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1 **TITLE:**
2 A Neonatal Imaging Model of Gram-Negative Bacterial Sepsis
3

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24 **KEYWORDS:**
25 bioluminescence, intravital imaging, *E. coli,* subscapular inoculation, neonates, sepsis,
26 inflammation, cytokines
27

28 **SUMMARY:**
29 Infection of neonatal mice with bioluminescent *E. coli* O1:K1:H7 results in a septic infection with
30 significant pulmonary inflammation and lung pathology. Here, we describe procedures to
31 model and further study neonatal sepsis using longitudinal intravital imaging in parallel with
32 enumeration of systemic bacterial burdens, inflammatory profiling, and lung histopathology.
33

34 **ABSTRACT:**
35 Neonates are at an increased risk of bacterial sepsis due to the unique immune profile they
36 display in the first months of life. We have established a protocol for studying the pathogenesis
37 of *E. coli* O1:K1:H7, a serotype responsible for high mortality rates in neonates. Our method
38 utilizes intravital imaging of neonatal pups at different time points during the progression of
39 infection. This imaging, paralleled by measurement of bacteria in the blood, inflammatory
40 profiling, and tissue histopathology, signifies a rigorous approach to understanding infection
41 dynamics during sepsis. In the current report, we model two infectious inoculums for
42 comparison of bacterial burdens and severity of disease. We find that subscapular infection
43 leads to disseminated infection by 10 h post-infection. By 24 h, infection of luminescent *E. coli*
44 was abundant in the blood, lungs, and other peripheral tissues. Expression of inflammatory

45 cytokines in the lungs is significant at 24 h, and this is followed by cellular infiltration and
46 evidence of tissue damage that increases with infectious dose. Intravital imaging does have
47 some limitations. This includes a luminescent signal threshold and some complications that can
48 arise with neonates during anesthesia. Despite some limitations, we find that our infection
49 model offers an insight for understanding longitudinal infection dynamics during neonatal
50 murine sepsis, that has not been thoroughly examined to date. We expect this model can also
51 be adapted to study other critical bacterial infections during early life.
52
53 **INTRODUCTION:**
54 Bacterial sepsis is a significant concern for neonates that exhibit a unique immune profile in the
55 first days of life that does not provide adequate protection from infection[1]. Neonatal sepsis
56 continues to be a significant U.S. healthcare problem accounting for greater than 75,000 cases
57 annually in the U.S alone[2]. To study these infections in depth, novel animal models that
58 recapitulate aspects of human disease are required. We have established a neonatal mouse
59 infection model using *Escherichia coli*, O1:K1:H7[3]. *E. coli* is the second leading cause of neonatal
60 sepsis in the U.S., but responsible for the majority of sepsis-associated mortality[4,5]. However, it
61 is the leading cause when pre-term and very-LBW babies are considered independently[5]. The
62 K1 serotype is most frequently associated with invasive bloodstream infections and meningitis
63 in neonates[6,7]. Currently, there are no other treatment options beyond antibiotics and
64 supportive care. Meanwhile, rates of antibiotic resistance continue to rise for many pathogenic
65 bacteria, with some strains of *E. coli* resistant to a multitude of antibiotics commonly used in
66 treatment[8]. Thus, it is imperative that we continue to generate methods to study the
67 mechanisms of sepsis and the host response in neonates. These results can help to improve
68 upon current treatments and infection outcomes.
69
70 The immune state of neonates is characterized by both phenotypic and functional differences
71 compared to adults. For instance, elevated levels of anti-inflammatory and regulatory
72 cytokines, such as interleukin (IL)-10 and IL-27, have been shown to be produced by cord blood-
73 derived macrophages and are present at greater levels in the serum of murine neonates[9-11].
74 This is consistent with lower levels of IFN-α, IFN-γ, IL-12, and TNF-α that are frequently
75 reported from neonatal cells compared with adult counterparts[10]. Additionally, the neonatal
76 immune system is skewed toward a Th2 and regulatory T cell response as compared to adults[12].
77 Elevated numbers of neutrophils, T cells, B cells, NK cells, and monocytes are also present in
78 neonates, but with significant functional impairments. This includes defects in expression of cell
79 surface markers and antigen presentation that suggest immaturity[13-15]. Additionally, neonatal
80 neutrophils are significantly deficient in their ability to migrate to chemotactic factors[16].
81 Myeloid-derived suppressor cells (MDSCs) are also found at elevated levels in neonates and
82 recently shown to be a source of IL-27[11]. MDSCs are highly suppressive toward T cells[17].
83 Collectively, these data demonstrate limitations in neonatal immunity that lend to increased
84 susceptibility to infection.
85
86 To study the progression of the bacterial burden and dissect protective host immune responses
87 during neonatal sepsis, we have developed a novel infection model. Neonatal mice at days 3-4
88 of life are difficult to inject in the intraperitoneal space or tail vein. In our model, day 3 or 4

89   pups are administered the bacterial inoculum or PBS subcutaneously into the scapular region. A
90   systemic infection develops and using luminescent *E. coli* O1:K1:H7, we can longitudinally image
91   individual neonatal mice to follow the disseminated bacterial burden in peripheral tissues. This
92   is the first reported model to utilize intravital imaging to understand the kinetics of
93   dissemination of bacteria during sepsis in murine neonates[3].
94
95   Here, we describe a protocol to induce septic *E. coli* infections in neonatal mice[3]. We describe
96   how to prepare the bacterial inoculum for injection, and how to harvest tissue for assessment
97   of pathology, measurement of inflammatory markers by gene expression analysis, and
98   enumeration of the bacterial burden. In addition, the use of luminescent *E. coli* for intravital
99   imaging of infected neonates and quantification of bacterial killing by neonatal immune cells is
100   also described. These protocols may also be adapted to study other important bacterial
101   infections in neonates. The data presented here represents an overall novel approach to
102   understanding infection dynamics in a translatable neonatal sepsis model.
103
104   **PROTOCOL:**
105   All procedures were approved by the West Virginia Institutional Animal Care and Use
106   Committees and conducted in accordance with the recommendations from the Guide for the
107   Care and Use of Laboratory Animals by the National Research Council[18].
108
109   **1.   Preparation of Bacterial Inoculum**
110
111   1.1 Streak a Tryptic soy agar (TSA) plate with an inoculating loop for isolation of a single colony
112   from a freezer stock of *E. coli* O1:K1:H7-*lux* that stably expresses luciferase and carries
113   kanamycin resistance[3]. Incubate overnight at 37 ˚C.
114
115   1.2 The following day allow Luria broth (LB) to come to room temperature (25 ˚C) in a biosafety
116   cabinet.
117
118   1.3 Under a biosafety cabinet hood, identify a single colony from the streaked plate and
119   inoculate it in 3 mL of LB supplemented with kanamycin (30 μg/mL). Incubate overnight at 37 ˚C
120   with shaking (220 rpm). This is the starter culture.
121
122   1.4 Dilute the starter culture 1:100 into a fresh 3 mL of LB under a biosafety cabinet hood and
123   return it to the incubator for 2-3 h at 37 ˚C with shaking (220 rpm). This is the stock culture.
124
125   1.5 Read the optical density (OD) of both the blank and stock culture at 600 nm using a
126   spectrophotometer. Add 100 μL of LB (containing no bacteria) into one well of a 96 well flat
127   bottom assay plate; this is the blank. Then add 100 μL from the stock culture to a separate well.
128   Repeat for two additional replicates. The absorbance is read using a plate reader.
129
130   1.6 Subtract the blank absorbance from the stock culture absorbance value (the OD value) and
131   compare to a previously generated and validated growth curve to determine an approximation
132   of the bacterial density in the stock culture for the preparation of infectious dose.

133
134   1.7 Generate target inoculums depending on the research question. Target inoculum of 2×10^6
135   (low) and 7×10^6 (high) colony-forming units (CFUs) per mouse (/mouse) were used for this
136   study.
137
138   1.7.1.   Divide the target dose per mouse (Dose$_T$) by the estimated concentration of bacteria in
139   the stock culture (Stock) to get the volume of bacteria needed from the stock tube (V_S).
140
141   1.7.2.   Multiply V_S by the number of mice (N_M) that need to be infected along with enough for
142   5-10 extras for the total amount of bacteria required for the infection plus 5-10 additional
143   doses. Remove this volume from the stock tube and add it to a new centrifuge tube.
144
145   1.7.3.   Use the equation below:
146
147    $DoseT / Stock\ =\ VS\ x\ NM$
148                $=\ total\ volume\ (VT)\ of\ bacteria\ to\ be\ removed\ from\ the\ stock\ tube.$
149
150   1.8 Centrifuge the bacteria at 2,000 $x\ g$ for 5 min at 4 ˚C and resuspend the bacterial pellet in 50
151   µL of PBS (pH 7.2-7.6) per mouse to be infected (e.g., for 10 doses of 2×10^6 bacteria each dose,
152   the pellet of 2×10^7 bacteria would be resuspended in 500 µL PBS). Again, it is recommended to
153   prepare more inoculum than is needed. Prepare an equal volume of PBS only for control
154   inoculations. Maintain the infectious inoculum and PBS control on ice until infection.
155
156   1.9 Perform seven ten-fold serial dilutions into PBS in a 96 well plastic bottom dilution plate,
157   and plate 25 µL of the dilutions in duplicate onto quadrant TSA plates supplemented with
158   kanamycin (30 µg/mL) to enumerate the actual amount of bacteria administered. Incubate at
159   37 °C overnight for colony formation prior to enumeration.
160
161   **2.   Animal identification**
162
163   2.1 Arrange a sufficient number of breeding pairs such that litters may be synchronized for age-
164   matched pups. Age variability of ± 1 day is acceptable.
165
166   2.2 Identify a pregnant C57BL/6 female mouse and monitor for birth of the litter in advance of
167   the planned experiment to accurately determine age.
168
169   2.3 To distinguish between control and infected 3- or 4-day old pups, use a pair of small, fine-
170   tipped, iris scissors to snip the ends of the tails of the control pups only. The infected pups do
171   not receive tail snips. Before cutting the tail, disinfect the skin with a cotton ball doused in 70%
172   ethanol. Apply pressure to the end of the tail with a cotton ball or gauze as needed.
173
174   NOTE: This procedure is performed under a biosafety cabinet hood. A tail snip of approximately
175   1/8 of an inch is sufficient.
176

177   2.4 To identify pups within the control and infected groups, use a 1 mL insulin syringe with a 28
178   G x ½'' permanent needle to tattoo the tails of the pups. Before tattooing, disinfect the skin
179   with a cotton ball doused in 70% ethanol. This procedure is performed under a biosafety
180   cabinet hood.
181
182   2.5 To tattoo the tail, apply animal tattoo ink to the tip of the needle. Next, carefully restrain
183   the pup with one hand, with their tail fully exposed. Gently insert the needle under the skin,
184   while maintaining a superficial level of depth, and move the needle parallel with the skin a few
185   millimeters until a small marking, or dot, has been created. Wait a few seconds before slowly
186   removing the needle from under the skin, to avoid excess ink releasing from under the skin.
187
188   2.6 Apply pressure to the wound with a cotton ball or gauze as needed. Remove excess tattoo
189   ink on the surface of the skin with 70% ethanol.
190
191   2.7 Repeat this process with subsequent mice in the infected and control groups, while adding
192   an additional dot with each successive pup tattooed (e.g., pup 1 will have 1 dot on their tail,
193   pup 2 will have two dots on their tail, etc.).
194
195   NOTE: For an additional layer of identification, it is recommended to use separate colors of
196   animal tattoo ink for the control and infected groups.
197
198   **3.   Subscapular inoculation**
199
200   NOTE: For this study, 2 experiments were performed with a low-dose and high-dose group
201   designated for each experiment. In the first experiment, 7 pups were given the low dose
202   inoculum (4 pups were used as controls), and 5 pups from a separate litter were given the high
203   dose (3 pups were used as controls). The pups from experiment 1 provided data for only the 24
204   h timepoint. In the second experiment, 8 pups were given the low dose inoculum (2 pups were
205   used as controls), and 6 pups were given the high dose inoculum (2 pups were used as
206   controls). Pups from experiment 2 provided data for the 0, 10, and 24 h timepoints.
207
208   3.1 Age match pups ≤ 1 day. Assign each litter as either a low dose or a high dose litter. Within
209   a litter randomly assign pups as a control or infected pup.
210
211   3.2 On day 3 or 4 postnatal, record weights of all pups prior to inoculation with *E. coli-lux* or the
212   PBS control. Separate the dam from the pups during this time to ensure they are not moved
213   during the infection.
214
215   3.3 Within a biosafety cabinet using an insulin needle, aspirate either PBS or the *E. coli-lux*
216   inoculum. For this work, inoculums of 2×10^6 and 7×10^6 CFUs per mouse were used. Keep both
217   infectious inoculum and PBS on ice until administration via subscapular injection.
218
219   3.4 Place the neonate on a clean surface in the biosafety cabinet hood and raise the skin at the
220   nape of the neck as if to scruff the pup.

3.5 In the space now created between the skin and the muscle of the animal, insert the needle, bevel up, just beneath the skin and inject 50 µL of PBS or *E. coli-lux*. Simultaneously release the pinched portion of skin to prevent injection backflow.

3.6 Remove the needle slowly and with care. Place pups back with dams after injections are finished.

NOTE: Due their anatomical stage in development, it is technically challenging to administer a tail vein or intraperitoneal injection to neonatal pups at day 3-4. Thus, the subscapular infection route was chosen for this study due to the ease of execution.

4.   Evaluation of disease and endpoint criteria

4.1 Monitor the pups twice daily throughout the duration of infection. Note any abnormalities in appearance.

4.2 Record weights as an objective measurement of morbidity.

4.3 In addition to weight changes, test the ability of the pups to right themselves by positioning the neonate on the dorsal side. Sick animals will be unable to turn over to the ventral side and onto the feet or will complete this action with difficulty.

4.4 Check for the following to mark the animals close to endpoint criteria: less than 85% of normal body weight; decreased movement and inability to right themselves; discoloration of the skin and amore grey or transparent appearance as opposed to pink; feeling cool to the touch, indicative of decreased body temperature and hemorrhagic bruising along the sides, also indicative of advance illness.

NOTE: If the neonates have failed to gain weight over two days and fit any of the descriptions in steps 4.4, they have met endpoint criteria. Pups that receive the high dose often meet endpoint criteria by 24 h. Control pups within the low and high dose litters will be euthanized at the same time to allow for comparative analysis between the control and experimental groups. Proceed to the euthanasia section below.

5.   In vivo imaging of bacterial burden

5.1 Use a microCT imager and software for imaging and subsequent analysis.

NOTE: Pup skin color does not impact imaging quality.

5.2 Place the cage with *E. coli-lux*-infected neonatal mice and dam into a BSL-2 level laminar flow hood. Remove mice to be imaged, and place into a transparent isoflurane chamber within

264   the hood. It is recommended to start with uninfected controls to gauge the amount of
265   isoflurane needed.

266

267   5.3 Open the software on the computer attached to the microCT. Initialize the system and wait
268   for the CCD temperature to lock at 37 °C.

269

270   5.4 Turn the isoflurane vaporizer on and adjust the dial to 5% isoflurane flow. Keep mice in the
271   chamber with this isoflurane mixture for 20-30 s until they stop moving; longer or shorter
272   anesthesia exposure times may be needed for some mice. Once mice stop moving, they are
273   sufficiently anesthetized, and can be imaged.

274

275   5.5 Move mice into the microCT imaging chamber and place them onto the imaging box in the
276   prone position, with noses facing perpendicular to nose cones. Use dental wax to gently
277   restrain the feet on the imaging box to limit any movement. Up to 4 neonatal mice can be
278   imaged at a time.

279

280   5.6 Turn the isoflurane vaporizer down to 2-4% flow to keep mice anesthetized during imaging.
281   Shut the microCT imaging chamber door. Check on the mice a few seconds later. If they begin
282   to move, douse a cotton ball in isoflurane and hold it to the nose of the animal moving for 5
283   seconds to anesthetize. Keep the cotton ball near the animals during imaging. Be careful not to
284   over anesthetize and terminate the mice.

285

286   5.7 Using the software, choose the **Luminescent** option for imaging. Use an excitation filter set
287   to **Block** and the emission filter set to **Open**, 500 nm, 520 nm, 560 nm, 580 nm, 600 nm, and
288   620 nm. There will be seven total emission filters set for luminescence.

289

290   5.8 Image the mice at each time point (0, 10, and 24 h post-infection [hpi]) and save all images
291   to a folder for each time point. Return the pups to the cage with the dam and check that all
292   pups have recovered from anesthesia.

293

294   5.9 To analyze 2D images, open images in the software. Change units to **Radiance (photons)**;
295   this will turn into the **Total Flux (photons/second)**.

296

297   5.10 Only analyze one image set with its multiple emission filters at a time. From each image
298   set, take note of the minimum and maximum radiance values located at the bottom right
299   corner of each image (e.g., if there are 7 emission filters, there will be 7 images, and 7 minimum
300   and maximum values). Repeat for each image set that is to be compared.

301

302   5.11 To determine a scale that will encompass the values and luminescence for all images,
303   locate the lowest minimum value and the highest maximum value for each image set. For this
304   study, the **Open** filter images were used as representative.

305

306   5.11.1. Highlight and open the image of choice to change the scale. On the **Tool Palette**, click on
307   the **Image Adjust** tab and change the **Color Scale** to the lowest minimum and highest maximum

308 values previously identified. Save each image set as a TIFF. Individually analyze each time point
309 in this manner to ensure the correct scale is displayed.

311 5.12 To quantify the total flux (amount of luminescent signal per mouse) for each individual
312 mouse, open an image as previously described in step 5.9-5.10. Open the **ROI Tools** tab on the
313 **Tool Palette** and select the circle tool. Choose **1** circle if analyzing one area of luminescence.

315 5.13 Move ROI to **Overlay** on the area of luminescence. Adjust the size of the ROI if necessary.

317 NOTE: If adjustment is necessary, adjust ROIs in other images comparably to maintain
318 consistency. Choose **Measure ROIs**. The ROI Measurements window will open displaying **Total**
319 **Flux (p/s)**, **Average Radiance (p/s/cm^2/sr)**, **Standard Deviation of Radiance**, **Minimum**
320 **Radiance,** and **Maximum Radiance**.

322 5.14 Record total flux measurements for each image set. This number is the quantified amount
323 of luminescence in the mouse in 2D images.

325 5.15 To make 3D reconstructed microCT images, open the **DLIT 3D Reconstruction** panel on the
326 **Tool Palette** and check all wavelengths to be included under the **Analyze** tab. Select
327 **Reconstruct**.

329 **6.   Euthanasia**

331 6.1 Prepare and label tubes for tissues/organs of interest for necropsy and appropriate
332 downstream applications.

334 6.2 Separate the neonates from the dam in a biosafety cabinet.

336 6.3 Soak a cotton ball in veterinary-grade isoflurane and place inside of a transparent
337 containment chamber.

339 6.4 If collecting blood, prepare a P200 micropipette with a tip and have a 1.5 mL tube with 10
340 μL of 5 mM EDTA as an anticoagulant. A volume of 50-200 μL of blood is expected.

342 6.5 Place a neonate in the chamber and monitor the pup until it becomes motionless.

344 6.6 Quickly, remove neonate and decapitate with scissors. If allowed to breathe fresh air for a
345 prolonged period, the pup can regain consciousness. Neonates have reduced lung capacity
346 relative to adult mice, and, therefore, do not breathe deeply enough for euthanasia by
347 isoflurane alone.

349 6.7 Collect blood from the trunk at the base of the head using a P200 micropipette. To
350 maximize the amount of blood collected, perform this step as quickly as possible following

351 decapitation. Enumerate bacteria in the blood by serial dilution and standard plate counting as
352 described in step 1.9.
353
354 6.8 Sterilize the entire neonate with 70% ethanol prior to excision of tissue samples.
355

7.   Tissue harvest

357

7.1 Within a biosafety cabinet, douse the neonate with 70% ethanol to prevent contamination. Lay the animal on its right side.

360

7.2 Using forceps, grasp the skin at a point between the abdomen and rear left leg and make an incision with fine-tipped surgical scissors. Continue to cut the skin away moving upwards towards the back. Progress until the entire spleen is exposed.

364

7.3 Use the forceps to grasp the spleen and remove it from the abdomen, using scissors to disconnect the connective tissue. Place the spleen in the solution appropriate for its downstream application.

368

7.4 To obtain the lungs, peel back the skin of the chest completely.

370

7.5 Entering at the base of the sternum with scissors held vertically, cut upwards until the rib cage is split.

373

7.6 Use forceps to grasp the right and left lungs individually and remove them from the thoracic cavity. Remove the heart from the lung tissue by cutting with scissors.

376

7.7 Place the lung in the solution appropriate for its downstream application. For RNA isolation, use 500 µL of guanidine thiocyanate/phenol (GTCP). For histopathology, use 5 mL of 10% neutral-buffered formalin.

380

8.   RNA isolation from lung tissue for gene expression

382

8.1 Pre-cool the microcentrifuge to 4 °C.

384

8.2 Mince the lung tissue in GTCP with scissors. Next, homogenize the tissue with a battery-powered homogenizer. Continue until the solution is as uniform as possible. Incubate at room temperature for 3-5 min.

388

8.3 Using filtered pipette tips, add 100 µL of chloroform. Invert the tube for 15 s and incubate 3-5 min at room temperature.

391

8.4 Centrifuge for 15 min at 12,000 $x\ g$.

393

394 8.5 During the spin, prepare 1.5 mL tubes with 500 µL of 70% ethanol. Assemble and label the
395 columns and collection tubes from the RNA isolation kit.
396
397 8.6 Carefully remove the top, aqueous layer without disturbing the interphase layer that
398 formed during centrifugation. Place the aqueous layer in the tubes containing 70% ethanol.
399
400 8.7 Move the ethanol and lysate mixture to the column in the collection tube.
401
402 8.8 From this point on, follow the RNA isolation kit commercial product protocol until the final
403 elution of RNA.
404
405 8.9 Analyze the RNA for purity and quantity. Use immediately or store at -80 °C until further
406 use.
407
408 **9.   cDNA synthesis**
409
410 9.1 Label PCR tubes and set aside.
411
412 9.2 Add 1 µg of RNA to the cDNA reaction mixture for each sample.
413
414 9.3 Add the reagents and template to the PCR tube as described in the cDNA protocol. Add the
415 enzyme to the mixture last.
416
417 9.4 Place PCR tubes in a thermocycler with the following run settings: 5 min at 25 °C, 40 min at
418 42 °C, 15 min at 85 °C and 4 °C final hold.
419
420 9.5 Remove PCR tubes from thermocycler and use immediately or store at -20 °C until further
421 use.
422
423 **10.   Real-time quantitative PCR (qPCR) cycle**
424
425 10.1 Prepare a reaction mix cocktail for each of the genes to be analyzed. Each 15 µL PCR
426 reaction requires 7.5 µL of 2x reagent mix, 0.75 µL of 20X 5'-FAM-labeled gene-specific
427 primer/probe, and 3.75 µL of nuclease-free water. Amplicons typically range from 60-120 bp.
428
429 10.2 Add 3 µL of cDNA template for each experimental group to the appropriate wells.
430
431 10.3 Add 12 µL of the gene-specific reaction mix cocktail to the appropriate wells.
432
433 10.4 Cover the plate with optical adhesive film and centrifuge for 1 min at 1,000 $x\ g$ to remove
434 any bubbles that may have formed in the wells.
435
436 10.5 Place the PCR plate in a real-time PCR thermocycler.
437

438 10.6 Set the run method as follows: 3 min at 95 °C, 40 cycles of 95 °C for 15 s followed by 60 °C
439 for 1 min.
440
441 10.7 Analyze data by normalizing the gene of interest to an internal control and express data
442 from infected samples relative to uninfected control samples using the 2^{-DDCt} formula and a $\log_2$
443 transformation of the numbers.
444
445 **11.   Lung histopathology**
446
447 11.1 Remove the lungs from the neonatal pup as described above.
448
449 11.2 Place the tissue in a volume of 10% neutral-buffered formalin so that the ratio of solution
450 to tissue is approximately 20:1 for 3-7 days.
451
452 11.3 Coordinate with an appropriate histology service for paraffin embedding, sectioning, and
453 hematoxylin and eosin (H&E) staining. For this work, the West Virginia University
454 Histopathology Core was utilized. Alternatively, follow previously described protocols[19].
455
456 **12.   In vitro bacterial killing assay**
457
458 12.1 Remove the spleen from the uninfected neonatal pup as described above and place it in a
459 40 μm nylon basket within a sterile 60 mm Petri dish. Repeat this and pool spleens into one
460 tube to be harvested and homogenized together.
461
462 12.2 Add 5 mL of PBS supplemented with 10% FBS.
463
464 12.3 Disaggregate the tissue using a sterile 3 mL syringe plunger until a single cell suspension is
465 created.
466
467 12.4 Collect the single-cell suspension outside of the nylon basket, transfer to a 15 mL
468 centrifuge tube, and pellet cells at 350 x g for 5 min.
469
470 12.5 Suspend the cells in red blood cell lysis buffer (2 mL for up to 7-8 spleens) and let it stand
471 for 5 min at room temperature to eliminate erythrocytes.
472
473 12.6 Wash splenocytes with PBS and pellet as above.
474
475 12.7 Suspend the splenocytes in 0.25 mL of PBS supplemented with 0.5% BSA and 2 mM EDTA
476 according to expected cell yield.
477
478 12.8 Count the splenocytes using a hemocytometer or other appropriate application.
479
480 12.9 Isolate Ly6B.2$^+$ (myeloid population of granulocytes/inflammatory monocytes) cells with
481 immunomagnetic beads according to manufacturer protocol.

10

482

483 12.10 Seed Ly6B.2$^+$ cells at a density of 1×10^5 cells per well in a black or white 96-well plate in
484 a volume of 0.1 mL of DMEM that contains 10% FBS, 2 mM glutamine, and 25 mM HEPES
485 (complete medium).

486

487 12.11 Enumerate bioluminescent *E. coli* as described in section 1 and prepare the bacterial
488 inoculum at the desired multiplicity of infection (MOI) in a final volume of 0.1 mL. This is best
489 done by making what is necessary for all wells at a common MOI in batch.

490

491 12.12 Add 0.1 mL of bacterial inoculum or complete medium alone as a control. Incubate the
492 multi-well plate at 37 °C and 5% CO_2 for 1 h.

493

494 12.13 Replace the media with 0.2 mL of fresh complete media that contains gentamicin (100
495 µg/mL) by gently removing media with a pipette and adding fresh media with a new pipette tip.
496 Return the culture to incubation for an additional 2 h.

497

498 12.14 At 3 h post-infection, measure the luminescence in each well of the lidded culture plate
499 from the bottom using a plate reader and then return the culture to incubation.

500

501 12.15 Repeat measurements of luminescence at other desired time points.

502

503 **REPRESENTATIVE RESULTS:**
504 This protocol induced bacterial sepsis in neonatal mice, and we used longitudinal intravital
505 imaging, enumeration of bacteria in the blood, histological assessments of pathology, and
506 inflammatory cytokine expression profiles to study the course of disease. Signs of morbidity
507 were observed in neonatal pups infected with both low (~2×10^6 CFUs) and high (~7×10^6
508 CFUs) inoculums of *E.coli* over time. Pups that received the greater inoculum displayed more
509 prominent signs of distress that included reduced mobility, the inability to correct their posture,
510 and impaired ability to maintain an upright position by 24 h post-infection (hpi). There was,
511 however, a range of morbidity as some pups appeared worse than others. Immediately
512 following infection, one low-dose animal died due to isoflurane exposure during an imaging
513 session to establish baseline. By 24 hpi, two of six high-dose animals succumbed to the systemic
514 infection (33.3% mortality). Infected pups that received either a high or low dose inoculum
515 weighed significantly less than their control littermates at 24 hpi (**Figure 1A,B**). All the pups that
516 received the higher inoculum met endpoint criteria at 24 hpi. As such, all the infected pups in
517 this group were euthanized following imaging. Bacteria in the blood were enumerated for a
518 subset of mice that received the lower inoculum, and for all animals that received the higher
519 inoculum since they were all euthanized. The results from two experiments performed similarly
520 indicate that while most animals had high levels of bacteria in the blood (CFUs/mL) at 24 hpi,
521 some animals did not have detectable bacteria in the blood (**Figure 1C**). The latter suggest that
522 they cleared the infection by this time point. As expected, pups that received the higher
523 inoculum had nearly three orders of magnitude more CFUs/mL at 24 hpi relative to pups that
524 received the low dose inoculum (**Figure 1C**).

525

526 Live animal imaging of luminescent bacteria further confirmed the dissemination of bacteria
527 and increase in growth in neonatal pups over time at 10 and 24 hpi (**Figure 2** and **Figure 3**).
528 Additionally, using intravital imaging with the microCT, we were able to identify infection foci,
529 including the brain **(Figure 2B)**, lungs **(Figures 2B, 3A,B)**, and other peripheral tissues **(Figure**
530 **2B)**. The lungs of some highly infected mice demonstrated opaque regions consistent with
531 inflammatory consolidation that co-localized to luminescent bacterial signal **(Figures 3A)**. These
532 regions of presumed inflammatory exudate are not found in uninfected control lungs **(Figure**
533 **3A)**. Further evidence of a pronounced inflammatory cytokine response within the lungs of
534 infected pups is demonstrated by gene expression analysis of IL-1β, IL-6, and TNF-α. A
535 significant increase in expression relative to controls was observed for all three cytokines in
536 both the low and high inoculum groups (**Figure 4A**). Histopathology of the lung was also
537 examined at 24 hpi in control and infected pups. Despite similar inflammatory cytokine profiles,
538 a progressive increase in pathology was commonly observed from the lower to the higher
539 inoculum. Compared with tissue from uninfected controls, the lungs of infected pups showed
540 notable inflammatory changes, thickening of the alveolar wall, increased alveolar
541 hemorrhaging, and inflammatory infiltration (**Figure 4B**). In the most severe infections, the
542 pulmonary congestion and areas of hemorrhage contributed to a massive reduction in open air
543 space (**Figure 4B**). Collectively, these results demonstrate that in our model of early onset
544 neonatal sepsis, dissemination of luminescent bacteria can be followed over time from a
545 subscapular inoculation site to important infection foci and cause significant inflammation and
546 pathology in severely infected animals.
547
548 To study host factors that contribute to bacterial killing by innate immune cells such as
549 monocytes, macrophages, and neutrophils, we developed a sensitive in vitro assay to measure
550 bacterial clearance. Ly6B.2$^+$ cells isolated from the spleens of neonatal mice were infected with
551 bioluminescent *E. coli* at a range of MOIs for 1 h and then treated with gentamicin to kill
552 extracellular bacteria. At 3, 6, 20, and 48 hpi, intracellular luminescence was measured with a
553 multimode reader. As expected, with increasing MOI, more luminescent signal was recorded at
554 3 h (**Figure 5**). Gradually, this signal was lost, indicative of bacterial clearance (**Figure 5**). This
555 assay is amenable to supplemented cytokines, neutralization of secreted effectors, and the
556 addition of pharmacological inhibitors of cellular pathways to study interventions that may
557 promote bacterial clearance and serve to improve outcomes in the neonatal sepsis model
558 described here.
559
560 **FIGURE AND TABLE LEGENDS:**
561
562 **Figure 1: Changes in body weight and bacterial replication in septic neonatal mice. (A,B)**
563 Individual mouse weights within a group (low and high) expressed as a percentage of the mean
564 weight of littermate control pups. Data are presented as mean percentage ± SEM. Individual t-
565 tests at each post-infection time point reveal significant differences at 24 h between control
566 pups and pups that received the low inoculum ($p<0.0001$) **(A)**, or between control pups and
567 pups that received the high inoculum ($p=0.0031$) **(B)**. **(C)** CFU/mL in the blood at 24 hpi were log
568 transformed and presented as the mean ± SEM. Mann-Whitney test reveals a trend towards
569 significance between the low and high dose inoculums ($p=0.0882$).

570

Figure 2: Intravital imaging demonstrates dissemination of bacteria in neonatal mice over time. (A) A representative neonatal mouse (#1) infected with an inoculum of ~2 x 10^6 CFUs is shown at time 0, 10, and 24 hpi. A colorimetric scale with the minimum and maximum radiance values per time point are displayed for each time point. Mice at 0 and 10 h arere displayed on both their time point scale and the 24 h scale to demonstrate changes in bacterial growth over time. **(B)** Representative 3D reconstructed microCT images of the same neonatal mouse at 10 and 24 hpi are shown. Each time point has images at overhead, transaxial, and coronal perspectives. In the transaxial image at 24 hpi, the plane has moved toward the periphery of the mouse to better display infection foci in the peripheral tissues. White arrows indicate the brain and kidney at 10 hpi and the kidney and lung at 24 hpi.

Figure 3: Lungs are a site of major infection during bacterial sepsis in neonates. (A) Representative 3D reconstructed microCT images of a neonatal mouse (#5) infected with an inoculum of ~7 x 10^6 CFUs are shown at 24 hpi compared to an uninfected control. Both mice are displayed in the transaxial perspective and lungs are indicated by white arrows. The infected mouse was placed on two radiance (photons/sec) scales. Scale #1 includes all 6 wavelengths (500, 520, 560, 580, 600, 620 nm) and scale #2 includes only 500, 520, and 560 nm wavelengths. This second scale allowed to visualize an increased signal in bacteria in the lungs because lower wavelengths are more highly absorbed by tissue and produce stronger signal. **(B)** Representative 3D reconstructed microCT images of a neonatal mouse (#4) infected with an inoculum of ~7 x 10^6 CFUs are shown at 24 hpi. This time point has images at the overhead, sagittal, transaxial, and coronal perspectives. White arrows indicate infection foci in the lungs.

Figure 4: Inflammation and associated histopathological findings in the lungs of septic neonates. At 24 hpi the lungs were harvested from pups that received ~2 x 10^6 or 7 x 10^6 CFUs or uninfected controls. **(A)** RNA was isolated and the expression of IL-1β, IL-6, or TNF-α as determined relative to uninfected controls by quantitative real-time PCR using the formula 2$^{-\Delta\Delta Ct}$. The data is shown as the mean log$_2$ transformed change in expression ± SEM for each inoculum as indicated. Statistical significance was determined using unpaired t-tests of ΔCt values between individual cytokine genes and the internal control in the 95% confidence interval. Asterisks indicate $p < 0.01$. **(B-D)** Histopathologic sections of H&E stained lung tissues (20x, area of interest constructed into clipping mask and enlarged for clarity) are shown. Lung tissues from a representative uninfected control **(B)** or infected neonate at the low **(C)** or high **(D)** inoculum are shown. Yellow arrows indicate alveolar thickening **(C)** or hemorrhaging **(D)**. Scale bar = 500 μm.

Figure 5: An in vitro assay for bacterial clearance. Ly6B.2$^+$ cells were isolated from the spleens of uninfected control neonates. Cells were seeded in 96-well plates and infected with luciferase-expressing *E. coli* O1:K1:H7 at a multiplicity of infection (MOI) of 10, 50, or 250 as indicated. After 1 h, the medium was replaced with fresh that contained gentamicin (100 μg/mL). Mean relative light units (RLU) ± SE for an individual experiment representative of multiple are shown. Statistical significance in the 95% confidence interval was determined using unpaired t tests with Welch's correction; asterisks indicate $p < 0.05$.

13

614
615 **DISCUSSION:**
616 Our subscapular infection model for inducing bacterial sepsis in neonatal mice is a novel
617 method to study the longitudinal spread of bacterial pathogens in real time. Intravital imaging
618 provides the opportunity to explore bacterial dissemination in real time in neonates. This is
619 critical to understand the kinetics of bacterial dissemination and to further study the host
620 response and damage at the appropriate phase of disease. Mouse pups are administered a
621 subcutaneous, subscapular injection of bacterial inoculum. This injection technique is simpler
622 than other commonly used alternatives, such as the tail vein and intraperitoneal infections, as it
623 requires less precision within an injection site. This is important given the small size of the pups.
624 The intravital imaging allows for a longitudinal assessment of bacterial proliferation and
625 dissemination into peripheral tissues and the central nervous system over time without the
626 need to sacrifice the animal. Similar imaging approaches and technologies have been used for
627 the study of cancer biology and metastasis[20,21]. Furthermore, while another study has cited the
628 use of bioluminescent imaging during an *E. coli* infection in neonatal rats[22], here, we have
629 applied the approach to neonatal mice, wherein our methodology allows evaluation of bacterial
630 kinetics during murine sepsis. Visualization of the bacteria is based on emission of
631 bioluminescent light at various wavelengths from bacteria (e.g., bacterial luciferase activity)
632 within the animal. Bioluminescence is then visualized through a cooled charged coupled device
633 (CCD) camera. The resulting visualized bioluminescence can then be reconstructed into a 3D
634 image that shows both spatial- and temporal-dependent effects of bacteria within an animal.
635 For an added, more nuanced layer of data acquisition, successful animal identification through
636 tail tattoo allows for a repeated measures assessment of individual pups across time and the
637 identification of possible outliers within a given experimental group.
638
639 The most successful application of the described model requires accuracy in preparation of the
640 bacterial inoculum. Here, we describe an optimized method for bacterial preparation using a
641 pre-established and validated *E. coli* growth curve that reduces variation between the target
642 and actual inoculum. This allows experimental reproducibility at an intended inoculum. The
643 inclusion of two inoculums in our model demonstrated dose-dependent outcomes in blood
644 CFUs, mortality, and lung pathology. However, some aspects of the disease trajectory were not
645 dose dependent. The failure to gain weight in infected animals was not dependent on the
646 inoculum at 24 hpi. Additionally, similar levels of inflammatory cytokine expression were
647 observed in the lung in response to infection with both inoculums. Whether or not this pattern
648 would be replicated in all tissues where bacteria were observed, such as the kidney, liver,
649 spleen and brain, remains to be determined. In addition to sepsis, *E. coli* O1:K1:H7 is associated
650 with meningitis in the neonatal population[23]. This brain infection occurs when bacteria from the
651 periphery invade and penetrate the blood brain barrier. Future studies will explore this aspect
652 of the model through analysis of changes in tight junction protein expression, as well as test
653 different ranges of bacterial inoculums. An additional modification during intravital imaging
654 includes the addition of a singular cotton ball, doused in isoflurane, which is placed
655 approximately 2-3 inches away from the mice during imaging. In response to previous
656 experiments wherein the neonatal pups have regained consciousness during the imaging
657 session, preventing accurate image acquisition, we now place the cotton ball close enough to

658 the mice to keep them continuously anesthetized during imaging. However, it is important this
659 is not done so close that they fail to recover from the anesthesia.
660
661 Although flexible and easily adaptable for the study of the kinetics of different bacteria in
662 various animal and disease models, our protocol has some limitations to consider. The first
663 limitation to consider is that the subscapular route of infection does not mirror a natural route
664 of transmission. However, a primary objective in the development of our model from the
665 outset was to establish an easily reproducible mode of delivery that could be used to establish a
666 systemic infection that replicates aspects of human disease. Therefore, in this report, we
667 describe a model of human early onset sepsis disease syndrome, not a model of natural
668 transmission. There is an established model of oral delivery in neonatal rats that replicates
669 some aspects of common human transmission, such as initial colonization of *E. coli* infection in
670 the alimentary canal and subsequent dissemination to the bloodstream and peripheral tissues,
671 including the brain[22]. The model established by Witcomb and colleagues also incorporates
672 bioluminescent *E. coli* and intravital imaging. Moreover, it is crucial to minimize isoflurane
673 exposure, as well as inject, tail tattoo, and handle pups as quickly as possible without
674 compromising accuracy and precision of the techniques in an attempt to mitigate stress levels
675 for both the neonates and the dams. In some cases, if the pups experience enhanced human-
676 induced and/or experimental manipulations, the dams can stop nursing and caring for the pups,
677 resulting in decreased survival unrelated to the infection. Similarly, pups that are exposed to
678 isoflurane for prolonged periods beyond the approximate 10 minutes of an imaging session
679 have an increased risk of death; thus it is crucial to supply just enough isoflurane to sufficiently
680 anesthetize the mice, but not enough to euthanize them. A final point of consideration is the
681 limit of sensitivity. Tissues in which less than 10^4 CFUs/mL *E. coli* were enumerated the
682 luminescent signal recorded falls at the low end of the detectable range, according to the
683 scaling method used in the imaging software[3]. Thus, some tissues may be colonized with low
684 levels of bacteria but appear without visible bioluminescence.
685
686 Currently, most studies utilize adult methods of bacterial dissemination, such as intraperitoneal
687 (i.p.) and tail vein injections for neonates. Pluschke and Plekonen analyzed the effect of *E. coli*
688 K1 on neonatal mice through i.p., tail vein, and oral infections[24]. This study demonstrated that
689 different genotypes of mice with immunodeficiencies are more susceptible to the K1 strain;
690 however, many aspects of host immune response to infection as well as the mechanisms for
691 bacterial spread are left unaddressed. Deshmukh and colleagues infected neonatal mice
692 intraperitoneally with *E. coli* K1 or *K. pneumoniae* and measured CFUs in the spleen and liver at
693 72 hpi[25]. This study also analyzed some aspects of host response to infection based on pre-
694 exposure of mice to antibiotics. However, thorough investigation of bacterial dissemination to
695 peripheral tissues and blood over time in parallel with inflammatory profiling in the same tissue
696 (other than granulocytosis) was not addressed. Other studies of neonatal sepsis in mice with
697 *Staphylococcus aureus*, *Staphylococcus epidermidis*, Group B *Streptococcus*, and *E. coli* explore
698 varying aspects of the host immune system in response to infection. However, none of these
699 studies utilize intravital imaging to explore the kinetics of bacterial dissemination or localization
700 of infection foci[23-26]. Our method of infection and intravital imaging, combined with bacterial
701 burden assessment and inflammatory profiling of peripheral tissues, allows us to

702 comprehensively examine aspects of both the host and pathogen during infection, providing a
703 more precise understanding of host-pathogen interplay during sepsis.
704
705 We intend to utilize this infection and imaging model to further our understanding of early-
706 onset neonatal sepsis using a variety of pathogenic bacteria commonly responsible for sepsis in
707 neonates, including Group B streptococci, *K. pneuomoniae*, and *Listeria monocytogenes*. This
708 infection model will allow us to longitudinally compare dissemination of different bacterial
709 pathogens in parallel with the host response in neonates. In addition, this model is adaptable to
710 the adoptive transfer of specific (fluorescently conjugated) immune cell types to study their
711 migration to sites of infection and subsequent influence on the host response and control of
712 bacteria. This grants the opportunity to better understand the host-pathogen interactions that
713 occur during sepsis in early life in ways that have not been previously demonstrated.
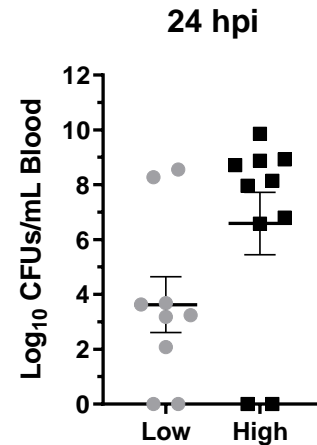714

717

718 **DISCLOSURES:**
719 The authors have no conflicts of interest to disclose.
720

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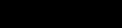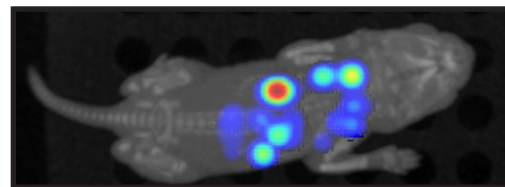785   infected tissues during neonatal bacterial sepsis. *Journal of Immunology.* **191** (9), 4759-
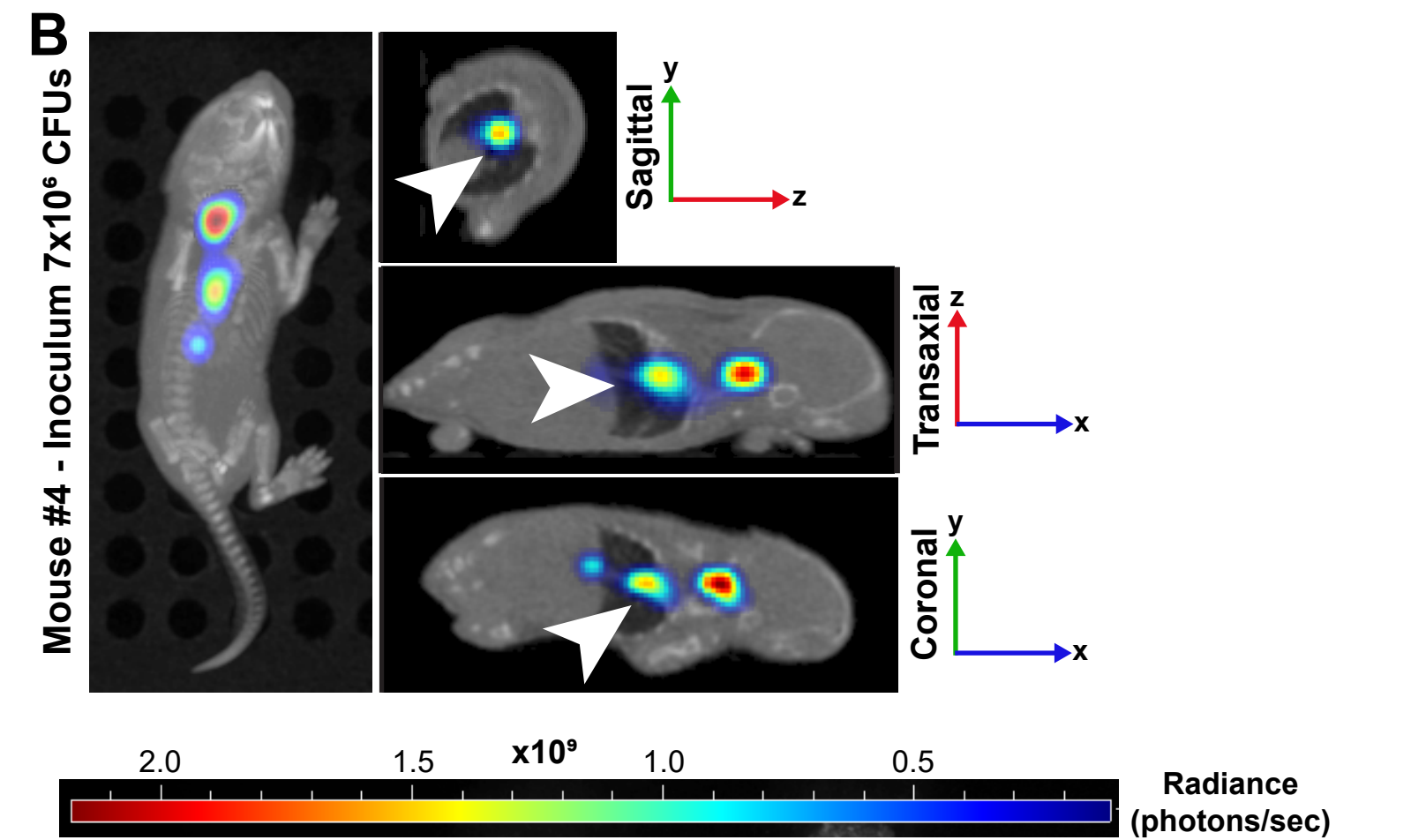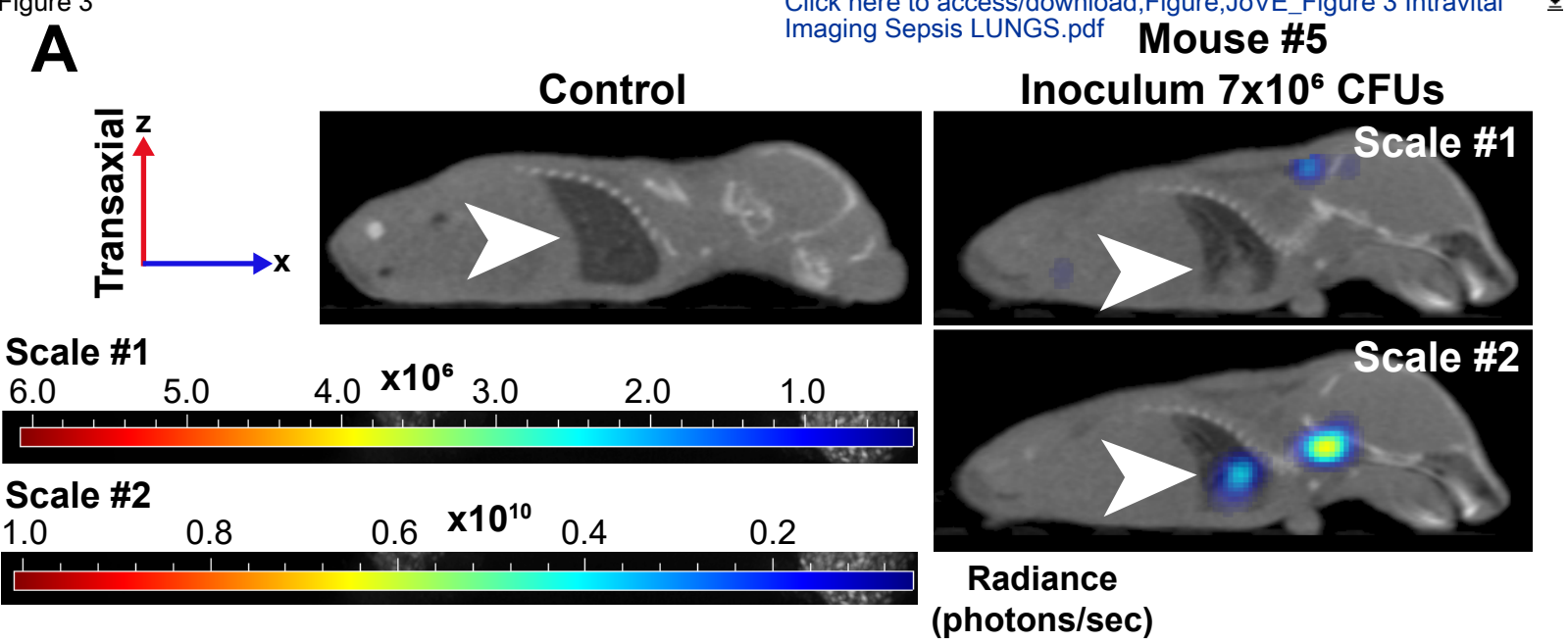786   4768 (2013).
787

Figure 1

A

B

C

Figure 2

A

0 hpi

10 hpi

24 hpi

Mouse #1 - Inoculum 2x10⁶ CFUs

x10⁷

x10⁹

x10⁹

x10⁹

Color Scale 0 h
Min = 7.53x10⁴
Max=1.03x10⁷
Radiance
(p/sec/cm²/sr)

Color Scale 24 h

Color Scale 10 h
Min = 2.82x10⁷
Max=8.81x10⁸
Radiance
(p/sec/cm²/sr)

Color Scale 24 h

Color Scale 24 h
Min = 6.24x10⁷
Max=2.84x10⁹
Radiance
(p/sec/cm²/sr)

B

Perspective

10 hpi

24 hpi

y / z / x

Transaxial

z / x

Coronal

y / x

Figure 3

A

Transaxial

Control

Mouse #5
Inoculum 7x10⁶ CFUs

Scale #1

Scale #2

Scale #1
6.0 5.0 4.0 **x10⁶** 3.0 2.0 1.0

Scale #2
1.0 0.8 0.6 **x10¹⁰** 0.4 0.2

Radiance (photons/sec)

B

Mouse #4 - Inoculum 7x10⁶ CFUs

Sagittal

Transaxial

Coronal

2.0 1.5 **x10⁹** 1.0 0.5

Radiance (photons/sec)

Figure 5

Figure 5

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
1 mL Insulin Syringe	Coviden	1188128012	Inoculum or PBS injection
10% Neutral Buffered Formalin	VWR	89370-094	Histopathology
ACK Lysis Buffer	Gibco	LSA1049201	Bacterial clearance assay
Animal Tattoo Ink Paste	Ketchum	KI1482039	Animal identification
Animal Tattoo Ink Green Paste	Ketchum	KI1471039	Animal identification
Anti-Ly-6B.2 Microbeads	Miltenyi Biotec	130-100-781	Cell isolation
Escherichia coli O1:K1:H7	ATCC	11775	
Escherichia coli O1:K1:H7-*lux* (expresses luciferase)	N/A	N/A	Constructed in-house at WVU
E.Z.N.A. HP Total Extraction RNA Kit	Omega Bio-tek	R6812	RNA extration
DPBS, 1X	Corning	21-031-CV	
Difco Tryptic Soy Agar	Becton, Dickinson and Company	236950	Bacterial growth
IL-1 beta Primer/Probe (Mm00434228)	Thermo Fisher Scientific	4331182	Cytokine expression qPCR
IL-6 Primer/Probe (Mm00446190)	Thermo Fisher Scientific	4331182	Cytokine expression qPCR
iQ Supermix	Bio-Rad	1708860	Real-time quantitative PCR
iScript cDNA Synthesis Kit	Bio-Rad	1708891	cDNA synthesis
Isolation Buffer	Miltenyi Biotec	N/A	Bacterial clearance assay
IVIS Spectrum CT and Living Image 4.5 Software	Perkin Elmer	N/A	Intravital imaging
LB Broth, Lennox	Fisher BioReagents	BP1427-500	Bacterial growth
EASYstrainer (Nylon Basket)	Greiner Bio-one	542 040	Cell strainer
SpectraMax iD3	Molecular Devices	N/A	Plate reader
Pellet Pestle Motor	Grainger	6HAZ6	Tissue homogenization
Polypropylene Pellet Pestles	Grainger	6HAY5	Tissue homogenization
Prime Thermal Cycler	Techne	3PRIMEBASE/02	cDNA synthesis
TNF-alpha Primer/Probe (Mm00443258)	Thermo Fisher Scientific	4331182	Cytokine expression qPCR
TriReagent (GTCP)	Molecular Research Center	TR 118	RNA extration

West Virginia University.
SCHOOL OF MEDICINE

July 6, 2020

Dear Dr. Bajaj:

Thank you for the review of our manuscript and the opportunity to submit a revised version of our previous submission (JoVE61609). All of the editorial comments were considered and addressed. The exception is for item 11 regarding permission to use a figure from a previous publication as this is not applicable. For simplicity sake, we did not list these since they were uniformly acknowledged without need for additional response. We have also carefully evaluated the reviewers' concerns and comments and have addressed these below. Our responses follow reviewer comments in red font. Changes to the text in the manuscript have been highlighted. We look forward to your reply regarding our revision.

Sincerely,

Cory Robinson

Reviewers' comments:

Reviewer #1:

Manuscript Summary: This method paper was very well written and mostly clear throughout. My suggestions below might help to clarify points for the reader but overall this is a good model for the neonatal sepsis field.

Major Concerns:

While this is not a 'major concern' the manuscript needs to contain further information about: Statement of ethics i.e. permissions required for the animal work.

This was added following the Discussion.

Methods Step 3. How many pups are used per experiment/treatment/timepoint? How are pups randomised - e.g. same litter has mix of control and infected pups, or are different litters treated separately. Are litters synchronised (e.g. using Whitten effect) or are dams checked for fertilisation and there are enough matings set up to anticipate sufficient litters together. How much variability in pup age is acceptable? What is the age limit of the infection model. Does pup skin colour have any effect, and at what age does hair start growing and does this impact imaging quality.

The total number of pups used per experiment, treatment (control, low dose, or high dose), and timepoint (0, 10, and 24 hours) has been included in section 3.1. As well, it has been clarified that each litter received its own treatment (i.e., one litter is designated to receive the high dose inoculum, and another, separate litter that is age-matched within a day is designated to receive the low dose inoculum. Within each litter, 2-4 mice were used as controls. For this work, timed-pregnant females were purchased from Jackson Laboratories. As mentioned in the third paragraph of the introduction in the manuscript, in this model the pups are infected on postnatal day 3 or 4. This detail was also added to section 3.2. Pup skin color does not impact imaging quality, nor does the growth of hair, although the latter is not relevant in the first 2 days of infection reported here, as C57BL/6 neonates do not begin to grow hair until approximately postnatal day 6.

Abstract - the authors should not include a 10^4 cfu limit on detection in the abstract if they do not demonstrate this in the methods. This would have been a helpful addition and would help to clarify the limitations of the model. Since this appears again in the discussion (line 535) the authors should include this data unless it is already published elsewhere.

The reference to a CFU limit has been removed from the abstract. The text in the Discussion has been modified and previous related work cited. Although a formal experiment to discern a strict limit of detection has not been performed, a range of bacteria in specific tissues and corresponding luminescent signal has been reported and can be used for an understanding of relative sensitivity.

Minor Concerns:

Title - add in 'imaging'

This was added as requested.

The following points are numbered according to the steps in the Methods.

Steps 1.7 and 1.8 are currently written in quite an unclear way to understand the protocol. 1.7 should start with the target dose specified - i.e. that each mouse will receive inoculums of $2x10^6$ or $7x10^6$, and then direct the reader to the calculation method. It should also state that the reader might need plenty of extra for the injections. It would not be a good idea to only prepare sufficient for the specific number of pups to be injected.

Bulleted steps along with an equation were added to step 1.7 to enhance clarity.

1.8. This should state that bacteria should be resuspended so that inoculum is in 50 μl dose per mouse. i.e. if preparing 10 doses at 2×10^6 per inoculum, then 2×10^7 bacteria should be suspended in 500 μl PBS. And again make the suggestion that the reader would need plenty of excess to enable sufficient for preparation of injections. What is pH of PBS used (and recipe?)

Additional text was added to enhance clarity. The pH for PBS was also added.

3. Can the authors comment on alternative infection routes and why they chose this one?

As mentioned in the third paragraph of the Introduction in the manuscript, there are technical difficulties surrounding alternative infection routes (i.e., tail vein, oral gavage, or intraperitoneal injections), and thus why subscapular injection was selected. Our method offers an ease of inoculation that results in a systemic infection that models aspects of human neonatal sepsis. We have also added brief text to section 3.6 of the methods.

4.6 - 'steps 4-6' Do the authors mean steps 4.4-4.6? Are pups euthanised if they reach any, or all, of the criteria? Authors use a 24 h timepoint as an endpoint in some experiments, can they expand on timepoint decisions. Authors should note at what timepoint control pups are also killed. They touch on this in the results section (line 390) but it would be helpful to flag up in the methods section.

The numbering has been corrected in the revised manuscript.

Pups are euthanized once they meet any of the endpoint criteria (which are now bulleted under step 4.4). The 24 h time point is a critical period for pups that receive either inoculum and peak disease is often observed. Pups that receive the high dose frequently meet endpoint criteria before 48 h post-infection. Control pups are also euthanized at this time point to allow for comparative analysis between the two experimental groups (control vs infected).

5.4 - this stage should be at 5.2, before the neonates are anesthetized, to allow stage to heat to 37°C.

This change was made as requested.

5.7 - Can the authors expand on the reason for choosing multiple emission filters - is this specific to the 3D microCT reconstruction and is there an optimal setting for the bioluminescence. It would be helpful for the authors to also give more settings here, e.g. do they use varying exposure times, binning preference, etc.

In general, firefly luciferase has a broad emission spectrum which ranges from 500 to 700 nm, which is why we choose to use so many emission filters so that we can get as much signal from throughout the mouse as possible. Exposure times are chosen by the imaging software during imaging and is based on how strong the signal is from the mice. Binning is also chosen by the software.

6.4 - it would be helpful to suggest size of pipette that might be needed. E.g. a P200 Gilson? How much blood do they expect to collect by this route.

We use a universal P200 pipette tip. It is likely to expect a volume of 50-200 µL of blood per animal. This has been added to the text in the manuscript in section 6.4.

7. Do the authors image the excised organs to get a specific bioluminescence measure of tissue distribution prior to downstream applications? This is a useful way to demonstrate that bioluminescence originates in a specific organ.

We have not imaged the excised organs directly, but this is a useful suggestion to incorporate into future studies. However, it is possible that the tissue could dry significantly during the approximate 10 min required to image and thus influence downstream applications. This would need to be carefully evaluated. Alternatively, harvested tissues could be placed in wells of a multi-well dish with equivalent amounts of PBS and bioluminescence quantified with a multi-mode reader or luminometer.

9.2 Authors should state use of BioRad iScript cDNA Synthesis Kit

The authors have removed the use of the BioRad iScrpit Synthesis Kit from the manuscript text (as with all other references to commercial items), but have included it in the Table of Materials.

10.1 - it might be useful to have a little more information on the authors' approach to qPCR, e.g. a quick introduction on what size fragments the authors generally aim for, or refer to prior publications for the same cytokine expression to direct the reader to more in depth methodology. The PCR recipe needs appropriate concentration of primer/probe. Do the authors use SYBR green or Taqman approach i.e. DNA dye or fluorogenic probe, and any particular reason for this.

We have provided the concentrations of all qPCR reaction components in section 10.1. As well, we have included primer information in the Table of Materials. Amplicons in these assays typically range from 60-120 bp in length. We prefer Taqman assays over SYBR green incorporation because it minimizes signal from non-specific amplicons and/or primer hybridization. However, this is only our laboratory preference and we do not consider ourselves experts on PCR chemistry. For this reason, we have not provided a recommendation for one chemistry over another.

12.1 'Pool each spleen' - do the authors collect all the spleens from different animals together? Is this just spleens from uninfected animals (before addition of further bioluminescent E. coli in 12.11). Or are there no spleen-localised E. coli in the infected animals?

For isolation of cells for in vitro assays, the spleens from uninfected animals are pooled. Bacteria are not added to the cultured cells until step 12.1.

How many experimental repeats do they recommend.

We recommend 3 independent experimental replicates to demonstrate reproducibility for most reported observations. However, this could vary and require additional replicates depending on the experimental details. We have established an in vitro assay that can be used for a variety of questions and is amenable to cytokine neutralizations, pharmacologic inhibitors of cellular pathways, other interventions, etc.

12.9 - what cell type is isolated by Ly6B.2+ extraction.

Ly6B.2$^+$ cells are a population of myeloid cells that include neutrophils and inflammatory monocytes.

12.10 - how long after seeding cells should the killing assay be performed. Is there a time limit on the viability of the Ly6B.2+ cell culture? Are there any stages at which the experiment can be paused - e.g. whole spleen in the fridge, splenocytes frozen (details of freezing media and for how long), etc.

The Ly6B.2$^+$ cells can be infected immediately after seeding. We have not empirically determined a time period at which the cells can be cultured resting before infection. Since neutrophils are present in this cell population and do not have extended viability, a significant waiting period is not recommended. We have not measured the viability of Ly6B.2+ cells over time. This can be done at reviewer request, but we will need sufficient time to generate a new litter of neonates, age them to at least 4 days, isolate cells, and perform the viability assay. Spleens should not be stored chilled or frozen for later primary cell isolation. This would result in significant loss of viability and selection of certain mononuclear populations that would better survive the freeze-thaw process. It is also not clear what effect this would have on cellular activity and phagocyte function.

12.12 should read 0.1 mL

This change has been made.

Lines 385-396. It would be helpful to know how many animals are referred to e.g. 2 high-dose animals succumbed to the infection within 24 hpi - what percentage of the group is this?

In this report, 2 of 6 mice that received the high dose were not viable at 24 h, so this is equivalent to 33.3% of this group. This percentage has been added to the text.

Fig 2 - this would be an ideal point to demonstrate how the different scales can be changed to accommodate all the images. At present the color scale is set to max/min for each individual image. If the color scales of the earlier time points were adjusted so that their scale matched the 24 hpi timepoint, then it would be clear how much more bacterial luminescence there is at this later time.

We have included images both ways. Panel A shows the same mouse over time on a common scale and as was previously submitted. It is clear that this mouse has an increase in bioluminescence by 24 h compared to earlier time points (the scale increases >2-fold), but we agree with the reviewer that a common scale simplifies the interpretation. We also think that including the images as presented previously is also important to emphasize the total bioluminescence at each time point.

Fig 3 - can the authors explain a little more what the difference in the wavelengths means, and how this changes the visible bioluminescence signals in the same mouse i.e. scale 1 has two bioluminescent spots in the head and neck region, while scale 2 has 2 different spots in lung and throat(?).

Absorption of photons in the far-red spectrum in mammalian tissues is low (this is 600-700 and beyond nm), though absorption is higher in the blue/green spectrum (500s nm). In these images, we chose to show emission filters from the blue/green spectrum (scale 2) which will give us higher absorption and will therefore provide a stronger signal (Kuo C, 2007, J. Biomed. Optics). This signal is utilized to show the localization of bacteria that is found within the lung and thymus regions of the mouse. In particular for panel A, when we use scale 1 (including all of the emission filters), a weaker signal is generated. This allows us to show the CT portion of the lung without the bacterial signal, and highlight the opaque region indicative of consolidation that colocalizes with bacteria observed with scale 2. The opaque region analogous to what is observed on chest film is not observed in control pups. This is an important feature to highlight since we focus on lung inflammation and pathology in Figure 4.

Given that E. coli O1:K1:H7 is associated with meningitis as well as sepsis, the authors should mention brain infection and how this might be investigated at some point in the manuscript (e.g. in section 7 or in discussion), even if they do not go into method detail.

We have briefly discussed the association between *E. coli* O1:K1:H7 and meningitis in the discussion, paragraph 2, and how we might investigate the effects of this strain of bacteria on the brain. This is an active area of research in our laboratory.

Table of materials needs a few additions: tattoo ink, tryptic soy agar and LB - are both of these purchased, if so, state where from. If not, give recipe. Ditto PBS, neutral-buffered saline, neutral-buffered formalin.

These have been added as requested.

One reference that should probably be included is Witcomb et al (2015) Infect. Immun. 83:4528-4540 Bioluminescent imaging reveals novel patters of colonization and invasion in systemic Escherichia coli K1 experimental infection in the neonatal rat.

We have included text that describes this publication in the Discussion. We thank the reviewer for alerting us to important literature that was not included.

Reviewer #2:

Manuscript Summary: This protocol thoroughly lays out methodology for a model of late-onset neonatal bacterial sepsis. It includes how to infect pups, monitor the infection during the course, and several protocols for endpoint assessment of infection.

Major Concerns:

While this is a very detailed protocol that includes an in depth discussion of the context of bacterial

infections in prematurely born populations, the authors should include a discussion of the limitations of this model. Notably, how does the subcapsular injection reflect the route of infection in clinical sepsis cases, particularly given the fact the authors are using E. coli, which is also associated with urinary tract infections.

We have added this point to the limitations paragraph of the discussion (paragraph 3). We agree with the reviewer that subscapular inoculation is not a natural mode of transmision. Our focus in the development of the model was to establish a reproducible infection across litters that recapitulates features of human disease and can be used to study the neonatal immune response to infection. We acknowledge this is a model of neonatal sepsis disease syndrome and not a model of natural transmission. This is discussed further in the manuscript.

Authors describe this model as "Early-onset neonatal sepsis" (line 557-558), however their model more resembles "Late onset neonatal sepsis". In short, early onset sepsis occurs within 72hrs of birth, is mostly associated with group B strep, which is thought to be transmitted from the mother to the infant either in utero or during delivery. Late onset sepsis occurs at least 72 hrs post-delivery and the causative bacterial pathogen is community acquired, introduced either from intravenous clinical work or via translocation from the GI tract.

We appreciate the reviewer's point that there is a small discrepancy in the age at which the neonates in our model acquire the infection relative to human neonates diagnosed with EOS. This is, however, minor. In our model the pups are infected on the 3rd day (which can technically fall within 72 h and sometimes does) or 4th day of life. The latter is only up to one day removed, but more important is the administration, trajectory of the infection, and pathogen. LOS is most frequently associated with invasive interventions such as mechanical ventilation and intravascular catheterization, invasion by skin commensals, or dissemination from early colonization of the GI tract. As a responsible pathogen, *E. coli* is significantly outnumbered by coagulase-negative staphylococci (CONS) and *S. aureus* in LOS. Moreover, LOS infections typically develop more slowly in association with biofilm formation and are often accompanied by underlying host conditions or low birth weight status. So, taken together and the minor discrepancy in day of infection aside, we respectfully disagree that this is a model of LOS. However, we thank you for this comment as it is important to distinguish. Although in humans group B *Streptococcus* is the most common etiologic agent, *E. coli* is a very close second. Also important are much higher mortality rates observed with K1-encapsulated *E. coli*, the pathogenic serotype we use in this study. These epidemiologic facts are stipulated in the first paragraph of the Introduction. We look at this mouse infection as a way to model the early human infection and to better understand how the neonatal host responds to early onset pathogens.

Minor Concerns: Protocol 1 (steps 1.5-1.7) Authors should include the target optical density and how that relates to the bacterial concentration they use. Of course target ODs and bacterial concentration are subject to variation in species growth patterns. Since the authors use target concentrations of around 2 to 7x10^6 CFUs, they should have a target OD of the bacterial stock after 2-3 hours.

There is no target OD and we respectfully decline to include since that would be a misrepresentation of our approach. The efficacy of our approach is rooted in a reliable 3 h growth curve that predicts the bacterial density of a 2-3 h culture with minimal variation. Although it is not the focus of the manuscript, we believe strongly that this curve more accurately predicts a bacterial density than a single OD reading. The reason is that target ODs often attempt to manufacture a density with the use of dilution or concentration because it is difficult to naturally stop a bacterial culture to pinpoint a target OD based on time of growth.

Reviewer #3:

Manuscript Summary: Seman et al describe a protocol for performing intravital imaging and various ex vivo analysis of tissues using a gram-negative model of neonatal sepsis. The protocol is clear and would be useful for dissemination to the broader scientific community. There are several instances where their methods could be more detailed and several clarifications that would improve the ease with which other investigators could implement this protocol in their own facility. I have also included several potential suggestions for improving methodology which are meant to be helpful as the authors continue to utilize this approach. These have been highlighted below.

General comments:

1. Please speak to precautions or protocols to maintain body temperature when pups are separated from their dams. At this age, pups are dependent on dams for maintaining body temperature. Example of things to include: Do you use a heat pad? Do you build a nest with bedding materials? Do you give pups back to dams as soon as infection is performed? Are control (uninfected) animals also separated during this time?

Neonates are taken from the dams temporarily for injection, and then immediately placed back with her so they are not given enough time to lose body temperature (now stated in 3.6 and was previously stated in 5.8 of the protocol). Both infected and uninfected controls are

housed together with the mother because all pups are still nursing during the period of infection, though control pup tails are snipped, and both control and infected pup tails are tattooed to individually identify each pup (stated in section 2, Animal Identification).

2. It would be helpful to provide more information about serial dilution technique. For example, what media do you dilute in? How many replicate plates are you using? For recovery from tissue, what controls are used to validate that CFUs are of the inoculated E. coli and not endogenous E. coli or other bacteria?

Dilutions are done in PBS (neutral pH) containing no calcium or magnesium. Briefly, bacteria are diluted 10-fold are then plated, in duplicate, onto TSA with kanamycin (30 µg/mL) quadrant plates. These plates are incubated at 37°C overnight before enumeration. The mobilized recombinant DNA that contains the lux operon contains a kanamycin resistance cassette. Growth on kanamycin distinguishes the infection strain from endogenous bacteria. We also plate blood or diluted tissue homogenates from control animals and find that there is no colony growth at any dilution or undiluted in tissues of uninfected mice. These details have been added.

3. There is not enough information detail regarding the materials used. Please go through the manuscript and make sure the materials section reflect what was described above. To start, here is an incomplete list of materials required that were not listed: homogenizer, medias, Spectramax plate reader, IVIS intravital imaging machine, real-time thermocycler, formalin, "nylon basket", etc.

This has been completed as requested.

Minor Concerns:

1. Some parts of the technique are described as being performed in a biosafety cabinet hood while others are not. Unless specified, is a biosafety cabinet not required?

We have added to the protocol the other times during which a procedure needs to be done under a biosafety cabinet hood. Generally, procedures such as inoculations of bacteria and necropsy of infected animals should be performed in a biosafety cabinet.

2. For the animal identification protocol, it is unclear if tail snipping and tail tattooing are separate approaches for identification or if all tails are snipped prior to tattoo ink injection. Protocol only says to cut tails of control animals.

Only control animals receive tail snips; infected animals do not. There are only so many unique patterns of tattoo that can be implemented on a neonatal tail. Snipping creates a first layer to uniquely identify. Both control and infected animals receive tail tattoos as an additional layer of identification. This has been clarified in the animal identification section of the protocol.

3. Are both infectious inoculum and PBS control kept on ice until infection?

Yes. This is added to section 3 in the protocol.

4. It would be helpful to explicitly list endpoint criteria in a listed fashion under its own subheading under   Section   4.   How   much   body   weight   loss   triggers   endpoint?
5. For in vitro assay, please include more detail about mixing and centrifugation of samples. Are samples centrifuged prior to removing media before adding gentamycin?

Samples do not need to be centrifuged prior to removing media as they are plated on cell-culture treated plates and adhere to the bottom of the dish. Media is gently pipetted out of the well and gentamicin-supplemented media is gently added back in. This is now clarified in step 12.13.

6. It might also be helpful to include images of the ROI measurement and subsequent data representation.

This is relatively minor and the instructions are pretty straight forward. This image may help demonstrate. This is a representative image of a mouse with the ROI luminescence

quantified (24 h). At the Reviewer or Editor's request we could include as a figure, but it seems an unnecessary use of figure space.
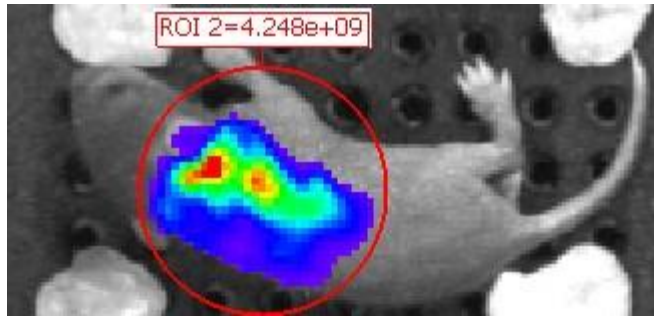
Potential areas for methodological improvements:

1. The current described approach to anesthesia could be further optimized to prevent adverse effects from direct inhalation of isofluorane soaked cotton balls. The discussion brings up another idea of using This reviewer would recommend incorporating a transparent isoflurane chamber that is directly compatible with imaging and using continuous flow through that container during imaging to maintain a consistent and safe amount of isoflurane throughout the entire imaging process. This could be accomplished by purchasing a pre-made manifold made for this purpose (https://www.environmental-expert.com/products/patterson-five-mouse-imaging-chamber-677727) or creating one in house.

Thank you for the suggestion. Our system has a continuous flow isoflurane delivery system, but due to the reduced inhalation with neonates, it is less effective.

2. There have been several previous reports using longitudinal intravital imaging after oral administration of bioluminescent bacteria in murine neonates to study the kinetics of bacterial dissemination. PMIDs for reference are 15774831, 25210785, 26351276, 31700190. That does not discount the utility of this protocol, but I would like to refer the authors to this previous work on the topic if they were not already aware of it.

Thank you for raising our awareness to these studies.