secure and then spray the abdomen with 70% ethanol.

2.3.2. Using sterile forceps and scissors, cut through the skin, loosen the skin from the peritoneal lining with the scissors, and cut open a rectangular region from groin to sternum, and left side to right side. Remove any fur from the peritoneum.

2.3.3. Switch to a new pair of sterile tools to cut through the peritoneum, making a rectangular opening as was done for the skin, switching tools if the ones used contact the skin.

2.3.4. Identify the cecum, which should be running left to right across the body. Disrupt connective tissue to identify the cecum branches from the intestines and cut the cecum away from the intestines. Place the cecum on a sterilized sheet of weighing paper.

NOTE: Weighing paper can be sterilized either by spraying it with 70% ethanol on both sides and leaving it to dry, or by UV irradiation. Alternatively, the cecum can be dissected on a sterile Petri plate.

2.4. Cecal content extrusion

2.4.1. In a BSC, use sterile tools to cut through both ends of the cecum.

2.4.2. Hold the middle of the cecum with sterile forceps and use a flat sterile metal spatula to

gently push the cecal contents out of the cut ends, using a rolling motion and avoiding a scraping motion that could tear the epithelium. Collect the contents and place them into a preweighed 15 mL centrifuge tube.

2.4.3. Pool the cecal contents from a maximum of five mice into the same tube. Weigh the tube again once all the contents have been added.

NOTE: Expect an average of 300 mg, and up to 390 mg of cecal slurry per mouse, requiring 1.8 to 2.4 mL of D5W for resuspension per mouse; therefore, using more than five mice during this step can result in overfilling the 15 mL centrifuge tube.

2.4.4. Wipe the tools clean with an ethanol-sprayed paper towel and resterilize them by repeating steps 1.2–1.6.

2.5. Cecal slurry filtration

2.5.1. Weigh the centrifuge tube filled with cecal contents and calculate the amount of D5W to add to the cecal contents by dividing the weight of the cecal contents by the desired stock concentration in milligrams per milliliter, as in the equation below.

$$volume\ D5W\ (mL) = \frac{weight\ of\ cecal\ content\ (mg)}{desired\ cecal\ slurry\ concentration\ (\frac{mg}{mL})}$$

2.5.2. In a BSC, add the required amount of ice-cold D5W to the 15 mL centrifuge tube containing the cecal contents.

2.5.3. Vortex the 15 mL centrifuge tube vertically and horizontally for 30 s. Check for particulate of more than 1–3 mm in diameter, and if present, continue vortexing until all large particulate has visibly disappeared.

2.5.4. Place a sterile 70 µm cell strainer into a 50 mL centrifuge tube that is placed on ice. Pipette 4 mL of resuspended cecal slurry into the cell strainer and, then to the collection tube. Resuspend the particulate by pipetting up and down 2x–3x. Gently extrude bubbles to increase the filtering speed while stirring the contents with the pipette tip until there are no more droplets being filtered.

NOTE: When mixing, there may be particulate large enough to plug the 5 mL pipette. In this case, repeat the vortexing from step 2.5.3, and if the solution still does not break apart, use the pipette to press the particulate against the wall of the centrifuge tube.

2.5.5. Repeat step 2.5.4, changing cell strainers between each tube of cecal slurry, and pool all contents into the same 50 mL centrifuge collection tube kept on ice, or into a second 50 mL centrifuge tube if the volume of the filtrate exceeds the ice level in the ice box.

2.6. Aliquot the cecal slurry.

2.6.1. If applicable, combine multiple 50 mL cecal slurry filtrate tubes from step 2.5.5 into a larger sterile container (e.g., a 1,000 mL storage bottle). Then, vortex for 15 s and place 20 mL into a new 50 mL centrifuge tube.

2.6.2. Vortex the cecal slurry stock that is in the 50 mL centrifuge tube for 5–10 s, and aliquot 500 µL into three 2 mL cryogenic vials that have a rubber seal, to prevent evaporation over time. Immediately place the master stock and aliquoted cryogenic vial on ice.

2.6.3. Repeat steps 2.6.1 and 2.6.2 until all of the cecal slurry has been aliquoted, vortexing the master stock after every three cryogenic vials to prevent the settling of any particulate and to maintain a homogeneous mixture.

2.6.4. Freeze the cecal slurry aliquots at -80 °C.

NOTE: Expect between three to four stock vials at 500 µL from each adult mouse. Each stock vial should be roughly enough to challenge one litter of eight mice at DOL 7.

3. Sepsis challenge of 7-day-old neonatal mice

3.1. Separate, identify, and weigh neonatal mice.

3.1.1. In a BSC, transfer the neonatal mice to a new cage to keep the mice away from the dam and to reduce stress to the dam.

3.1.2. Remove and rub part of the nesting material with gloves to transfer the cage's smell to the gloves. Then, mold the nesting material into a smaller nest and place it into a new cage without the dam.

3.1.3. Transfer the neonatal mice to the nesting material in the new cage.

3.1.4. Transfer more nesting material to make a second, empty nest in the new cage.

3.1.5. Close and remove the dam's cage from the hood so that the dam is not stressed from hearing any of the neonatal mice's distress.

3.1.6. To track individual neonatal mice within the litter over time, use an ethanol-proof marker to mark one to five dots on the front or reverse of the tail, reapplying every 12–24 h as needed.

3.1.7. Weigh each mouse that will be challenged, placing each into the secondary nest after weighing, and repeat this for all the mice.

3.1.8. Return the entire litter to the dam before preparing the cecal slurry challenge aliquot.

3.2. Calculate the individual weight-adjusted doses of cecal slurry and required dilution with D5W by completing this step for each litter separately, using the calculations below or using the provided worksheet (see **Supplemental File**).

3.2.1. Calculate the milligrams of cecal slurry (*a*) to be administered to each mouse by multiplying the weight of the mouse in grams (*b*) by the desired challenge dose in milligrams of cecal slurry per gram of mouse (*c*).

$$a = b \times c$$

3.2.2. Calculate the individual volume of undiluted cecal slurry stock required per mouse in microliters (*d*) by dividing the milligrams of cecal slurry needed per mouse from step 3.2.1 (*a*) by the stock cecal slurry concentration, 160 mg of cecal slurry per milliliter of D5W (*e*), and multiplying by 1,000 µL per milliliter to convert from milliliters to microliters.

$$d = a \div e \times 1{,}000\ \mu\text{L per mL}$$

3.2.3. Average the stock volume of cecal slurry required per mouse (*g*) by summing the volume of cecal slurry stock (*d*) per mouse in a litter of *n* mice, divided by the number of mice (*n*).

$$g = sum\ (d_{i \to n}) \div n$$

3.2.4. Calculate the average dilution factor for the cecal slurry stock (*h*) by dividing the average injection volume (100 µL) by the average stock volume of cecal slurry required per mouse (*g*).

$$h = 100\ \mu\text{L} \div g$$

3.2.5. Calculate each mouse's specific injection volume in microliters (*j*) by multiplying each mouse's volume of stock cecal slurry required (*d*) by the average dilution factor (*h*), and then round it off to the nearest ten (to match the 10 µL increments of the injection syringe).

$$j = d \times h$$

3.2.6. Calculate the average required volume of D5W to dilute the cecal slurry stock (*k*) by subtracting the average cecal slurry stock (*g*) from the average injection volume (100 µL).

$$k = 100\ \mu\text{L} - g$$

3.2.7. Calculate the total amount of cecal slurry stock in microliters (*l*) by multiplying the average stock cecal slurry per mouse in microliters (*g*) by the number of mice in this litter (*n*) and multiplying by 1.4 to create extra.

$$l = g \times n \times 1.4$$

3.2.8. Calculate the total amount of D5W in microliters (*m*) required to dilute the cecal slurry stock by multiplying the average required volume of D5W (*k*) by the number of mice (*n*) and multiplying by 1.4 to create extra.

$$m = k \times n \times 1.4$$

3.3. Prepare the challenge aliquot after calculating the amount of stock cecal slurry required (*l* from step 3.2.7). In a BSC, thaw the required number of cecal slurry stock vials at room temperature, pipetting its contents to mix.

3.3.1. When there are no more visible ice crystals present in the thawed cecal slurry, transfer the calculated amount of cecal slurry stock (*l* from step 3.2.7) to a sterile 1.8 mL microcentrifuge tube.

3.3.2. Dilute to the required concentration by adding ice-cold D5W as calculated in step 3.2.8 (*m*). Store the challenge aliquot on ice.

3.3.3. Before loading the syringe, mix the microcentrifuge tube by flicking it 20x, followed by 3x of drawing up and expelling 300–500 µL of cecal slurry with a 500 cc 28 G ½ inch insulin syringe.

3.3.4. Draw up roughly 150 µL of diluted cecal slurry into the same syringe.

3.3.5. Flick the syringe to dislodge bubbles from the plunger, draw back slightly on the syringe, and then expel the bubbles.

3.3.6. Dispense the excess cecal slurry back into the microcentrifuge tube until the correct amount of cecal slurry for one mouse, as was calculated for individual mice in step 3.2.5 (*j*), is loaded in the syringe.

3.4. Intraperitoneally inject cecal slurry, according to relevant local animal care institution guidelines, or use the steps outlined below.

3.4.1. In a BSC, separate the neonatal mice from the dam as described in step 3.1.

3.4.2. Scruff the mouse by the back of the neck, using the thumb and index finger.

3.4.3. Secure the mouse's tail across the back of the middle and ring fingers, or on the front of the ring and pinky fingers.

3.4.4. To minimize leaks, tilt the neonatal mouse so that it faces downward and insert the needle bevel of the needle facing up, between the leg and the genitalia, keeping the needle shallow and subcutaneous.

3.4.5. When the needle is inserted for 1 cm, press downward and forward to feel the needle puncture the peritoneum. Slowly depress the plunger, keeping the tip of the needle as steady as possible, as lateral movements could damage the mouse's organs.

3.4.6. Carefully withdraw the needle over 5–10 s, following the same route out as in, relaxing the middle finger during the removal to reduce tension in the mouse's body.

3.4.7. To check for leaks, hold the mouse for a few seconds after the removal of the needle, to allow time for the injection site to close, and observe any leakage or bulging at the injection site, at which point the mouse should not be used in the analysis.

NOTE: Bulging of the skin at the injection site indicates a failed intraperitoneal injection, with the injectant being subcutaneous.

3.4.8. Place the mouse on a paper towel and allow the mouse to take a step. If the mouse is immobile for 5 s, then lightly press the tail.

3.4.9. Pick up the mouse and check for any leakage of cecal slurry at the injection site. If there is a leak, exclude the mouse from the analysis and euthanize the mouse.

4. Mouse monitoring

4.1. Monitor the mice regularly to check them for arriving at a humane endpoint.

4.1.1. Observe the mice 2 h postchallenge for any injection-related complications.

4.1.2. Monitor the mice 12 h postchallenge for sepsis-related morbidity and the identification of mice at a humane endpoint (see steps 4.2–4.3 for criteria).

4.1.3. Subsequently monitor every 4–6 h for the first 2 days, except for 8 h overnight, when the neonatal mice are unattended.

4.1.4. Beyond 2 days postchallenge, monitor 1x–2x per day. If sick mice or mice whose health score decreases are observed, then increase the monitoring frequency to every 4–6 h.

4.2. Monitoring neonatal mice

4.2.1. For any procedure involving neonatal mice, transfer the bedding material to a new cage as described in step 3.1 (for the same reasons as mentioned there). Carefully check for any neonates that are dragged from the nest while nursing. Any mice that are dragged out of the litter while nursing should not be considered to be scattered mice.

4.2.2. When removing the top of the nest, identify any scattering of neonatal mice either away

from the nest or stuck in the nesting material but away from their littermates, with the exception of mice dragged away from the litter while nursing. Refer to the humane endpoint criteria in step 4.5 if a mouse is found scattered.

4.3. Measure the mice's righting reflexes and mobility.

4.3.1. On a paper towel, place a mouse on its back and monitor for its ability to right itself within a maximum of 4 s. When placed on its back, the mouse will fall to either the left or right side, which is when the 4 s count begins.

NOTE: To be classified into the "Rights" group, the mouse must be able to get at least three of four paw pads on the paper towel for 1 s. It is still grouped as being able to right itself if it falls over.

4.3.1.1. If the mouse can right itself, then wait for 8 s to determine its level of mobility.

4.3.1.2. Categorize the mouse as "Rights–Mobile" if it can right itself and explore its environment by taking multiple steps in a row.

4.3.1.3. Categorize the mouse as "Rights–Lethargic" if it can right itself and take a few steps to explore its environment. The mice in this group may fall over while taking a step, look shaky on their feet, and pause between steps.

4.3.1.4. Categorize the mouse as "Rights–Nonmobile" if it can right itself but does not move around a lot. It may still fall over, and if it does not take any steps within 8 s, it is grouped as Rights–Nonmobile.

4.3.2. If the mouse could not right itself, then categorize its mobility based on the observed hip movement.

NOTE: Avoid repeating the monitoring or increasing the length of time the mouse spends on their back because this could affect the scoring system and humane endpoint, as a mouse that fails to right itself within 4 s can sometimes do so if given more time.

4.3.2.1. Categorize the mouse as "Fail to right (FTR)–Mobile" if it is unable to right itself and displays hip movement that exceeds 90° angle from horizontal. Some mice can right themselves if given more than 4 s but should still be categorized as FTR, with mobility scores based on hip movement.

4.3.2.2. Categorize the mouse as "FTR–Lethargic" if it is unable to right itself and displays hip movement below 90° angle from horizontal.

4.3.2.3. Categorize the mouse as "FTR–Nonmobile" if it is unable to right itself and has legs that shake or vibrate but no hip movement. Limbs may extend or retract but do not have lateral

movement. The mouse is visibly ill and has reached the humane endpoint.

4.4. Repeat step 4.3 on the other side of the mouse, recording both sides.

NOTE: See the **Supplementary File** for recording observations.

4.5. Determine whether the mouse is at a humane endpoint and requires euthanasia as outlined in **Table 1**, and below.

4.5.1. Categorize mice into different righting and mobility levels based on the monitoring observations noted in steps 4.3 and 4.4. The mouse's mobility is measured for each side, and the mobile behavior is used to determine whether the mouse requires euthanasia.

4.5.2. Assign any mice with a righting reflex of either (a) FTR–Nonmobile or (b) FTR–Lethargic and found separated from the nest to be at a humane endpoint.

4.5.3. In monitoring time points beyond 20 h postchallenge, classify any mouse with a righting reflex of "fail to right" on both sides as being at a humane endpoint, because the presented data predict with high accuracy that these mice eventually succumb to disease, and do not recover.

4.6. Separate mice that are to be euthanized, as determined in step 4.5. If the monitored mouse is not seen as at a humane endpoint, place it into the second empty nest in the new cage without the dam, and continue with the other neonatal mice.

4.7. Once the entire litter has been monitored, move half of the nesting material into the cage with the dam, reforming a nest with room in the middle for the neonatal mice.

NOTE: An improperly formed nest could cause the mice to scatter and reduce the amount of available care that the dam can offer.

4.8. Transfer the neonatal mice back into the cage with the dam.

4.9. Enclose the litter in the nest by putting the leftover nesting material over the litter and gently pinching it around the lid to secure the nesting material in place.

4.10. Euthanize the neonatal mice separated in step 4.6 according to local institution requirements.

5. Titration of the cecal slurry

5.1. Challenge the mice at the desired challenge dose (section 3) and monitor the outcomes (section 4).

5.2. Observe whether the final outcome results in the desired LD, and if not, repeat sections 3

and 4 with a new litter at a higher or lower challenge dose, adjusting it by 5%–10%.

NOTE: Challenge doses may be similar to **Figure 1B** but need to be titered in each facility and strain of mice.

5.3. Also, observe whether the mice achieve a humane endpoint faster or slower than the expected kinetics in **Figure 1B**, and repeat sections 3 and 4 with a new litter at a higher or lower challenge dose, adjusting it by 5%–10%.

REPRESENTATIVE RESULTS:

Cecal slurry viability stored at -80 °C can be tested over time by serially diluting and plating aliquots of cecal slurry stock on 5% sheep's blood tryptic soy agar followed by 24 h of aerobic incubation at 37 °C. Subsequent counting of culturable colony-forming unit (CFU) content of a cecal slurry preparation was found not to change over a 6 month period, and the viability was not affected by prolonged storage at -80 °C (**Figure 2**). Each donor mouse resulted, on average, in enough cecal slurry to challenge three to four litters (data not shown).

Mice challenged at DOL 7 with cecal slurry to induce polymicrobial sepsis began to reach the humane endpoint within 12 h of the challenge, and polymicrobial sepsis was mostly resolved by 48 h postchallenge, as observed in a Kaplan-Meier survival curve combined from data from over 200 challenged mice (**Figure 1A**). The lethality was dependent on the challenge dose administered, with a 5% change in challenge dose resulting in a roughly 15% difference in survival rate (**Figure 1B**). The mouse body weight was measured at each monitoring visit. Weight loss was seen in all challenged animals, being nondiscriminatory between mice that ended up surviving and those that did not during the initial 24 h postchallenge (**Figure 1C**). After 24 h, most surviving animals began to regain their weight, while all nonsurvivors continued to lose weight and moved to their humane endpoint. However, a small proportion of surviving animals that had retained their righting reflex also continued to lose weight or failed to gain weight, until the end of the experiment, even losing as much as 20% of their initial body weight within 40 h of the challenge. As there was an overlap of weight loss between mice that ended up surviving and those that did not, the change in weight or a threshold of weight loss could not be used as a criterion for humane endpoint while still maintaining the goal of accurately dividing survivors from nonsurvivors.

The behavior of mice was monitored as outlined in the protocol and in **Table 2**. Snapshots of the health categories are displayed (**Figure 3A-C**). These photos show the different health categories of mice who failed to right themselves after being placed on their back and outline the difference between FTR–Mobile and FTR–Lethargic, which is an important distinction. Unchallenged healthy mice of this age do not display FTR-Lethargic activity; therefore, this health category is a marker of disease and a response to challenge. Sick mice displayed FTR-Lethargic symptoms (**Figure 3B**) and could regress toward FTR–Nonmobile (**Figure 3C**), where the upper leg remains parallel with the bottom leg, with little to zero hip rocking movement, which is one of the criteria for humane endpoint. The mice might also recover, gaining increased hip movement and becoming FTR-Mobile (**Figure 3A**). The righting reflex and mobility scores were determined for both the left and right side of each mouse, and the highest score was utilized to determine whether the mouse

had reached a humane endpoint. Behavioral information was collected from over 240 animals challenged with a lethal dose 60 (LD_{60}) of cecal slurry, and 144 humane endpoints were observed (**Figure 3D-F** and **Table 1**). This evidence-driven approach was used to define and refine the humane endpoint across four disease stages, categorized by the experimenters based on both behavioral differences between survivors and nonsurvivors and by the fraction of humane endpoints reached during each time frame. During early experiments, FTR-Nonmobile mice that had no hip movement were consistently found dead within 4–6 h of this behavior being observed. In the collection of the presented information, an FTR-Nonmobile health score was used as criterion for a humane endpoint. From 12–21 h postchallenge, while FTR-Nonmobile mice were euthanized, both surviving and nonsurviving animals displayed very similar behavioral patterns and could not be distinguished in any other way (**Figure 3D**). From 21–48 h postchallenge, the majority of surviving mice regained their righting reflex, while fewer than 1% of the FTR behaviors observed were in animals that would go on to survive the experiment (**Figure 3E**). Thus, mice that failed to right themselves from both sides became an additional criterion for humane endpoint during this time. Between 12 and 20 h postchallenge, 12.5% of the total number of humane endpoints were observed, versus 80.5% between 20 and 48 h, and 7% after 48 h (**Table 1**). A distinguishing feature between mice that ended up surviving and that eventually worsened to a humane endpoint was the loss of the righting reflex, independent of hip mobility (**Figure 3F**). Indeed, between 20 and 48 h after the challenge, a total of 121 mice had failed to right themselves from both sides, with 116 of these mice eventually progressing to a humane endpoint (which represents a 96% accuracy in identifying mice that would not recover). Beyond 48 h after the challenge, 11 mice were observed to fail to right themselves from both sides, and 10 of these progressed to a humane endpoint (a 91% accuracy). Beyond 20 h after the challenge, the number of mice that lost the righting reflex for both sides predicts the final outcome with an accuracy of more than 90%; therefore, this has been added to the humane endpoint criteria, to identify nonrecovering mice earlier and reduce mouse suffering (**Table 1**).

The frequency that mice need monitoring changes over time, due to different rates of death postchallenge, and is outlined in **Table 1**. A mouse was considered to be at its humane endpoint at any point if it had failed to right itself and displayed nonmobile hip movement on both sides, or if the mouse was found scattered from the nest, was unable to right itself, and had lethargic hip movement. Mice with either of these conditions were not expected to be able to rejoin the litter and have been observed to be FTR–Nonmobile within 4–6 h. Starting 20 h after the challenge, a new humane endpoint was added because the presented information shows that the vast majority of mice that FTR from both sides ends up succumbing to disease.

The videos, tables, and resources presented in this manuscript are an effective teaching resource for the correct behavioral assignment of challenged mice. Seven experimenters were asked to watch the training video and read both the protocol and the tables before assigning behaviors to 60 challenged animals. The identification of humane endpoint assignment was accurate both for distinguishing FTR-Nonmobile mice from mice that displayed the other behaviors (**Figure 4A**) and FTR mice from mice that were able to right themselves within the allowable time frame (**Figure 4B**).

FIGURE AND TABLE LEGENDS:

Figure 1: Kaplan-Meier survival curve, cecal slurry dose titration, and weight change following the cecal slurry challenge. (**A**) Survival outcome of neonatal C57BL/6J mice challenged with an intraperitoneal cecal slurry injection at DOL 7. The data for this figure were combined from independent experiments using multiple challenge doses, ranging from 0.7 to 1.3 mg of cecal slurry per gram body weight was administered to these mice. (**B**) Neonatal mice challenged with 0.80 to 0.95 mg of cecal slurry per gram body weight from one cecal slurry preparation display a dose-dependent relationship between the amount of cecal slurry given and the percentage of survival. (**C**) The percentage of change in weight compared to the challenge weight, with the dotted line denoting a 20% loss of weight from the time of the challenge.

Figure 2: CFU concentration in cecal slurry stock stored at -80 °C does not change over a 6 month period. The effect of the cecal slurry age on CFU concentration was tested using linear regression. Each point represents one aliquot of the same cecal slurry preparation, serially diluted and plated over a 6 month period.

Figure 3: Hip mobility categories of mice that fail to right themselves and of animal behaviors at various times postchallenge. Mice that have been challenged with sepsis, when placed on their back, will display signs of morbidity that can be measured by the degree of hip movement. (**A**) A fail to right (FTR)-Mobile mouse shows hip rocking movement of their upper leg exceeding 90° angle from horizontal. (**B**) An FTR-Lethargic mouse shows hip rocking movement but does not exceed 90° angle from horizontal at any point during the 4 s of monitoring. (**C**) Some FTR-Nonmobile mice will extend their leg, bending at the knee, but will show very little (less than 10° angles) to zero hip rocking movement, and the legs will remain parallel to each other. (**D**) Animal behaviors 12–21 h postchallenge show that only FTR-Nonmobile behaviors separate survivors from nonsurvivors. (**E**) From 21 to 48 h postchallenge, only 4 out of the 592 observed FTR behaviors (0.67%) belong to survivors, allowing the righting reflex to predict the final outcome and be used as a new criterium for humane endpoint. (**F**) Beyond 48 h postinfection, 6 out of 131 mice (4.55%) that had a righting reflex went on to become part of the FTR group and were sacrificed by the end of the experiment, justifying sustained monitoring throughout the course of recovery.

Figure 4: Instructional resources result in accurate behavioral classification by independent experimenters. Experimenters trained by watching video accompanying this protocol categorized videos of 60 neonatal mice into different health groups. (**A**) The ability to distinguish a humane endpoint was determined and an average of 97% of behaviors was accurately categorized as FTR-Nonmobile or not, while only 1% of FTR-Nonmobile mice were misidentified. Two percent of the mice were falsely identified as FTR-Nonmobile. (**B**) The identification of the second humane endpoint criterium of correctly distinguishing between FTR mice or those having the ability to right themselves within 4 s of being placed on their back was assigned correctly in 97% of the scorings, while only 0.96% of the mice were incorrectly assigned as righting themselves and 2% of mice were incorrectly assigned as FTR.

Table 1: Frequency of monitoring and humane endpoint criteria in the different stages of disease. Monitoring frequency, humane endpoints observed, the percentage of humane endpoints, and humane endpoint criteria during different stages of disease.

Table 2: Monitoring table and criteria in determining the health score of mice. The provided criteria were used to define health category groups to mice, and to reduce individual variance in assigning health scores.

DISCUSSION:

Postnatal neonatal mice have very limited mobility and fail to right themselves after being placed on their back, even when unchallenged. By DOL 7, the age of mice challenged in this model, a range of movement spanning from Rights–Mobile to FTR–Mobile was observed in unchallenged mice, with an important difference, namely that an unchallenged mouse at this age did not display FTR-Lethargic behavior. Only mice challenged with polymicrobial sepsis were observed to become FTR-Lethargic; therefore, this response can be a marker of disease severity. Being attentive to the cutoff of a 90° angle from horizontal for hip movement allows for the consistent and accurate assignment of lethargic or mobile hip movement in mice. The time frame of 4 s to see if a mouse can right itself was selected because unchallenged mice were able to consistently right themselves within this time frame. Repeated measurement of the same mouse was avoided, while the time to right themselves and the measurement of hip mobility was limited to 4 s, to avoid excessively tiring the mouse, which could otherwise affect its ability to obtain food and warmth and could affect its prognosis to get better. Righting itself from both the left and the right side were observed, and the higher of the scores was used to determine if the mouse was at a humane endpoint, because some mice were found to display FTR-Nonmobile on one side yet have a higher mobility on the other side and be able to recover eventually.

The scoring system used to evaluate mouse health relied on the application of categorical cutoffs to what is a spectrum of movement and, therefore, could be prone to individual bias. Staff was trained together to ensure each person scored the mice the same; however, there will likely remain a level of subjectivity leading to variation. The consistency of scoring was evaluated by having seven researchers who had not previously performed the neonatal mouse monitoring learn the requirements outlined in this protocol and video and, then, independently assign behaviors and determine humane endpoint. A 97% accuracy was observed with scoring performed on 60 challenged mice, suggesting that individual bias does not play a substantial role in the behavioral assignments of this model. The presented behavioral monitoring protocol is based on observations of animals challenged on DOL 7, yet mice younger than 6 days in an unchallenged healthy state cannot consistently right themselves. Thus, the described humane endpoint criteria could not be applied directly to younger mice. If younger mice are used in this experimental model or if a different challenge model with different disease kinetics is applied, then suitable humane endpoint criteria must be developed and piloted to avoid the euthanasia of mice that would otherwise, eventually, recover. The scoring system displays a robust method of improving humane endpoint classification that, with testing and confirmation, could potentially be applied to other models.

Each preparation of cecal slurry or the use of a new mouse strain required the retitration of the cecal slurry dose to administer to achieve a similar lethal dose. Each preparation was standardized by the readout of interest, namely survival, rather than giving the same bacterial count. Each cecal slurry preparation's viable bacterial concentration varied slightly, potentially due to differences in the donor's commensal bacteria or due to variances in the weight left in the cell strainer of the cecal slurry stock postfiltration. During the titration of the cecal slurry, the first two litters were divided into two groups and each half of the litter were challenged with one of the two doses so that each of the doses would be tested in two litters. If the resulting survival rate did not match the required level, then the challenge dose was either increased or decreased by 5%–10% and the experiment repeated. Multiple litters were used to account for litter-to-litter differences that could cause resistance or increased susceptibility to sepsis across a litter. It was important to accurately titer the cecal slurry stock with each new preparation to ensure that the new titration of cecal slurry was comparable to previous cecal slurry preparations. Periods of excess noise and vibration, specifically during the compacting of asphalt and the construction of a nearby building and road, were observed to increase stress in the dams. This correlated with increased rates of cannibalization, and affected the mortality of the survival experiments, even affecting unchallenged mice, indicating that there can be extraneous impacts on neonatal survival that also need to be controlled for.

Prior methods for cecal slurry stock preparation included either the use of fresh cecal slurry or the preparation of frozen cecal slurry, using a variety of methods, including the storage in glycerol that would inevitably be transferred during the challenge. While the use of fresh cecal slurry provides the advantage of having a bacterial composition closest to original cecal contents, there is the risk of variance between individual donor mice due to the variation of commensal bacteria. While this was minimized by using cecal donors from the same vendor with minimal time between arrival and progression of the experiment, this could become a cost-prohibitive option for some laboratories and presented another timing logistics challenge in having age-matched mice available when commencing a cecal slurry experiment in neonatal mice that were 7 days old. An alternative method to using fresh cecal slurry was utilized, where multiple adult donors' cecal contents were pooled, resuspended in D5W, frozen at -80 °C without glycerol, and thawed one aliquot at a time for experiments. The utilization of adult donor cecal slurry to study neonatal sepsis could potentially transfer species of bacteria present in the cecal slurry that the neonatal mouse has not been exposed to, but it is a strategy that allows for the study of sepsis in neonatal mice and has been used to study neonatal mouse biology in the past[13–15]. Cecal slurry was diluted in D5W to provide nutrition to the bacteria, which allowed the establishment of an active infection once the bacteria were injected, and was done to mimic the availability of nutrients in the peritoneal cavity during necrotizing enterocolitis. Glycerol was not included as a stabilizing agent in freezing bacteria because of the potential negative side effects that could arise from glycerol injection alone. If glycerol had been included in the cecal slurry preparation, then the potential damage that glycerol alone could induce would need to be tested for by including a glycerol-only (lacking cecal slurry) injection in mice, which would have increased mouse usage. The bacteria viability of the cecal slurry stocks was tested after freezing the cecal slurry stock without glycerol and was found to be constant, with no change in bacteria concentration in separate aliquots of the same cecal slurry preparation stored at -80 °C over a 6 month period.

This suggests that the storage without glycerol is feasible in providing a consistent biological outcome. The use of a bulk-prepared frozen cecal slurry stock also allowed for the use of mice bred in-house, reducing cost and utilizing male mice that would otherwise be excess from breeding, therefore reducing mouse wastage.

The identification of failed challenges in mice was important to avoid adding extra noise to the system. After undergoing an intraperitoneal injection of cecal slurry, the mice were observed for the presence of a bulge underneath the skin, which indicated a failed injection that was actually subcutaneous. Mice were observed for leaks at the injection site, both immediately after needle removal and after allowing them to take a step after the injection, because mice would sometimes (rarely) leak only after moving the limb of the injection site by taking a step. The presence of a bulge or leak following the injection resulted in removing the mouse from the analysis. After all, either of these could result in a different outcome due to the incorrect amount of cecal slurry injected as a 5% difference in challenge dose has been observed to affect subsequent survival.

Cecal slurry challenge experiments often required varying target lethal doses with varying weight-adjusted doses. Due to this, injection volumes can range from as little as 20 µL and up to 100 µL. The proportionate experimental error associated with dead needle volume also changes along with the injection volume, increasing the difficulty to directly compare different doses. With the simple modification of standardizing the injection volume, this source of variance is removed from the experiment.

The neonatal mouse's behavioral monitoring system used in this protocol is the first of its kind. Researchers intent on conducting ethical research with newborn mice are often faced with the challenging lack of resources to assess the animal's well-being at this age. The presented intuitive and consistent monitoring system begins to address this knowledge gap. Importantly, this evidence-driven approach not only increases the quality of the experimental data obtained but, at the same time, also reduces the suffering of the experimental animals.

ACKNOWLEDGMENTS:

Special thanks to Claire Harrison and the Animal Care Facility at British Columbia Children's Hospital Research Institute (BCCHR) for their support in the animal work, as well as to Dr. Po-Yan Cheng for their guidance and input on animal monitoring and well-being.

DISCLOSURES:

Dr. James Wynn receives support from the National Institutes of Health (NIH)/National Institute of General Medical Sciences (R01GM128452) and the NIH/*Eunice Kennedy Shriver* National Institute of Child Health and Human Development (NICHD) (R01HD089939).

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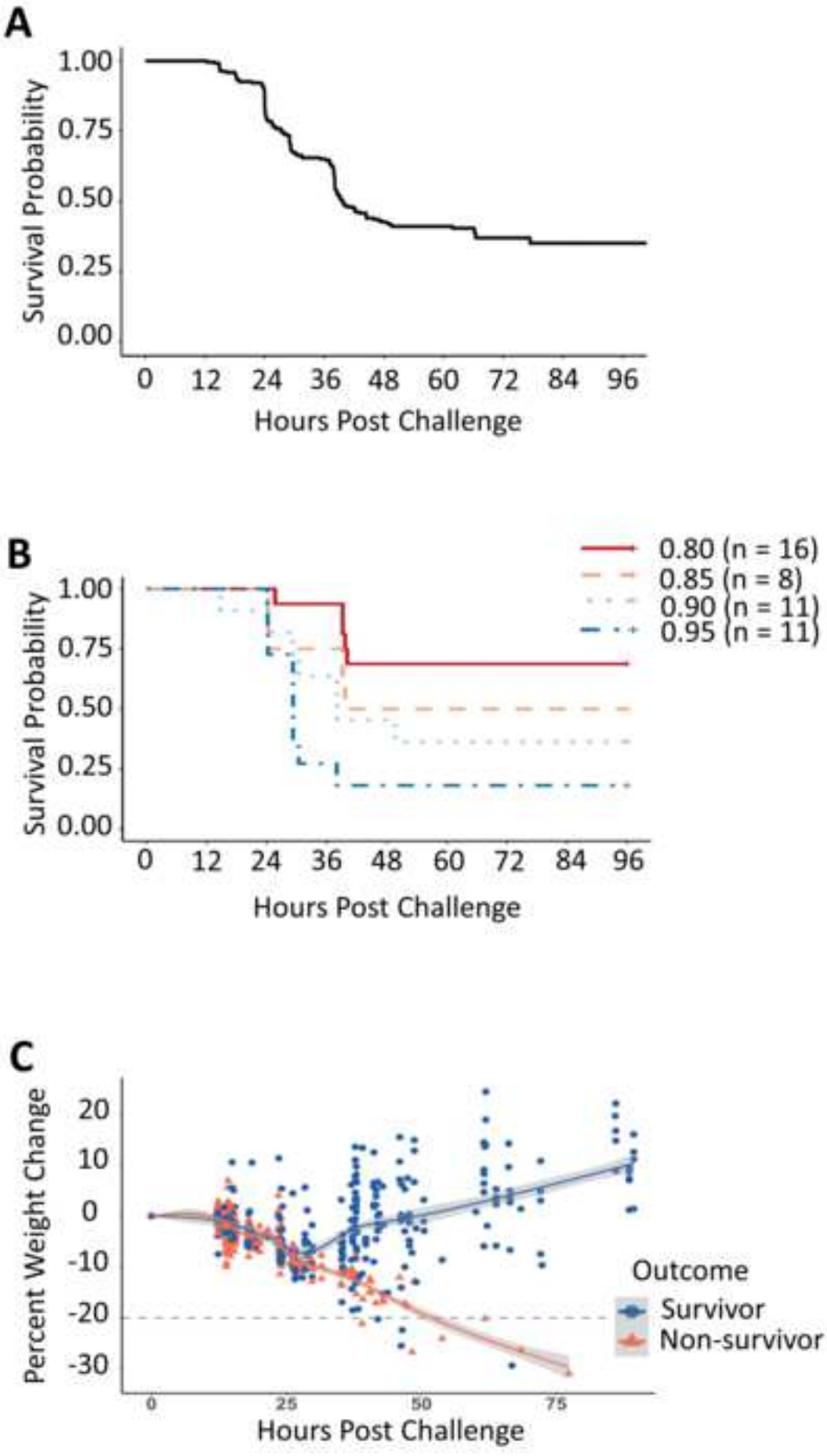
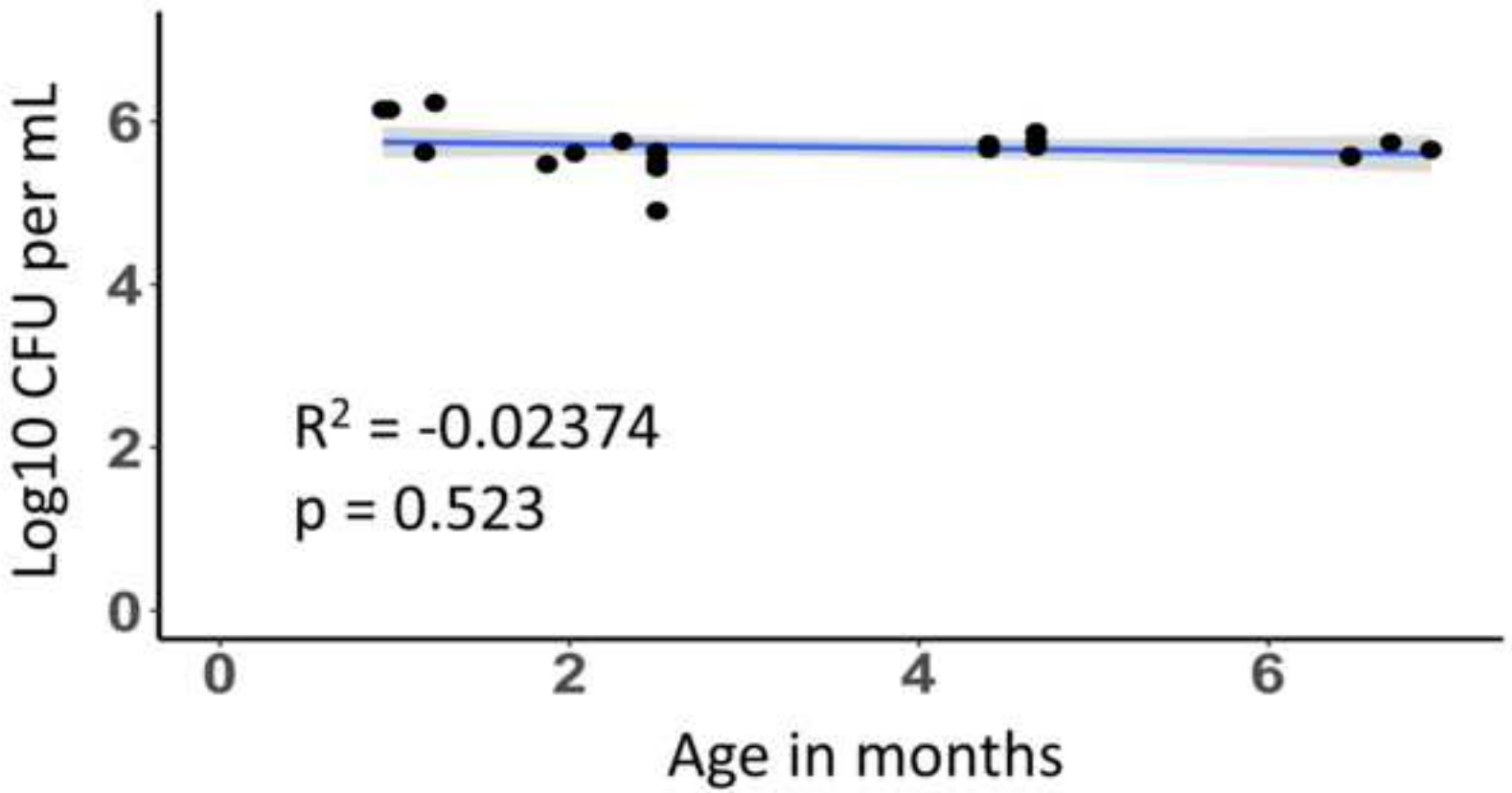
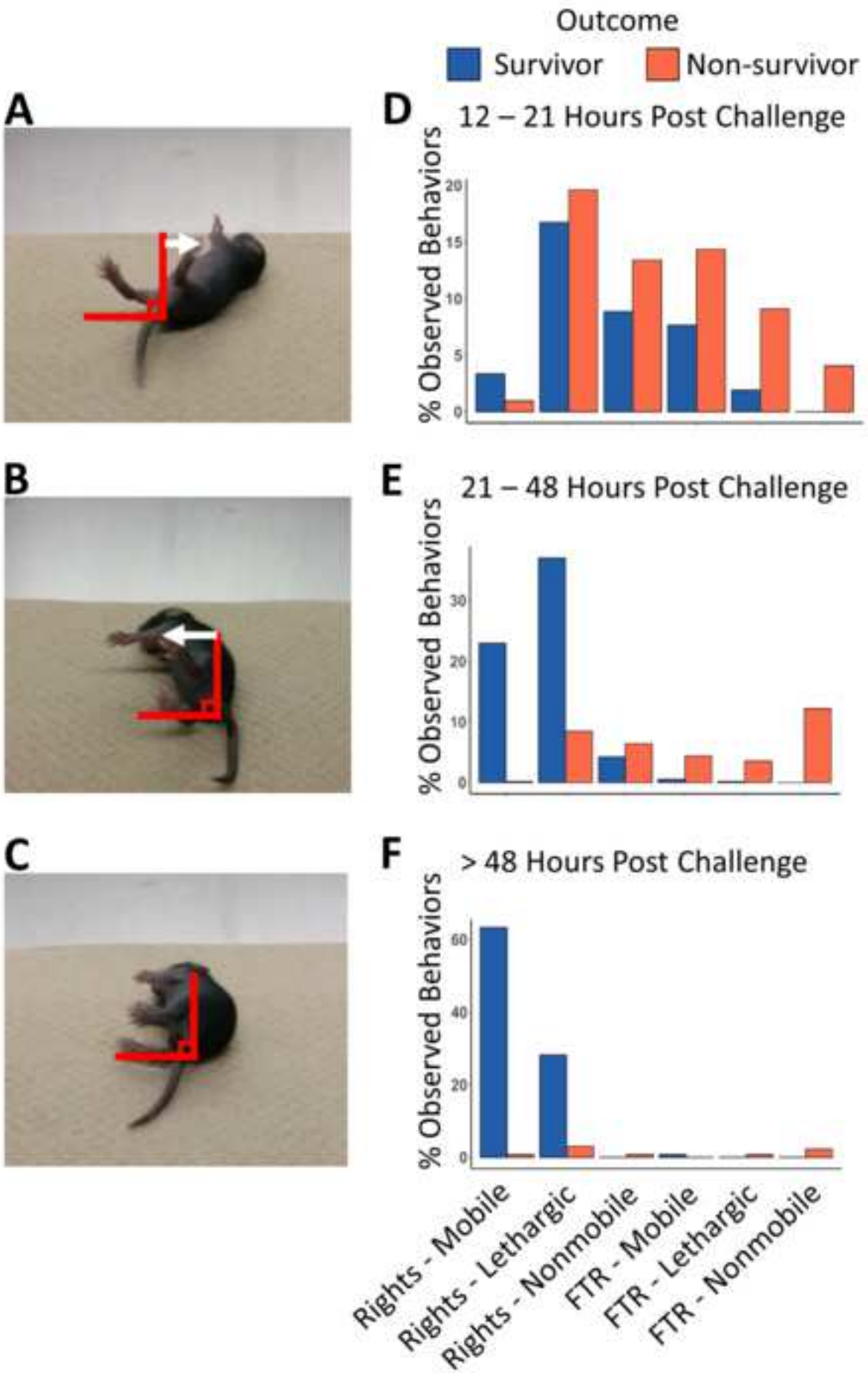
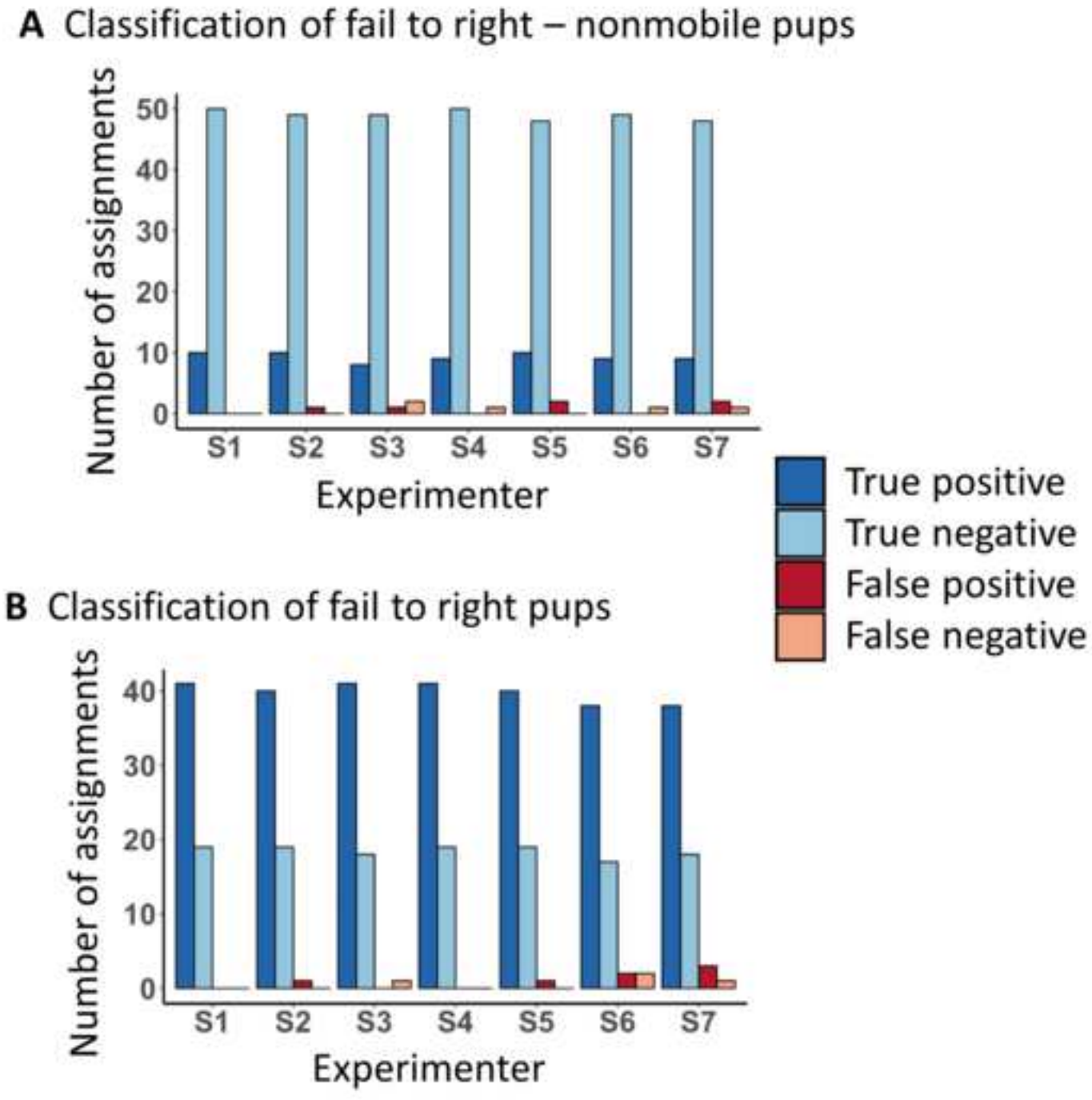

A
Survival Probability
1.00
0.75
0.50
0.25
0.00
0 12 24 36 48 60 72 84 96
Hours Post Challenge
B
Survival Probability
1.00
0.75
0.50
0.25
0.00
0 12 24 36 48 60 72 84 96
Hours Post Challenge
0.80 (n = 16)
0.85 (n = 8)
0.90 (n = 11)
0.95 (n = 11)
C
Percent Weight Change
20
10
0
-10
-20
-30
0 25 50 75
Hours Post Challenge
Outcome
Survivor
Non-survivor



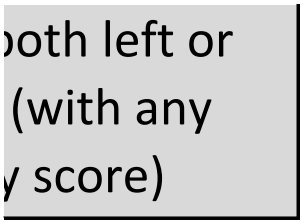




	A: High morbidity, no mortality	B: High morbidity, low mortality	C: High morbidity, high mortality
Hours post challenge	0–12	12–20	20–48
Monitoring frequency	2 h post challenge	every 4–6 h	Every 4–6 h, 8 h, unattended overnight
Proportion of total humane endpoints observed	0/144	18/144	116/144
Percentage of humane endpoints observed	0%	12.5%	80.5%
Humane endpoint	1. FTR–Nonmobile on both sides 2. Scattered from nest and is FTR–Lethargic		1. FTR–Nor both 2. Scattered and is FTR-

criteria		3. FTR on b right side mobilit

D: Low morbidity, low mortality
>48
1–2 times daily, more if needed
10/144
7%
mobile on sides
d from nest –Lethargic


oth left or
(with any
y score)

Righting Reflex	Mobility	Time limit to right after being placed on back
Rights	Mobile	4 s
	Lethargic	
	Nonmobile	
Fail to right	Mobile hips	
	Lethargic hips	
	Nonmobile hips	

Time limit to measure amount of movement (mobile / lethargic / nonmobile)
An additional 8 s
The same 4 s used to measure righting reflex

Mobility scoring Criteria
The mouse takes multiple steps in a row, maintaining forward momentum, and explores its environment. Pup will not fall over.
The mouse can take a step but will stop and pause before taking another. Pup may fall over.
The mouse does not take any steps after righting itself. Pup may fall over.
Has energetic hip movement with the upper leg rotating beyond 90° from horizontal at least once within 4 s.
Hip movement up to but not beyond 90° from horizontal.
Limbs may move by extending and retracting but the hips will not rotate. Pup looks very sickly.

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
0.1 - 20 µL pipette tips	VWR	732-0799	
1.8 mL Microcentrifuge tube	Costar	3621	
100 - 1000 µL pipette tips	VWR	732-0801	
1 - 200 µL pipette tips	VWR	732-0800	
15 mL Centrifuge tube	FroggaBio	TB15-25	
23G1 needles	Becton Dickinson	305145	only the needle, not the syringe, used for pinning mouse to styrofoam
28G 0.5 mL Insulin syringe	BD	329461	
2 mL Cryogenic vial	Corning	430488	
50 mL Centrifuge tube	Fisher scientific	14-432-22	
5 mL pipette	Costar	4487	
6 - 10 week old C57BL/6J adult mice	Jackson Laboratories	664	
7 + day old C57BL/6J neonatal mice	Bred in house	n.a	
70 µm Cell strainer	Falcon	352350	
Defibrinated Sheep's Blood	Dalynn	HS30-500	
Dextrose 5% Water (D5W)	Baxter	JB0080	
Dissecting forceps	VWR	82027-386	
Dissecting Scissors, Sharp Tip	VWR	82027-592	
Dissecting Scissors, Sharp/Blunt Tip	VWR	82027-594	
Ethanol (HistoPrep 95% Denatured Ethyl Alcohol)	Fisherbrand	HC11001GL	diluted to 70% with double distilled water
Ethanol-proof marker; Lab marker	VWR	52877-310	

EZ Anesthesia Vaporizer	EZ Anesthesia	EZ-155	
Germinator 500, Dry sterilize surgicial instrument (Hot bead sterilizer)	Braintree Scientific	GER 5287-120V	
Isoflurane	Fresenius Kabi	CP0406V2	
Micro Spatula	Chemglass	CG-1983-12	
Pipette-Aid	Drummond	4-000-100	
Rainin Classic Pipette PR-1000	Rainin	17008653	
Rainin Classic Pipette PR-20	Rainin	17008650	
Rainin Classic Pipette PR-200	Rainin	17008652	
Scale	Sartorius	BL 150 S	
Specimen forceps	VWR	82027-440 / 82027-442	
Square 1000 mL Storage Bottle	Corning	431433	
Styrofoam board	Any	n.a	
Sure-Seal Mouse/Rat euthanasia chamber	Euthanex	EZ-178	
Tryptic Soy Agar	Sigma-Aldrich	22091-2.5KG	
VX-200 Lab Vortex Mixer	Labnet International	S0200	
weigh paper	Fisherbrand	09-898-12B	

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Article Title:	Neonatal polymicrobial sepsis: A guide for a controlled mouse model
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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.
2. 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 ^{o}C which suggests that the lack of glycerol in the storage media is inconsequential.
3. 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 ^{o}C 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.
4. 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.
5. 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

<u>Reviewer 2's concerns:</u>

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
2. 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.

3. 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.
4. 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.

5. Regarding the minor concern of data used as a singular:
    - we have gone through the manuscript and corrected these

General Information Per Litter **Protocol No. A17-0110**

Experimental Details:

Experiment Type:

Cage label:

Mouse Litter Information

DOB Mother:		Experienced/ Not:	
DOB Pups:		No. pups:	

Treatment Details

Pre-Treatment:		Date/Time:
CS Challenge:		Date/Time:

Weighing Day + Time:

Experimental group:	tail marking	first experiment day weight

Experimental Details:

Experiment Type:

Cage label:

Mouse Litter Information

DOB Mother:		Experienced/ Not:	
DOB Pups:		No. pups:	

Treatment Details

Pre-Treatment:		Date/Time:
CS Challenge:		Date/Time:

Weighing Day + Time:

Experimental group:	tail marking	first experiment day weight

Experimental Details:

Experiment Type:

Cage label:

Mouse Litter Information

DOB Mother:		Experienced/ Not:	
DOB Pups:		No. pups:	

Treatment Details

Pre-Treatment:		Date/Time:
CS Challenge:		Date/Time:

Weighing Day + Time:

Experimental group:	tail marking	first experiment day weight

Routine Monitoring Sheet - Cecal Slurry Peritonitis
Emergency contacts:

Make cecal slurry by:	dose 1:	vol slurry (uL) vol D5W (uL)	(if doing) dose 2	vol slurry (uL) vol D5W (uL)	pup DOB:	Genotype:

pg ______ of ______

Brief Exp Outline:		Cage label:	

Pup No.	Visit Date + Time:					Visit Date + Time:					Visit Date + Time:				
	Who monitored:					Who monitored:					Who monitored:				
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	chal.vol	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right

Pup No.	Visit Date + Time:					Visit Date + Time:					Visit Date + Time:				
	Who monitored:					Who monitored:					Who monitored:				
	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
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	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
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	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right
	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right	Order:	Weight:	Temp:	Score Left	Score Right

<u>*notes on monitoring*</u> each thick-linned box is a monitoring visit. We monitor following challenge at 2hr, 12hr, 18hr, 24hr, 28-30hr, 36-38hr, 44hr, 48hr, 54hr, 62hr, 74hr, 86hr, and 98hr post-challenge. If the mice are quite sick and could degress to humane endpoint we increase monitoring to every 4 hours. The times vary, therefore the date and time of each bolded box is left blank

<u>*note on humane endpoints (HE)*</u> mice are evaluated on whether they meet humane endpoint (HE) criteria at <u>each visit</u>. HE's are measured by ability to righten after being placed on back, and degree of mobility that the mouse shows, and is the earliest moment that we know a mouse would not recover. The criteria are as follows: (any time point) fail to right (FTR) nonmobile mice, or mice scattered from the litter and FTR lethargic, (21 hr - end experiment) fail to right on both sides.
***Score left and score right are mobility scores that measure the behaviour of the mouse.**

58574_Litter and weight adjusted cecal slurry excel calculation sheet

Click here to access/download;Supplemental File (Figures, Permissions, etc.);cecal slurry calculations sheet.xlsx

Cecal slurry dose calculations by stock concentration and weight-adjusted dose

Your experiment date:

[slurry Stock] (mg/ml)	
Dose (mg/g body weight)	

Cage ID:

Pup ID	Pup weight (g)	Inject vol (ul)
		#DIV/0!
		#DIV/0!
		#DIV/0!
		#DIV/0!
		#DIV/0!
		#DIV/0!
		#DIV/0!
		#DIV/0!
		#DIV/0!
		#DIV/0!
Average:	#DIV/0!	

100/Av CS

#DIV/0!

CS / pup	#DIV/0!
D5W / pup	#DIV/0!
No. pups	0

Total (ul):

Slurry stock	#DIV/0!
D5W	#DIV/0!