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A Precise Pathogen Delivery and Recovery System for Murine Models of Secondary Bacterial Pneumonia

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Jaydev Upponi, Ph.D.
Science Editor | Immunology and Infection
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Dear Dr. Upponi,

Our video and manuscript submission titled: *A Precise Pathogen Delivery and Recovery System for Murine Models of Secondary Bacterial Pneumonia,* describes methods to improve secondary bacterial pneumonia studies. We demonstrate and provide a written description of a non-invasive route of instillation into the lower respiratory tract of mice followed by pathogen recovery and transcript analysis.

The manuscript and video are original material and have been carefully revised based on the reviewer's comments. All authors have contributed, seen, and approved the submitted version of the video and manuscript.

Thank you in advance for your time and consideration with the video and manuscript. Please contact me if you have any questions.

Sincerely,

Jovanka M. Voyich, Ph.D.

TITLE:

A Precise Pathogen Delivery and Recovery System for Murine Models of Secondary Bacterial Pneumonia

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KEYWORDS:

Intubation, intratracheal, lower respiratory tract, intranasal, pneumonia, influenza, *Staphylococcus aureus*, co-infection, superinfection

SUMMARY:

Here, we present methods to improve secondary bacterial pneumonia studies by providing a non-invasive route of instillation into the lower respiratory tract followed by pathogen recovery and transcript analysis. These procedures are reproducible and can be performed without specialized equipment such as cannulas, guide wires, or fiber optic cables.

ABSTRACT:

Secondary bacterial pneumonias following influenza infections consistently rank within the top ten leading causes of death in the United States. To date, murine models of co-infection have been the primary tool developed to explore the pathologies of both the primary and secondary infections. Despite the prevalence of this model, considerable discrepancies regarding instillation procedures, dose volumes, and efficacies are prevalent among studies. Furthermore, these efforts have been largely incomplete in addressing how the pathogen may be directly influencing disease progression post-infection. Herein we provide a precise method of pathogen delivery, recovery, and analysis to be used in murine models of secondary bacterial pneumonia. We demonstrate that intratracheal instillation enables an efficient and accurate delivery of controlled volumes directly and evenly into the lower respiratory tract. Lungs can be excised to recover and quantify the pathogen burden. Following excision of the infected lungs, we describe a method to extract high quality pathogen RNA for subsequent transcriptional analysis. This procedure benefits from being a non-surgical method of delivery without the use of specialized laboratory equipment and provides a reproducible strategy to investigate pathogen contributions to secondary bacterial pneumonia.

INTRODUCTION:

Secondary bacterial pneumonia following influenza infection is a leading cause of death in the United States and an active area of research[1, 2]. Despite numerous studies using murine models of secondary bacterial pneumonia, inconsistencies regarding pathogen instillation and interrogation remain[3–5]. In addition, while many previous efforts have focused on the immunomodulatory effects of influenza that lead to an increased susceptibly to secondary bacterial infection, more recent data suggests virulence regulation of the bacterial pathogen is an equal contributor towards the establishment of disease[6–9]. These new data necessitate a more precise method to explore secondary bacterial pneumonia in murine models of co-infection that facilitate investigation into the pathogen response.

Unique to influenza co-infection models, host organisms are intentionally immunocompromised by a primary influenza infection prior to the administration of the secondary bacterial agent. In order to best replicate the disease pathogenesis observed in human hosts, it is imperative that the pathogen load of both primary and secondary agents be controlled so as to observe the individual and combinatorial effects of each infectious agent. Most commonly, respiratory infections in mice have been established through an intranasal administration[3–6, 10]. Inasmuch as this route is noted for being technically simple and can be appropriate in some single-agent infection applications, it is unsuitable for co-infection models, as instillation procedures, dose volumes, and efficacy are highly variable within published literature[3–6].

To gain a more complete understanding of secondary bacterial pneumonia pathogenesis, contributions of both the host and the pathogen must be considered. To that end, we have developed a straightforward and reproducible approach for recovery of viable bacteria and pathogen RNA from infected lungs. This method uses a simplified, non-invasive intratracheal instillation procedure followed by subsequent isolation of bacterial RNA. The intratracheal instillation procedure described herein is similar to previously described methods and is not limited to pathogen delivery[11–13]. Use of this particular procedure benefits from being low-cost and does not require the use of specialized equipment such as cannulas, guide wires, or fiber optic cable; furthermore, because this procedure is non-invasive it insures minimal stress on murine subjects, minimizes an inflammatory response from the inoculation mechanics, and provides an efficient delivery route for the infection of multiple subjects. Briefly, isoflurane anesthetized mice are suspended from the incisors. Forceps are used to gently grasp the tongue followed by insertion of a pre-loaded bent, blunt-tipped, 21-gauge needle into the trachea and delivery of pathogen load. Validation of this procedure is demonstrated by visual confirmation of dye equally distributed into the pulmonary compartment and recovery of bacterial load. We then demonstrate how to recover viable *Staphylococcus aureus* (*S. aureus*) from infected lungs and describe a reproducible method to isolate high quality pathogen RNA.

PROTOCOL:

All methods conform to the National Institutes of Health guidelines and were approved by the Institutional Animal Care and Use Committee (IACUC) at Montana State University.

1. Intratracheal Instillation

1.1. Prepare a workspace containing the following supplies: an intubation platform, a sterile 1 mL syringe, sterile blunt-tipped forceps, sterile 21-gauge blunt-tipped needle, and two conical tubes to store the syringe and forceps.

NOTE: Intubation platforms can be purchased commercially or constructed in-house. The intubation platform used here was constructed using 0.25 inch plexiglass. Briefly, heat was applied to the plexiglass and the board is bent to an approximate 65° interior angle. On the exterior side of the intubation platform, two machine screws were seeded 3 inches apart and a rubber-band was suspended between the two screws.

1.2. Prepare the pathogen inoculum by serially diluting the sample so that the desired final concentration is obtained in a total volume of 50 µL. Store the inoculum on ice.

1.3. Wearing sterile gloves, bend a 21 G blunt-tipped needle to approximately 35°.

NOTE: A separate needle is needed for each mouse.

1.4. Fix the bent 21 G blunt-tipped needle to a 1 mL syringe. Draw a 100 µL air cushion into the syringe followed by 50 µL of the infectious agent. Place the loaded needle and syringe in a location easily accessible by the dominant hand.

1.5. Deeply anesthetize a mouse using a 4% isoflurane/oxygen mixture or similar IACUC approved method of anesthesia. Proper anesthesia depth is typically obtained when respiration rates slow to approximately 1 inhalation per 5–8 s and can be confirmed by pinching an appendage and observing no reaction from the mouse.

NOTE: This method of anesthesia provided a sufficient anesthetization duration of approximately 1.5 min. This was sufficient for members in our lab to learn and perform this instillation procedure; however, other institutionally approved anesthesia methods may be employed[11–13].

1.6. Remove the anesthetized mouse from the anesthesia chamber and suspend the mouse on the intubation platform from the maxillary incisors.

1.7. To open a clear passage into the trachea, use the blunt-tipped forceps to gently grasp and extend the tongue outside of the mouth. Transfer the tongue from the forceps into the thumb and index finger of the non-dominant hand.

1.8. Remain holding the tongue in the non-dominant hand and pick up the pre-loaded syringe. Point the bent end of the needle away from the body and insert the needle into the oral cavity to the base of the tongue.

1.9. With the needle at the base of the tongue, gently angle the wrist to cause a slight push of the needle away from the body. This step insures the needle will be inserted into the trachea and not the esophagus.

1.10. Slowly guide the needle down into the trachea; often a slight tick is felt as the needle passes through the vocal fold. Pass the needle through the trachea until slight resistance is felt as the needle encounters the carina.

1.11. Slightly lift the needle in the proximal direction to suspend the needle above the carina. This enables delivery into the two primary bronchi. Fully depress the syringe's plunger to deliver the infectious load. Remove the needle from the trachea by lifting upward and discard.

1.12. Remove the mouse from the intubation platform by depressing the rubber band but maintain holding the mouse in an upright position. Obstruct the nasal airways by placing a finger directly over the nares. Hold this position for approximately 1 min or until several deep breathes are observed.

NOTE: This last step ensures that the total inoculum volume is delivered into the lower respiratory tract.

1.13. Return the mouse to its cage and observe a complete recovery from the anesthesia.

2. Excision of Infected Lungs

2.1. Working within a laminar flow hood to maintain sterility, prepare a workspace with the following items: a scale and sterile weigh boat, a dissection platform, a dissection kit containing scissors and forceps, sterile RNAse-free PBS, and sterile gauze.

2.2. Euthanize an infected mouse with CO_2 or similar IACUC approved method of euthanasia.

2.3. Place an infected mouse on a dissection platform and secure the mouse using pins at the end of each appendage. Spray the mouse with ethanol to help maintain sterility of the working surfaces.

2.4. Beginning at the umbilicus, use a pair of forceps to lift the skin and make an incision up to the base of the trachea. Grasping the skin on either side of the initial incision, pull the skin away from the body and cut through the tissue connections.

NOTE: It can be helpful to make several lateral cuts across the skin to reveal more of the thoracic region.

2.5. Starting at the base of the xiphoid process, make a small incision with the intention of puncturing the diaphragm. This results in an increase in pressure in the thoracic cavity that causes the lungs to retract. With the lungs retracted continue the incision to free the diaphragm.

2.6. Remove the ribs by making an incision along both sides of the rib cage. This will fully expose the thoracic cavity and lungs.

2.7. To excise the lungs, grasp the base of the heart with the forceps and lift upward. Place the scissors behind the lungs and begin to make small incisions through the tissue connections while continuing to the lift the heart upward.

2.8. Once the lungs have been excised, place them onto the sterile gauze and remove the heart. The heart can be easily removed by lifting it away from the lungs with the forceps and cutting the remaining tissue connections.

2.9. Transfer the lungs to a pre-tared weigh boat and record the weight.

2.10. After the lungs have been weighed, transfer them into sterile phosphate buffered saline (PBS) and temporarily store them on ice until moving forward to the pathogen recovery and analysis steps.

3. Pathogen Recovery and Analysis

3.1. Continuing to work within a laminar flow hood, prepare a workspace with the following supplies: a tissue grinder, sterile RNAse-free PBS, Buffer RLT supplemented with ß-mercaptoethanol (1:100), and 1.5 mL RNAse-free microcentrifuge tubes.

CAUTION: ß-mercaptoethanol can be hazardous in the case of skin contact, ingestion, eye contact, or inhalation. Dilution of ß-mercaptoethanol into buffer RLT should be done in a fume hood.

3.2. Begin by first adding 1 mL of sterile RNAse-free PBS to the tissue grinder. Once filled, store the tissue grinder on ice.

3.3. Prepare a series of sterile RNAse-free serial dilution tubes that will be used to quantify the bacterial load recovered from the infected lungs. This is most easily accomplished by aliquoting 900 µL of sterile water into each microcentrifuge tube followed by serial dilution of the pathogen inoculum.

NOTE: The number of dilution tubes necessary for accurate pathogen enumeration will vary on initial pathogen input and duration of infection. This must be empirically determined.

3.4. Using sterile forceps, transfer the lungs into the prepared tissue grinder and thoroughly homogenize the tissue by pressing down on the pedestal while rotating.

3.5. Open the tissue grinder and aliquot 100 µL of the homogenized sample into the first of the prepared serial dilution tubes. Serially dilute the samples and enumerate CFUs by plating onto

nutrient agar. CFUs can be recorded as CFUs/mL or CFUs/mL/mg of lung tissue.

NOTE: At this step an additional 200 µL of the homogenized sample can be removed for purification of viral RNA (see **Table of Materials**) or stored at -80°C for future analysis.

3.6. After the aliquots have been removed for CFU determination and/or viral RNA isolation, replace the grinding pedestal and pellet the homogenized tissue by centrifugation at 4,000 rpm (3724 x *g*) for 10 min at 4 °C.

3.7. Using a pipette, remove the supernatant from the pelleted sample and place into a 1.5 mL microcentrifuge tube.

NOTE: The supernatant is free of bacteria and can be stored at -80 °C for analysis of soluble host and secreted pathogen factors.

3.8. Resuspend the homogenized tissue pellet by pipetting or vortexing in 700 µL of buffer RLT-ß-mercaptoethanol and transfer the sample to a sterile RNAse-free 1.5 mL microcentrifuge tube.

NOTE: At this point the samples can be stored at -80 °C for several months.

3.9. Purify RNA using slight modifications to the manufacturer's protocol of a purification kit (see **Table of Materials**); see Voyich et al. 2008[14].

3.9.1. Transfer the homogenized lung slurry into a 2 mL microcentrifuge tube containing 0.1 mm silica beads and processed via a bead beater for 20 s at 6 m/s.

3.9.2. Centrifuge the sample at 1,500 rpm for 3 minutes. After centrifugation, pipette the supernatant into new 1.5 mL microcentrifuge tube.

3.9.3. Add 350 µL of 96%–100% ethanol to the sample and thoroughly mix by pipetting or inversion.

3.9.4. Transfer 700 µL of the sample to a purification column placed in a 2 mL collection tube. Centrifuge the sample at ≥ 8,000 x *g* for 15 s and discard the flow-through. Run any excess sample through the same column as described above.

3.9.5. Wash the column with 700 µL of buffer RW1 and centrifuge sample at ≥ 8,000 x *g* for 15 s. Discard the flow-through and transfer the silica membrane column into a new 2 mL collection tube.

3.9.6. Apply 500 µL of buffer RPE to the column. Wash the column by centrifugation at ≥ 8,000 x *g* for 15 s. Discard the flow-through.

3.9.7. Apply an additional 500 µL of buffer RPE to the RNeasy column and centrifuge at 8,000 x *g*

for 2 min. Discard flow-through and centrifuge the column at ≥10,000 x *g* for 1 min.

3.9.8. To elute purified RNA, begin by transferring the column to a new 1.5 mL RNAse-free microcentrifuge tube. Pipette 50 µL of RNase-free water directly onto the silica-gel membrane of the column and centrifuge at ≥8000 x *g* for 1 min.

NOTE: The eluted RNA can be run over the same column again to increase RNA yield.

3.9.9. Add 50 µL of RNase-free water to the purified RNA to bring the total volume to 100 µL. Aliquot 350 µL RLT-ß-mercaptoethanol followed by 250 µL of 96%–100% ethanol to the sample and mix thoroughly by pipetting or inversion.

3.9.10. Apply the sample to a purification column placed in a collection tube and centrifuge at ≥8000 x *g* for 15 s. Discard the flow-through and replace the collection tube.

3.9.11. Wash the column by adding 350 µL of buffer RW1 followed by centrifugation at ≥8000 x *g* for 15 s.

3.9.12. Prepare a DNase solution containing approximately 27 Kunitz units by adding 10 µL of DNase stock (750 Kunitz units/mL) to 70 µL of buffer RDD. Add 80 µL of DNase solution directly onto the silica-gel membrane and incubate the sample for 15 min at room temperature.

3.9.13. Wash the column with 350 µL of buffer RW1 and centrifuge at ≥8000 x *g* for 15 s and discard the flow-through.

3.9.14. Repeat steps 3.9.6–3.9.8 to purify RNA.

NOTE: It is recommended to repeat the RNA clean-up procedure by following steps 3.9.9–3.9.10 and 3.9.6–3.9.8 (skip DNase steps 3.9.11–3.9.13). This additional clean-up it results in very high-quality RNA.

3.10. RNA yield can be quantified using a spectrophotometer with readings at 260 nM for determining concentration and 260:280 nM for purity. After RNA yield is obtained, standardize the concentration of each RNA sample to 50 ng/µL.

NOTE: It is recommended to make multiple aliquots at a concentration of 50 ng/µL to avoid freeze/thaw cycles that can lead to RNA degradation.

3.11. Store the purified RNA at -80 °C or use immediately for transcript analysis.

NOTE: In previous publications RNA from 3–5 mice was pooled for transcript analysis. Through optimization of the above technique, RNA from 1 mouse has been empirically determined as sufficient for transcript analysis (80–120 ng/µL).

REPRESENTATIVE RESULTS:

Figure 1 utilizes a 0.1% weight/volume Coomassie brilliant blue solution to demonstrate that an intratracheal instillation delivers the inoculum directly and evenly within the lower respiratory tract. **Figure 2** shows that the bacteria (*S. aureus*) CFUs recovered directly from homogenized lung tissue. **Figure 3** demonstrates the use of this system for precise delivery and recovery of inoculum in the lower respiratory tract by plotting the input and recovery CFUs from individual mice. **Figure 4** shows the qRT-PCR amplification curve of the bacterial housekeeping gene *gyrB* to demonstrate that bacterial RNA can be extracted directly from infected lung tissue with minimal DNA contamination. **Figure 5** shows the construction of a standard curve using qRT-PCR amplification of the influenza A virus M-segment to demonstrate viral RNA can be extracted directly from infected lung tissue.

FIGURE LEGENDS:

Figure 1: Intratracheal instillation enables even distribution into the lower respiratory tract. (**A**, **B**) Uninfected lungs were excised from a healthy mouse following intratracheal instillation of sterile PBS and photographed from (**A**) dorsal and (**B**) ventral perspectives. (**C**, **D**) 50 µL of a 0.1% Coomassie brilliant blue solution were administered to an anesthetized mouse via intratracheal administration. (**C**) Dorsal. (**D**) Ventral.

Figure 2: Representative recovery of bacterial CFUs from infected lung homogenate. Lungs were excised one day post-challenge with *S. aureus.* Following homogenation, 100 µL of the lung slurry was serially diluted through 10^{-6}. To enumerate CFUs recovered, 10 µL drops were plated from the 10^{-5} and 10^{-6} dilutions onto tryptic soy agar (TSA) and incubated at 37 °C with 5% $C0_2$ overnight.

Figure 3: Precise delivery and recovery of the pathogen inoculum following intratracheal administration. Mice were divided into two groups containing three mice per group. Mice were subjected to intratracheal instillation of *S. aureus* at 1 x 10^8 (low) and 2 x 10^8 (high) CFU/mL. One hour post-infection, mice were euthanized and the lungs were excised to demonstrate the precision of the instillation and recovery. Bacterial inoculum and bacteria recovered from the lung homogenate were plated on TSA (tryptic soy agar). No significant differences were reported between bacterial input and recovery.

Figure 4: Representative bacterial RNA recovery and purity. Six-hours following intratracheal instillation with 1 x 10^8 CFU/50 µL of *S. aureus*, mice were euthanized. Lungs were excised and homogenized followed by resuspension of the lung slurry in buffer RLT-ß-mercaptoethanol. RNA was purified as described in step 3.9[14]. qRT-PCR was used to detect transcripts of the bacterial housekeeping gene *gyrB*. A control containing no reverse transcriptase (nRT) was included to demonstrate the purity of the RNA recovered. At a threshold of 0.1, *gyrB* transcripts were detected at an average cycle of 21.1, 20.3, and 20.5. nRT controls were not detected until cycle averages 35.9, 35.5, and 35.0. n = 3 biological replicates, containing 3 technical replicates/biological replicate.

Figure 5: Representative viral RNA recovery. Six days following intratracheal instillation with 100 PFU/50 µL of influenza A/PR/8/1934(H1N1), mice were euthanized. Lungs were excised and homogenized, and 200 µL of the homogenized slurry was collected and passed through a 70 µm cell strainer prior to viral RNA purification. Purified RNA was serially diluted (10^{-1}-10^{-4}) followed by amplification of influenza A M-segment. (**A**) Amplification plot of influenza A RNA recovered from an infected lung and diluted 10^{-1}–10^{-4}. (**B**) Standard curve of influenza A M-segment. Threshold = 0.2, R^2 = 0.994, slope = -3.46.

DISCUSSION:

Use of this model provides a highly efficient and reproducible method to study secondary bacterial infections. The ability to tightly control the delivery of the pathogen inoculum enables more precise observations of the individual and combinatorial effects of each pathogen. Inefficiencies in the more common intranasal instillation route have likely contributed to the discrepancies in dose volumes and concentrations present in the literature. It is reasonable that the lack of a precise murine system to study secondary bacterial pneumonia has delayed findings identifying bacterial specific responses that contribute to the severity of pulmonary co-infections. Developing a reproducible model to study virulence expression during secondary bacterial infections could lead to the identification of vaccine or drug targets to ameliorate these infections.

The intratracheal instillation step is critical to successfully establish a lower respiratory tract infection and any down-stream analysis of the pathogens. When learning this technique, it may be helpful to practice using a dye (as described in the methods) prior to administering infectious material. Using a dye allows for the direct visualization of the inoculum into the respiratory tract. A common mistake that can occur is insertion of the blunt-needle into the esophagus rather than the trachea. This will result in delivery of the inoculum into the stomach rather than the lungs. To correct this mistake, angle the needle further away from the body and pass it down into the trachea. Once mastered, this procedure is very efficient and can be used to conduct experiments with large numbers of mice. Working in batches to anesthetize mice, the intratracheal instillation can be completed in approximately 30 seconds per mouse. In addition, the excision of the lungs can be completed in 2 to 3 minutes per mouse.

Recovery of viable and pure bacterial RNA from infected tissues is critical for transcript analysis. RNases are ubiquitous and can quickly ruin an experiment[15]. Some methods include using RNase inhibitors; however, we have found that freezing the sample at -80 °C in RLT-ß-mercaptoethanol or immediately processing the sample for RNA isolation using all RNase free tubes and reagents are effective at reducing RNase contamination. Additionally, we recommend that a maximum of six samples be purified at one time. Including more than six samples can result in prolonged latencies between protocol steps that can culminate in RNA degradation. Once purified, care should also be taken to avoid any unnecessary freeze-thaw cycles. Thus, if multiple analyses will be done on one sample, aliquoting purified RNA for storage at -80 °C is recommended.

In addition to the techniques reviewed herein, this method can be supplemented by

performing bronchial alveolar lavage prior to the excision and homogenation of the lungs[16]. This can be accomplished by lavage of the entire lower respiratory tract or by using suture thread to restrict one branching arm of the bronchial tree followed by lavage through the remaining branch. Often this leads to a reduction in the recovery of the pathogen load but provides a sample whereupon information such as lactate dehydrogenase activity, cellular population identity, and cytokine profiles can be obtained[16]. Together these data can form a more complete understanding of the host-pathogen interactions occurring during secondary bacterial pneumonia.

While the methods discussed have been within the context of secondary bacterial pneumonia, they are suitable to be extended to any murine model of lower respiratory infection; specifically, those that would benefit from tightly controlled delivery and recovery of the installed inoculum. Furthermore, like many other infection routes, the intratracheal instillation can be utilized in non-infectious applications, such as the administration of therapeutics and environmental compounds[12].

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DISCLOSURES:

The authors have nothing to disclose.

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Figure

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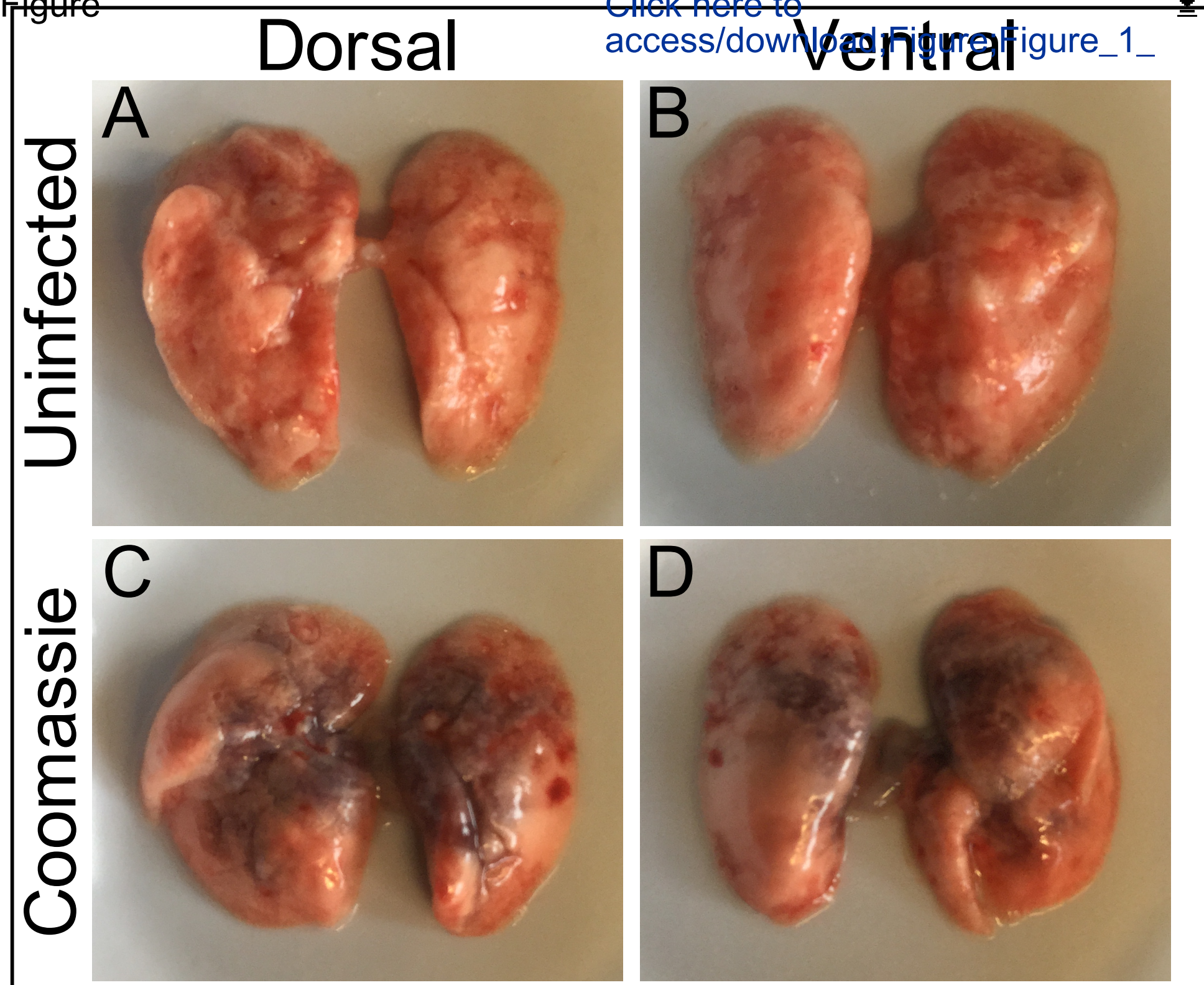

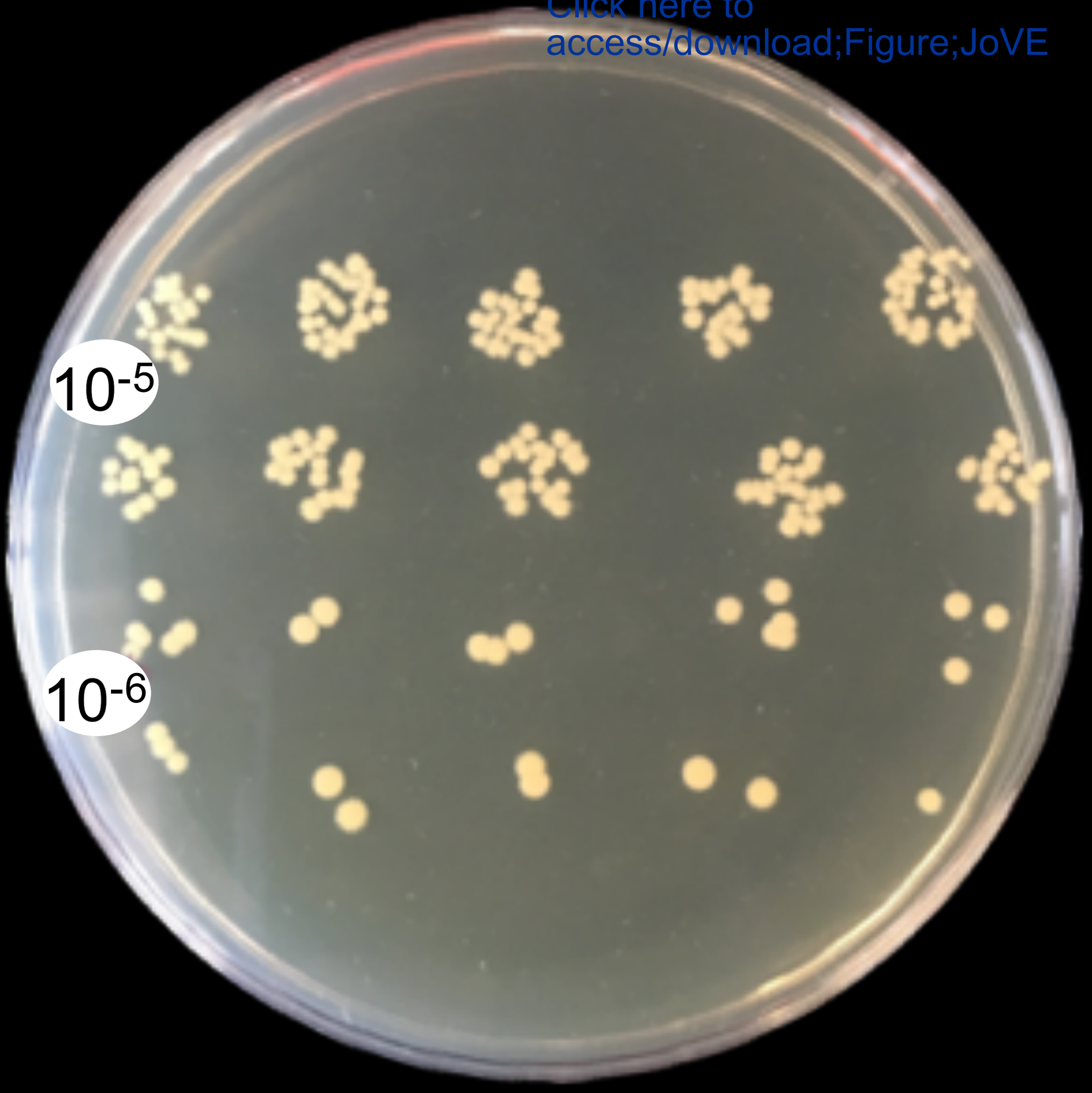

Figure

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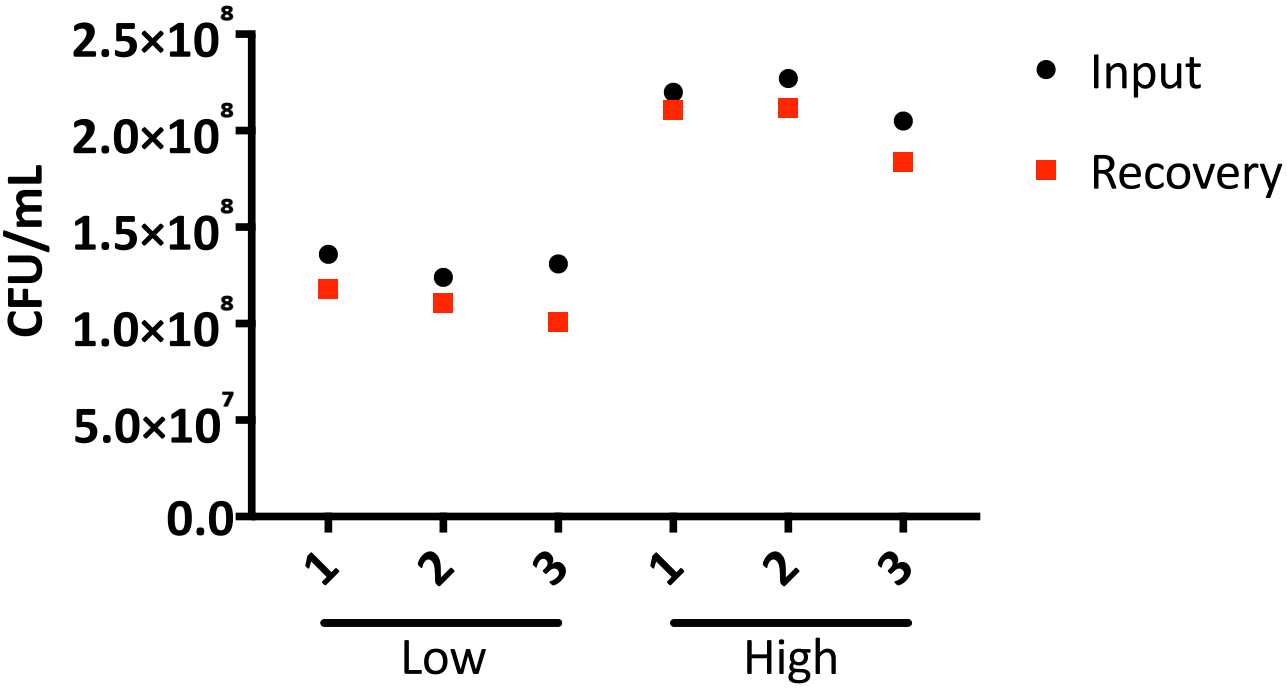
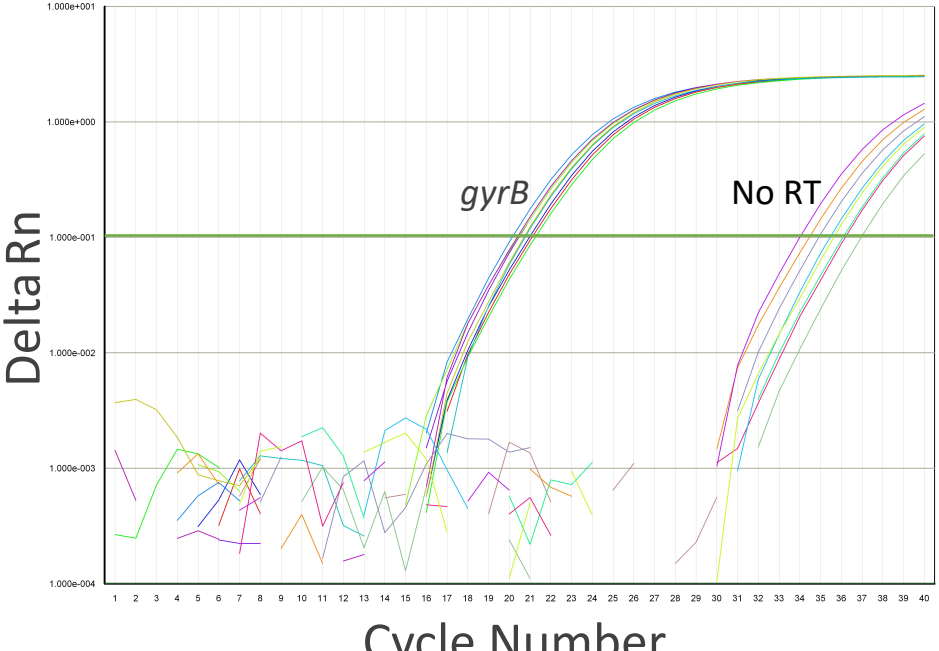
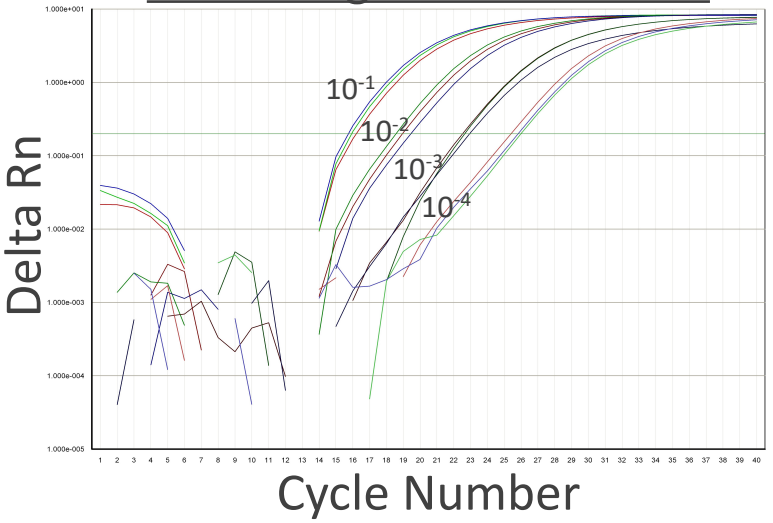


gyrB Transcript Detection



A

IAV M-Segment Detection



B

M-Segment Standard Curve

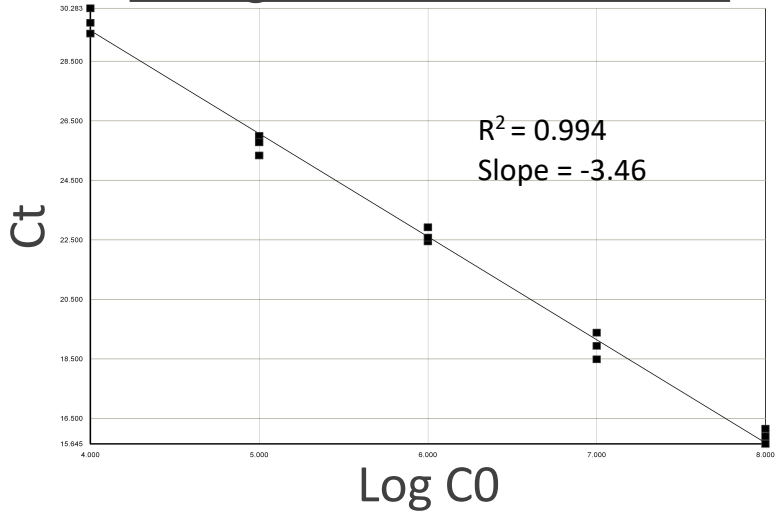


Table of Materials

Name of Material/ Equipment	Company	Catalog Number	Comments/Description
Lysing Matrix B	MP Biomedicals	6911100	Referred to in text as "0.1 mm silica beads"
21-gauge blunt needle	SAI	B21-150	1.5" is recommended.
RNase-Free DNase Set	Qiagen	79254	DNase used in the accompanying text.
FastPrep-24 Classic Instrument	MP Biomedicals	116004500	FastPrep FP120 is no longer available. Referred to in text as "Bead Beater"
TaqMan AIV-Matrix Reagents	Applied Biosystems	4405543	Influenza A M-segment qRT-PCR kit.
Intubation Stand	Kent Scientific	ETI-MES-01	Referred to in text as "intubation platform." Intubation platform used in the accompanying video was made in house.
RNeasy Mini Kit	Qiagen	74106	RNeasy columns, Buffer RLT, Buffer RW1, and Buffer RPE
QIAamp Viral RNA Mini Kit	Qiagen	52904	Viral RNA purification kit.
Tissue Grinders	Thermo Fisher Scientific	02-542-08	
2-Mercaptoethanol (β-Mercaptoethanol)	Calbiochem	UN2966	

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Author(s):	Timothy R. Borgogna, Adrian Sanchez-Gonzalez, Kelly Gorham, Jovanka M. Voyich

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To Whom It May Concern:

We would like to thank the editorial and production team along with the reviewers and veterinarians for their time and helpful critique of our manuscript submitted to *JoVE*. We were pleased at the enthusiasm for our studies. Both the editor, reviewers, and veterinarians had suggestions to improve our manuscript. After careful consideration of the comments, we have revised our manuscript and provided amendments to specifically address each of these concerns. Specific comments and corresponding manuscript revisions have been summarized below.

Editorial and production comments

General: "JoVE cannot publish manuscripts containing commercial language. This includes trademark symbols (™), registered symbols (®), and company names before an instrument or reagent. Please limit the use of commercial language from your manuscript and video and use generic terms instead. All commercial products should be sufficiently referenced in the Table of Materials and Reagents.
For example: Qiagen, QIAamp, FastPrep, TaqMan, Nanodrop, etc."
Response: We have limited the use of commercial language throughout the manuscript and corresponding video. The following are the specific changes that have been included:

- "Qiagen" has been removed from section 3.1.
- "QIAamp Viral RNA Mini Kit (Qiagen)" has been removed from the section 3.5 note.
- "(Qiagen)" has been removed from section 3.8
- "Lysis Matrix B tube (MP Biomedicals)" has been removed and replaced with "a 2 mL microcentrifuge tube containing 0.1 mm silica beads" in section 3.9.1.
- "FastPrep ® FP120" has been removed from section 3.9.1.
- "in the Lysing Matrix B tube" has been removed from section 3.9.2.
- "TaqMan™" has been removed from the Representative Results Figure 4 and Figure 5 descriptions.
- "with QIAamp Viral RNA Mini Kit (Qiagen)" has been removed from the Figure 5 Legend.
- "with TaqMan™ AIV-Matrix Reagents (Applied Biosystems)" has been removed from the Figure 5 Legend
- "such as RNAprotect Bacteria Reagent (Qiagen)" has been removed from the discussion.

Protocol: "For each protocol step, please ensure you answer the "how" question, i.e., how is the step performed? Alternatively, add references to published material specifying how to perform the protocol action. If revisions cause a step to have more than 2-3 actions and 4 sentences per step, please split into separate steps or substeps."
Response: Clarifications to the steps throughout the protocol have been made to provide sufficient instruction on how to complete each step. The following are the specific changes that have been included:

- "by serially diluting the sample" was added to section 1.2
- "Wearing sterile gloves" was added to section 1.3
- added "by lifting upward" was added to section 1.11
- "using pins at the end of each appendage" was added to section 2.3
- "This is most easily accomplished by aliquoting 900 µL of sterile water into each microcentrifuge tube" was added to section 3.3

We added the following note to section 3.3 “Note: The number of dilution tubes necessary for accurate pathogen enumeration will vary on initial pathogen input and duration of infection. This must be empirically determined.

- “Using sterile forceps” was added to section 3.4
- 3.7 has been amended to say “Using a pipette, remove the supernatant from the pelleted sample and place into 1.5 mL microcentrifuge tube.”
- “by pipetting or vortexing” was added to section 3.8.

Specific Protocol Steps: “1.5: Please mention how anesthesia is confirmed. Also, how long will anesthetization last?”
Response: Section 1.5 has been amended to clarify how anesthesia is confirmed as well as its duration. We have included the phrase “… can be by confirmed pinching an appendage and observing no reaction from the mouse.” In addition, we have included a note within section 1.5 providing a description of anesthesia duration as well as providing three references to alternative anesthesia procedures.

Figures: Figure 5: Please label this as it is in the video (e.g., concentrations shown in A).
Response: Figure 5 has been labeled as it is in the video.

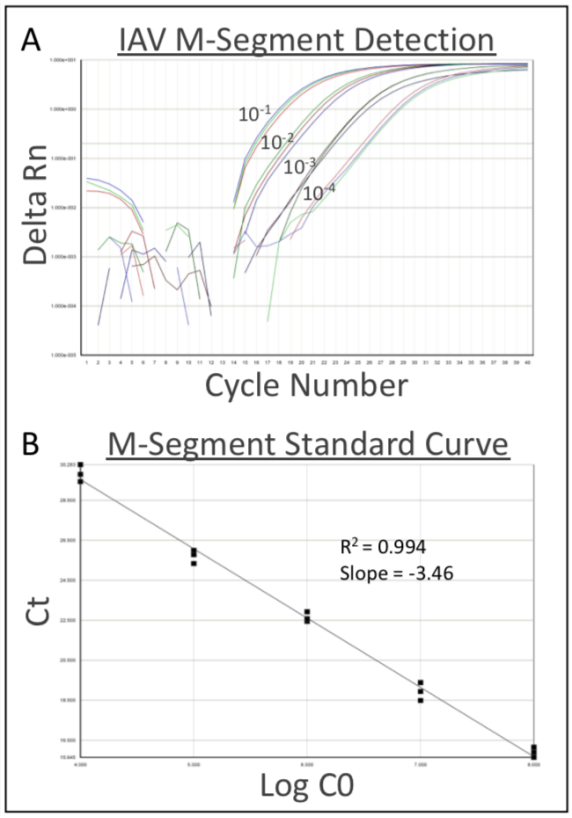


Table of Materials: “Please ensure the Table of Materials has information on all materials and equipment used, especially those mentioned in the Protocol.”
Response: The Table of Materials has been expanded to include reference information on the following items:

- Intubation Stand
- 21-gauge blunt needle
- Tissue Grinders
- RNeasy Columns
- Buffer RLT
- Buffer RW1
- Buffer RPE

Comments and descriptions have been included to align with references in the text.

Video: “1. Please ensure that the video content aligns with the written content; e.g., don’t include any information in the video that is not in the manuscript.

2. Future submissions should include the article ID number (59566) in the video file name.
3. 0:51-0:53 - A Qiagen RNEasy Kit is mentioned specifically here in the narration. If this block of narration is going to stay, that sentence will need to be rerecorded with a more generic term.
4. 3:16, 3:55, 6:29 - The edits here are jump cuts, which tend to have a jarring effect on the viewer. They should be smoothed out with crossfades instead.
5. 6:47 - There is a hard cut to a few frames of black, followed by a hard cut back to the video. This is a pretty jarring edit. I recommend using a crossfade here, instead.
6. 7:39 - The brand name 'Qiagen' is written and/or spoken here. Brand references should be removed.
7. 9:27 - 'Qiagen RNEasy method' is in the narration here. This should be removed and replaced with a more generic term.
8. 9:47 - 'Nanodrop' is mentioned in the narration here. This should be removed and replaced with a more generic term."

Response:

1. No information is included that in the video that in not in the accompanying manuscript.
2. The article ID number (59566) has been added to the video file name.
3. Commercial language has been removed from display and narration at the original times of 0:51-0:53, 7:39, 9:27, and 9:47.
4. The edits at the original time points of 3:16, 3:55, and 6:29 have been corrected.
5. The hard cut at the original time of 9:27 has been corrected.

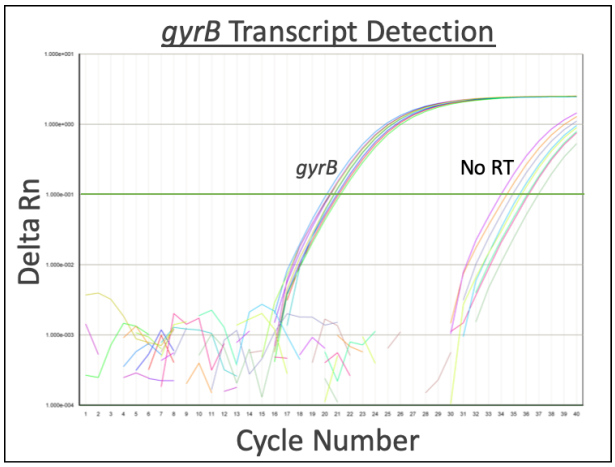
Reviewer #1

Minor Concern 1: "In protocol 1 and protocol 2, a simple diagram of the mouse anatomy discussed may be helpful. The forthcoming video will obviously help in this regard, but a simple figure would benefit the reader."
Minor Concern Response 1: We agree that simple figures would enhance the forthcoming video. To that end, we have included several figures in the introduction of the video to aid the visualization of the upcoming procedure.

Minor Concern 2: "In protocol 3, the authors should include steps at which RNAse-free reagents and supplies are to be used."
Minor Concern Response 2: We agree and thank Reviewer 1 for identifying that specific instruction regarding the use of RNase-free reagents is critical to the success of the protocol described. We have amended protocol steps 3.1, 3.2, 3.3, 3.8, and 3.9.8 to include direction on RNase-free materials. In addition, we have included clarifications in the narration accompanying protocol steps.

Minor Concern 3: "Figure 3 - define TSA."
Minor Concern Response 3: We have included "tryptic soy agar" to accompany "TSA" in the legend of Figure 3. TSA is also defined in the legend of Figure 2.

Minor Concern 4: "Figure 4 - how many experimental repeats are represented here? This is important because the authors conclude that their methods are more repeatable and vary less than published methods."
Minor Concern Response 4: We thank Reviewer #1 for identifying this error in Figure 4. The original figure submitted displayed only 1 experimental replicate which included 3 technical replicates. Figure 4 has been amended to include 3 experimental replicates containing 3 technical replicates per experimental replicate. The figure legend accompanying Figure 4 has been amended to reflect these changes and the revised figure (shown below) has been inserted into the video.



Reviewer #2

Concern 1: "The first part of the manuscript "intratracheal instillation" is similar to another publication on JoVE "Non-invasive intratracheal intubation to study the pathology and physiology of mouse lung" from Yan Cai and Shioko Kimura.
Both publications describe the same idea of a non-invasive intratracheal mouse model so that I conclude that the first part of the method described in this manuscript can also be used for other applications and is not limited for the controlled delivery of pathogens. It would be helpful if the authors can refer to this previous publication."

Response 1: We agree that there are similarities in the intratracheal installation protocol described herein and previously described methods. We have included a citation of the suggested "Non-invasive intratracheal intubation to study the pathology and physiology of mouse lung" at line 73 and inserted the following statement: "The intratracheal instillation procedure described herein is similar to previously described methods and is not limited to pathogen delivery[11–14]."

Minor Concern 1: "The authors use 4% isoflurane/oxygen mixture, remove the mouse from the anesthesia chamber without any further intranasal isoflurane inhalation.
Is this anesthesia long and deep enough also for less experienced researcher?
I would also mentioned as option the application of vet ointment to the eyes of the mouse in order to prevent drying of the eyes during anesthesia if the procedure takes longer."

Minor Concern Response 1: We thank Reviewer #2 for the concerns regarding the method of anesthesia described herein. Use of a 4% isoflurane/oxygen mixture for anesthesia does provide a long enough and deep enough window for a less experienced researcher to learn and accomplish this procedure. When developing this method our lab had no prior experience with intratracheal instillations however we were able to gain an extremely high rate of proficiency when using the aforementioned anesthesia procedure. In our experience using a 4% isoflurane/oxygen mixture did not require the addition of vet ointment to the eyes of mice.

In the revised manuscript, we have highlighted possibility of using alternative anesthesia procedures. A note has been added at section 1.5 clarifying our use of isoflurane and providing three citations that support the use of alternative anesthesia methods.

Minor Concern 2: "Highlighting the advantages of the non-invasive instillation (other than no requirement of specialized equipment) over direct installation into the trachea would further support the manuscript. "

Minor Concern Response 2: We thank Reviewer #2 for the recommendation to highlight the non-invasive aspect of this procedure. We have amended lines 75-76 to emphasize the benefits of a non-invasive intratracheal instillation.

Veterinarian #1

Concern 1: "This is a very wordy introduction. Is there a way to include diagrams or figures to emphasize the key points?"

Response 1: We agree that the original introduction was wordy and did not highlight key steps of the procedure. In the revised video we have provided several still shots and a diagram to emphasize points throughout the protocol and break apart the monotony of the monologue.

Concern 2: “The narration states to “remove an anesthetized mouse” to perform inoculation. It would be helpful to state what methods were used to anesthetize the animal. **“**
Response 2: Several methods of anesthesia are appropriate for the forthcoming procedure that is described. For this reason, we chose to retain the non-specific phrase “remove an anesthetized mouse” in the narration as it implies the method of isoflurane anesthesia can be modified to accommodate alternative anesthesia procedures. We have included a note and 3 references under section 1.5 to emphasize that alternative anesthesia procedures may be appropriate. In addition, to provide guidance on evaluating the depth of anesthesia used in the procedure described herein the following sentence was added at step 1.5: “Proper anesthesia depth is typically obtained when respiration rates slow to approximately 1 inhalation per 5-8 seconds.”

Concern 3: **“**The correct pronunciation of “nares” is /ˈnerēz/ , not as how it is said in the video.”
Response 3: The pronunciation of “nares” has been corrected in the narration.

Concern 4: “The narrator states that the abdomen should be cut at the beginning of the peritoneum.”
Response 4: The word “peritoneum” has been changed to “umbilicus” in both the narration and the text.

Concern 5: “Removal of the heart and lungs should be performed using standardized technique as described by mouse pathologists.”
Response 5: We developed the methods described in the manuscript and accompanying video to enable accurate and repeatable pathogen recovery. Therefore, we have decided to not modify our methods since they have been refined for a specific purpose. Furthermore, the reference provided by Veterinarian #1 demonstrates tissue removal for the purposes of necropsy not for the preservation of pathogens.

Concern 6: The words “ventral” and “dorsal” are meaningful only as the terms relate to relative anatomic locations. You cannot lift the lungs in the ventral direction outside of the body. You can say “upwards” or “away from the body”, but you are not lifting anything out of the body in a ventral direction.
Response 6: “The incorrect usage of the words ‘ventral’ and ‘dorsal’ have been corrected in both the text and the narration and been replaced with the suggestion of ‘upwards’ and ‘away from the body.’

Concern 7: “Removal of the heart. The connections between the heart and lungs are not simply ‘connective tissue.’ You can cut the connections between the heart and lungs, but those structures include major blood vessels.”
Response 7: “Connective tissue” has been changed to “tissue connections” in both the text and the narration.

Concern 8: “You state that previous work to determine needle placement was performed using dye. These pictures would be useful to someone learning this technique to fully understand where the installation should be.”
Response 8: In the discussion, we highlight that it may be helpful to install a dye via intratracheal instillation while learning this technique as the distribution of a dye through the lungs can be easy visualized upon dissection. Pictures of this technique are provided in Figure 1.

Veterinarian #2

Video Concern 1: “2:38-2:45 illustrates the needle being bent using examination gloves. Examination gloves are typically not sterile. This procedure requires sterile needles (otherwise other bacteria would be introduced into the lungs); therefore, the narration should clearly state that sterile gloves must be used to bend the needle. *Alternatively, the procedure would have to be demonstrated using surgical gloves.*”
Video Response 1: The narration accompanying the original time frames from 2:38-2:45 has been changed to clearly state that sterile gloves must be used.

Video Concern 2: “5:41-5:49 narrator states, ‘Beginning at the **peritoneum** use a pair of forceps to lift the skin and make an incision towards the base of the trachea.’ Narration should say ‘**umbilicus**’ given that the peritoneum is a body cavity and they are certainly not opening into the peritoneal cavity at that point. It may be worthwhile saying ‘*towards the larynx*’ rather than base of trachea.”
Video Response 2: The incorrect use of the word “peritoneum” has been corrected by replacing it with the correct word “umbilicus.” In addition, we have amended the narration to say “towards the base of the trachea.”

Video Concern 3: “5:56-5:59 narrator states, ‘…it can be make lateral cuts across the **peritoneum** to reveal more of the thoracic region…’ Use ‘**skin**’ instead of peritoneum (see above).”
Video Response 3: The incorrect use of the word “peritoneum” has been corrected by replacing it with the correct word “skin.”

Text Concern 1: “Line 99: Modify (clarify) the sentence ‘1.3) Bend a 21-guage blunt-tipped needle to approximately 35° to ‘1.3) **Use sterile gloves to b**end a 21-guage blunt-tipped needle to approximately 35°’ or *equivalent* to highlight the need for sterility during the process.”
Text Response 1: The original line 99 (now 104) has been modified to state: “Wearing sterile gloves, bend a 21-guage blunt-tipped needle to approximately 35°.”

Text Concern 2: “Line 155: Correct the sentence ‘Beginning at the peritoneum …’ to ‘Beginning at the **umbilicus** …’”
Text Response 2: The original line 155 (now 166) has been modified to state: “Beginning at the umbilicus…”

Text Concern 3: “Line 159: Correct the sentence ‘… make several lateral cuts across the peritoneum …’to ‘… make several lateral cuts across the **skin** …’”
Text Response 3: The original line 159 (now 170) has been modified to state: “… make several lateral cuts across the skin…”

Additional Changes
The labels on Figure 2 in the video have been changed to match the labels of Figure 2 in the manuscript.