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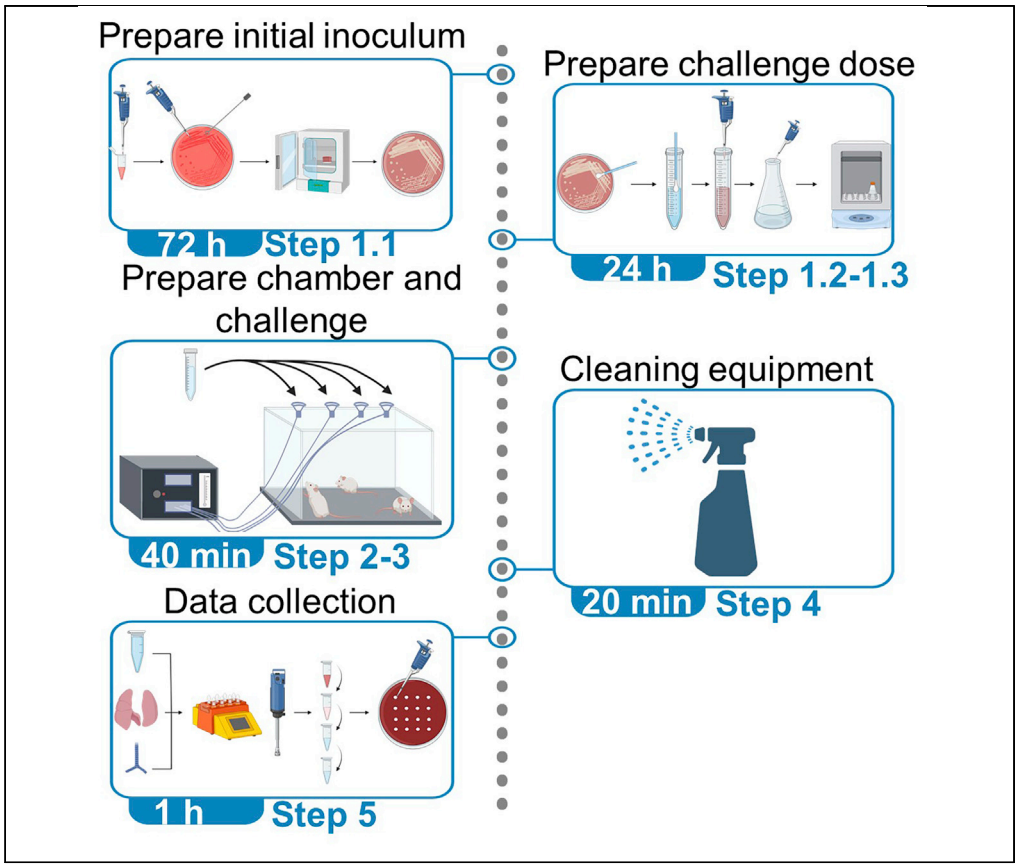
Protocol for aerosolization challenge of mice with *Bordetella pertussis*



Bordetella pertussis causes whooping cough and is transmitted via respiratory droplets. Here, we present a protocol to challenge mice with *Bordetella pertussis*. We describe bacteria preparation and long-term storage, followed by manufacturing a challenge dose for use in a commercial exposure chamber with controlled nebulization of *B. pertussis* into aerosols. We then detail the aerosol challenge of mice through a more natural administration than intranasal instillation and post-challenge data collection. This protocol allows for better comparisons between preclinical pertussis studies.

Publisher's note: Undertaking any experimental protocol requires adherence to local institutional guidelines for laboratory safety and ethics.

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Highlights

Protocol for an aerosol challenge of mice with *Bordetella pertussis*

Long-term storage of *Bordetella pertussis* and preparation of a challenge dose for use

Aerosol challenge of mice through a more natural administration

Post-challenge data collection and quantification of bacterial burden

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Protocol for aerosolization challenge of mice with *Bordetella pertussis*

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SUMMARY

***Bordetella pertussis* causes whooping cough and is transmitted via respiratory droplets. Here, we present a protocol to challenge mice with *Bordetella pertussis*. We describe bacteria preparation and long-term storage, followed by manufacturing a challenge dose for use in a commercial exposure chamber with controlled nebulization of *B. pertussis* into aerosols. We then detail the aerosol challenge of mice through a more natural administration than intranasal instillation and post-challenge data collection. This protocol allows for better comparisons between preclinical pertussis studies.**

BEFORE YOU BEGIN

The human disease pertussis, also known as whooping cough, is caused by the bacterium *Bordetella pertussis* (*Bp*) and was identified in 1906 by Jules Bordet and Octave Gengou.[1] During the century after the identification of *Bp,* several different animal and challenge models were implemented to study pertussis pathogenesis and aid in vaccine development efforts. The only known reservoir for *B. pertussis* is humans and it is transmitted through the spread of respiratory droplets. This is a highly contagious disease with a R° value of 12–18 in unvaccinated populations and a R° value of 5–6 in populations that are fully vaccinated.[2] In the baboon model of pertussis, animals are able to transmit bacteria through respiratory droplets to naive animals housed together and in separate cages up to 7 feet away.[3]

The intranasal droplet instillation method (IN) was published in 1937 by Burnet and Timmins in which 25 μL–50 μL of a bacterial solution was pipetted directly on the external nares of mice to induce infections.[4,5] Although, at the time, this method had some concerns about the efficiency of establishing uniform infections in test mice; it has since been shown to produce reliable results in our lab and others.[6–8] In addition to our group, other groups have also examined *Bp* pathogenesis upon IN challenge with doses between 2×10^2–2×10^7 CFUs/mL and have shown a difference in timing of infection and severity of bacterial burden, leukocytosis, and pulmonary proinflammatory cytokine levels[4,6] (publication in progress). However, given the challenge volume utilized in the IN method, a large quantity of bacteria is deposited in the lungs of challenged animals which is not observed in a natural pertussis infection. Therefore, other groups took this "natural infection" a step further and developed an aerosol challenge model of pertussis.[4,9–13] The current aerosol model typically utilizes a 1–3 × 10^9 CFU/mL dose of bacteria aerosolized for 15–30 min for 5–100 mice at a time.[9,10,13] The aerosol challenge model allows for multiple animals to be challenged at once. This decreases the chance of human error when instilling a challenge dose such as in the IN method. While infections established through these studies cited above are more uniform from study to study, most studies

use custom aerosol chambers and commercial nebulizers. We sought to establish a standardized protocol using a commercial chamber, nebulizers, and a controller unit to create a consistent protocol that can be implemented across laboratories.

The protocol below lists detailed steps for a standardized aerosol challenge model using the DSI Buxco® FinePointe™ mass dosing controller with a mass-dosing aerosol Chamber. The controller allows the researcher to set airflow, nebulizing time, and duty cycle (percentage of time the nebulizer operating during a 6-s cycle). In this protocol, *Bp* is grown first on BG agar plates, transferred to SSM liquid media to get log-phase growth, and then diluted in SSM to a specific aerosol dose. Next, mice are placed in the chamber, and 20 mL of the challenge dose is administered through aerosolized droplets. The mice inhale the infectious droplets, and a respiratory infection is established. This protocol may be used to test bacterial pathogenesis, host immune response, and vaccine efficacy. This protocol can be adapted to examine the effects of different challenge doses and could be applied to other animal models not listed here, e.g., other murine models (data not shown), guinea pigs, and rabbits.[14]

Institutional permissions

All animal work done in this protocol was performed in strict accordance with recommendations outlined in the *Guide for the Care and Use of Laboratory Animals* of the National Institutes of Health. West Virginia University's Institutional Animal Care and Use Committee (IACUC) approved these protocols under IACUC protocols #1602000797R1 and #1901021039. Any work done with *Bordetella pertussis* was completed in Biological Safety Level-2 (BSL-2) conditions under the approved Intuitional Biosafety Committee (IBC) protocol #17-01-11. Please ensure that when attempting to follow this protocol, all care is given to following your own IACUC and IBC guidelines, and work must be done under approved protocols.

Preparation and storage of *B. pertussis* bacterium

Timing: 3 days

1. Prepare 8 plates (100 × 15 mm) with 15 mL of Bordet-Gengou (BG) agar supplemented with 15% defibrinated sheep's blood and an appropriate antibiotic (e.g., Streptomycin 100 μg/mL for *Bp* strain UT25sm1).
   a. Solidify and dry the plates in a biosafety cabinet with the lids cracked open for 15 min.
2. Obtain a frozen stock of bacteria and spray off the tube with 70% ethanol before moving it into the biosafety cabinet (Figure 1A).
   a. Sanitize the working area inside the biosafety cabinet before opening the stock.
3. Scrape a frozen chunk of the bacterial stock (approximately 5 × 2 × 2 mm) using a sterile 20-gauge needle to onto a BG agar plate (Figure 1B).
   a. Discard the needle into an approved sharps container and use a fresh needle for each plate.
   b. Re-cap the bacterial stock tube to avoid introducing contaminants to the tube.
   c. Close the plate and set it aside until all plates have received the bacterial stock.

⚠ CRITICAL: Once all plates have received an inoculum of the stock bacteria, place the bacterial stock back into −80°C, so it does not thaw.

4. Next, spread the bacterial stock in a 3-phase streak on the plate using a cooled flame-sterilized metal inoculating loop (Figures 1C–1F).
5. Invert the plates and place them in a 36°C incubator for 48–72 h.
   a. *Bordetella pertussis* grows optimally at 36°C but will also grow at 37°C.

⚠ CRITICAL: To obtain the growth necessary for saving stocks of *Bp*, the temperature in the incubator needs to remain at 36°C (Figure 1G).

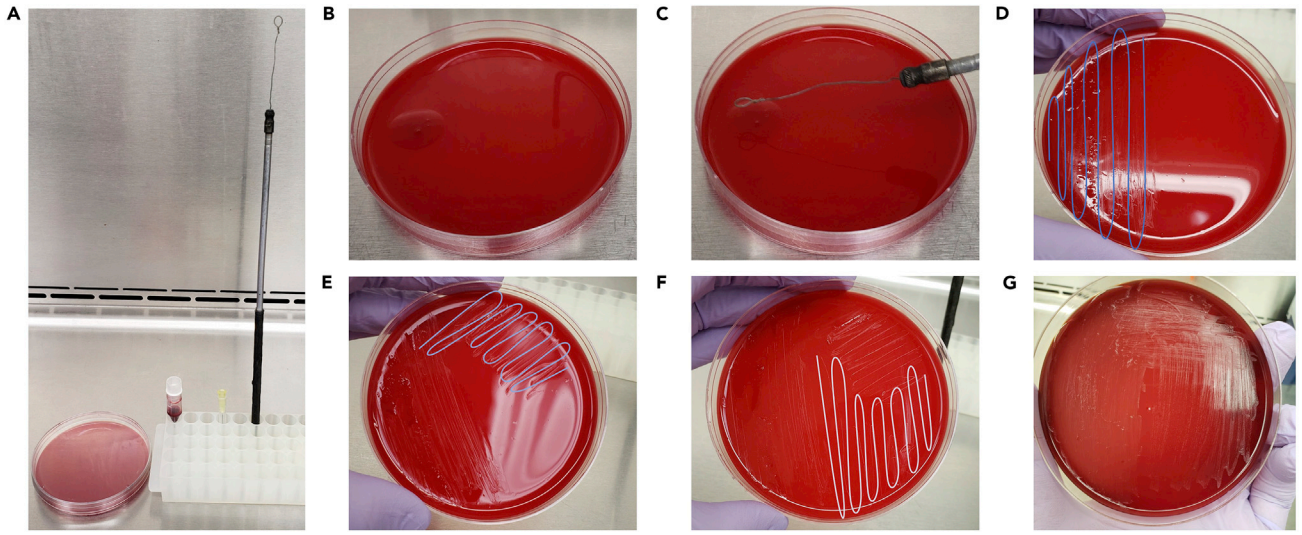

Figure 1. Representative images of performing a three-phase streak

B. pertussis was grown on BG agar supplemented with 15% defibrinated sheep's blood and 100 μg/mL of Streptomycin using a three-phase streak.
(A and B) The materials needed are shown (A) and a chunk of frozen *Bp* is scooped onto a plate and allowed to thaw (B).
(C) A cooled flame-sterilized loop is then used to streak the bacteria on the plate.
(D–F) The three phases of the streak are graphically portrayed with the first streak in dark blue (D), the second streak in light blue (E), and the third streak in white (F).
(G) Once incubated, the plate will have the optimal amount of *Bp* growth needed to use in the next steps.

6. Following incubation, set 4 mL of defibrinated sheep's blood in the biosafety cabinet and allow it to reach room temperature (20°C–25°C).
   a. Remove the plates from incubation and set them in a biosafety cabinet.
7. Swab all bacteria off the plate using a polyester swab and deposit with a swirling motion into the blood.
   a. Use a new polyester swab for each plate.

   Note: 2 plates per 1 mL of blood will give an inoculum dose of ~ 5.5 × 10^9 CFU/mL.

   ⚠ CRITICAL: Be sure to use only polyester swabs for *Bp* because the fatty acids in cotton swabs will inhibit *Bp* survival.

8. Gently vortex the blood with the bacteria to mix and aliquot 150 μL into new sterile 1.5 mL Eppendorf tubes.
9. Label the tubes with the corresponding bacteria and place in a −80°C freezer to be used for challenge dose preparation.

   Note: In our experience, saving aliquots at −80°C in defibrinated sheep's blood results in bacterial viability for at least 10 years.

Preparing the challenge dose

Timing: 4 days

10. Pour plates (100 × 15 mm) with 15 mL of BG agar supplemented with 15% defibrinated sheep's blood and an appropriate antibiotic (e.g., Streptomycin 100 for *Bp* strain UT25sm1).
    a. Dry the plates covered overnight (16–20 h) at room temperature (20°C–25°C) or 15 min in a biosafety cabinet with the lids cracked open.

11. Pipette 20 μL of *Bp* aliquot (from the preparation described above) onto 6 individual BG agar plates.
    a. Perform a three-phase streak on the BG agar using a sterilized metal inoculating loop (Figures 1C–1F).
    b. Place the inoculated BG plates inverted in an incubator set to 36°C for 48–72 h.

⚠ CRITICAL: Avoid freeze/thaw cycles with *Bp* and aliquots should be disposed after use.

12. Remove the BG plates from the incubator.
    a. Swab the entire plate using sterile polyester swabs, paying attention to not pull up chunks of agar with the swab.

Note: One polyester swab used per plate.

    b. Deposit the bacteria from the swabs in Stainer-Scholte Medium (SSM) with a swirling motion and discard the swab once completed.
        i. The swab should be disposed of in biohazard bag.

Note: 1 plate per 1 mL of media will give an inoculum dose of $\sim 10^{10}$ CFU/mL.

    c. Dispense 18 mL of SSM into a new, autoclaved 125 mL Erlenmeyer flask.
    d. Gently vortex bacterial suspension and pipette 2 mL into the 18 mL (1:10 dilution) of SSM from the previous step.
    e. Place inoculated flasks into a shaking incubator set at 180 RPM and 36°C for 22–24 h.

Note: Small footprint shaking incubators dedicated to bacterial growth and rarely interrupted result in the best growth rate (e.g., Benchmark Incu-shaker mini).

13. Once the bacterial solution reaches an OD_{600nm} ~0.6, remove the flasks from the incubator.
    a. Dilute the solution in SSM to an OD_{600nm} of 0.240 ± 0.05 using a UV-VIS standard spectrophotometer (e.g., Beckman Coulter DU-530 with 1 cm pathwidth cuvette).

Note: SSM used as the blank.

⚠ CRITICAL: The OD_{600nm} of 0.240 ± 0.05 gives us a reliable 10^9 CFU/mL of viable bacteria, but a pilot study should be conducted with each spectrophotometer to determine the proper OD for 10^9 CFU/mL.

    b. Quantify the challenge dose by gently vortexing bacterial suspension and pipette 100 μL of the challenge dose into 900 μL of 1× Phosphate Buffered Saline (PBS) for the first serial dilution.
        i. Prepare serial dilutions by mixing 100 μL of the previous dilution with 900 μL of sterile 1× PBS for 8 dilutions.
        ii. Pipette 10 μL of each dilution onto the BG with four technical replicates per dilution.
        iii. Once dots dry, place the plated BG agar inverted in a 36°C incubator for 48–72 h.
        iv. Remove plates and count the number of *Bp* colonies to calculate CFU per mL of the dose.

⚠ CRITICAL: Do not place the challenge dose on ice and use it within 1 h of preparation.

⚠ CRITICAL: Investigate antibiotic resistance for the challenge strain used to ensure proper antibiotic use.

Figure 2. Representative images of the Buxco® Mass Dosing Controller and chamber

(A and B) The chamber has the leur-lok fresh air supply connected (red oval) (A) and the mass dosing controller is set to 2 LPM of fresh air flow (B).

(C) The entire equipment setup is portrayed (C) and shows the nebulizers properly seated (blue square) and the 0.45 μm PVDF membrane filters attached (green oval) opposite the fresh air flow intake.

Setting up the dosing chamber

Timing: 10 min

14. Clean inside the chamber with a non-alcohol-based cleaning solution (peroxiguard®).
15. Connect plastic tubing to fresh air intake valves located on the chamber's side (Figure 2A).
16. Connect PVDF 0.45 μm membrane filters to the exhaust ports on the opposite side of the chamber (Figure 2C).
17. Place autoclaved nebulizers in the top ports of the chamber lid and ensure a snug fit (Figure 2C).
18. Connect power cords from DSI Buxco® FinePointe™ mass dosing controller to the nebulizer heads.
19. Plug in the DSI Buxco® FinePointe™ mass dosing controller and power on the unit.
    a. Set fresh air flow to 2 LPM (Figure 2B).
    b. Set Duty cycle to 100%
    c. Set nebulizing time to 10 min.

⚠ CRITICAL: Ensure that fresh air is being pumped into the chamber and air can flow out of the chamber to avoid the suffocation of animals.

KEY RESOURCES TABLE

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Bacterial and virus strains		
Bordetella pertussis UT25sm1	Dr. Sandra Armstrong (Univ. of Minnesota)	N/A

(*Continued on next page*)

Continued

REAGENT or RESOURCE	SOURCE	IDENTIFIER
Chemicals, peptides, and recombinant proteins		
Bordet-Gengou Agar	Difco	Ref# 248200
Defibrinated sheep's blood	HemoStat	Cat# DSB500
Streptomycin sulfate	Thermo Fisher Scientific	Cat# 11860-038
Glutamic acid monosodium salt·1H_2O	Thermo Fisher Scientific	Cat# AC15621-0010 Cas #56-86-0
Tris base	Thermo Fisher Scientific	Cat# BP152-500 Cas# 77-86-1
Sodium chloride	Thermo Fisher Scientific	Cat# P304-500 Cas# 7647-14-5
Potassium sulfate monobasic	Thermo Fisher Scientific	Cat# P284-500 Cas# 7778-77-0
Potassium chloride	Thermo Fisher Scientific	Cat# BP366-500 Cas# 7447-40-7
Magnesium chloride·6 H_2O	Thermo Fisher Scientific	Cat# BP214-500 Cas# 7786-30-3
Calcium chloride	Thermo Fisher Scientific	Cat#C614-500 Cas# 10043-52-4
L-Cysteine	Sigma-Aldrich	Cat# 168149 Cas# 52-90-4
Ferric sulfoxide·7 H_2O	Thermo Fisher Scientific	Cat# I146-500 Cas# 7752-63-0
L-Ascorbic acid	Thermo Fisher Scientific	Cat# A61-100 Cas# 50-81-7
Nicotinic acid (niacin)	Thermo Fisher Scientific	Cat# AC12829-1000 Cas# 59-67-6
Glutathione (reduced form)	Thermo Fisher Scientific	Cat# AC12000-0250 Cas# 70-18-8
L-Proline	Thermo Fisher Scientific	Cat# BP392-100 Cas# 147-83-3
Hydrochloric acid 10N	Thermo Fisher Scientific	Cat# SA49
Phosphate buffered saline 10× solution	Fisher Bioreagents	Cat# BP399-4
Pentobarbital sodium (Euthasol®)	Patterson Veterinary	Cat# 07-805-9296
Peroxiguard®	Lighthouse Life Sciences	EPA reg# 74559-9
Ethanol absolute 200 proof	Thermo Fisher Scientific	Cat# BP2818-4
Experimental models: Organisms/strains		
Mouse: CD-1; 10-week-old, Female	Charles River	CR: 022
Other		
DSI Buxco® FinePointe™ Mass Dosing Controller	DSI	PN: 011283-001
Mass dosing chamber	DSI	PN: 601-2036-001
Nebulizer	Aerogen	Ref# AG-AL1000
PVDF 0.45 µm membrane filter	Thermo Fisher Scientific	Cat# SLHVM33RS
125 mL Erlenmeyer flask	Thermo Fisher Scientific	Cat# FB-500-125
GentleMACS™ Octo Dissociator	Miltenyi Biotec	Cat# 130-096-427
GentleMACS™ C-tubes	Miltenyi Biotec	Cat# 130-094-334
Inoculating loop	United Scientific	Cat# INL703AL
15 mL polystyrene culture tubes	VWR	Cat# 60818-703
Polytron benchtop PT 2500 E homogenizer	Kinematic	PT# 9158168
20 gauge precision guide needle	BD	Ref# 305175
18 gauge catheter	BD	Ref# 381544
1.5 mL Eppendorf tube	Thermo Fisher Scientific	Cat# S13082
Puritan® Polyester Swab	Thermo Fisher Scientific	Cat# 22-029-574

MATERIALS AND EQUIPMENT

Bordet-Gengou Agar with 15% Defibrinated Sheep's Blood and Streptomycin		
Reagent	Final concentration	Amount
Bordet-Gengou agar	85%	76.5 mL
Defibrinated sheep's blood	15%	13.5 mL
200× Streptomycin	100 μg/mL	45 μL
Total	N/A	90 mL

Note: Wait until agar cools enough to be handled with gloved hands before blood and antibiotics are added, ~45°C.

Note: Swirl to mix blood and antibiotic in to minimize bubbles present in the plate.

Note: Discard any leftover media.

Note: Plates may be poured up to 3 days before use and stored inverted in a container at 4°C.

Basal Stainer-Scholte Media		
Reagent	Final concentration (mM)	Amount
Glutamic acid monosodium salt, 1 H_2O	58.5	10.72 g
Tris base	12.7	1.525 g
NaCl	43.6	2.5 g
KH_2PO_4	3.7	500 mg
KCl	2.7	200 mg
$MgCl_2$	1.1	100 mg
$CaCl_2$	183.9	20 mg
Distilled H_2O		970 mL
Total	N/A	980 mL

Note: Basal SSM should not be heated to dissolve contents.

Adjust pH to 7.6 using 10N NaOH, then bring volume up to 980 mL.

Note: Basal SSM should be autoclaved on a liquid cycle for 15 min at 121°C and 15 PSI.

Note: Media stored at 4°C for 3 months, or −20°C for 6 months.

SSM Supplement- step 1		
Reagent	Final concentration (mM)	Amount
L-Cysteine	825.4	200 mg
1N HCl		500 μL
Distilled H_2O		1.5 mL
Total	N/A	2 mL

Note: Add L-cysteine to 1N HCl and vortex to dissolve, once dissolved add distilled H_2O.

Note: Be sure to use L-cysteine and not L-cystine.

Note: This solution cannot be stored.

SSM Supplement- step 2		
Reagent	Final concentration	Amount
Step 1 solution	3%	1.5 mL
Distilled H_2O	97%	48.5 mL
Total	**N/A**	**50 mL**

Note: Mix solution by vortexing.

Note: This solution cannot be stored.

SSM Supplement- step 3		
Reagent	Final concentration (mM)	Amount
Step 2 solution		50 mL
$FeSO_4 \cdot 7H_2O$	3.6	50 mg
Ascorbic Acid	11.4	100 mg
Nicotinic Acid (Niacin)	3.3	20 mg
Glutathione (Reduced Form)	32.5	500 mg
Total	**N/A**	**50 mL**

Note: Mix solution by vortexing.

Note: Filter-sterilize and aliquot 600 µL into sterile 1.5 mL Eppendorf tubes.

Note: Solution may be stored up to 6 months at −20°C.

L-Proline Supplement		
Reagent	Final concentration (mM)	Amount
L-Proline	208.5	1.2 g
Distilled H_2O		50 mL
Total	**N/A**	**50 mL**

Note: Mix solution by vortex.

Note: Filter-sterilize and aliquot 600 µL into sterile 1.5 mL Eppendorf tubes.

Note: Solution may be stored up to 6 months at −20°C.

Stainer-Scholte Media		
Reagent	Final concentration	Amount
Basal Stainer-Scholte media	98%	49 mL
SSM supplement	1%	500 µL
L-proline supplement	1%	500 µL
Total	**N/A**	**50 mL**

Note: Supplements should be added right before use.

Note: Supplemented media may be stored at 4°C for 1 day if needed.

⚠ CRITICAL: 1N HCl- Corrosive to the eyes, skin, and mucous membrane. Appropriate PPE (e.g., gloves, lab coat, and goggles) should be worn when handling this substance.

Alternatives: We do not substitute any of these materials; therefore, we cannot comment on alternatives.

1× Phosphate Buffered Saline (PBS)		
Reagent	Final concentration	Amount
PBS 10× solution	10%	100 mL
Distilled H_2O	90%	900 µL
Total	N/A	1,000 mL

Note: 1× PBS should be autoclaved on a liquid cycle for 15 min at 121°C and 15 PSI.

Note: 1× PBS solution may be stored at room temperature (20°C–25°C) for 6 months.

STEP-BY-STEP METHOD DETAILS

Aerosol challenge of mice

Timing: 30 min

Upon completion, the aerosol challenge of mice will accomplish the goal of instilling bacteria into their respiratory system. 10-week-old, female CD-1 mice were used in this challenge model and afterward housed in filtertop cages with 5 mice per cage and food and water ad libitum. Setup and usage of the chamber followed the manufacturer's instructions in their application manual found here: Mass Dosing System Application Manual (011283-001).pdf.

1. Move mouse cages into the biosafety hood and remove the lids of the cage and dosing chamber.
2. Moving one mouse at a time, transfer mice from the cage into a compartment inside the mass dosing chamber.
   a. Once an entire cage has been transferred, replace the lid, and move the cage from the biosafety hood.
   b. Repeat this process for all mice being challenged.
3. Open the silicon stoppers on top of the nebulizer heads and carefully pipette 5 mL of the challenge dose into the nebulizer basin.

   Note: 5 mL will be nebulized in each of the 4 nebulizers for a total of 20 mL of liquid challenge dose.

   Note: This dose is approximately 10^{11} CFU per aerosol chamber volume (36.6 L).

4. Seal the silicon stoppers on top of the nebulizer heads.
5. Set a 5-min timer to allow the animals to acclimate to the new environment.
6. Ensure that all settings are correct on the DSI Buxco® FinePointe™ Mass Dosing Controller.
   a. Duty Cycle: 100%.
   b. Aerosolization time: 10 min.
   c. Air Flow Rate: 2 LPM.
   d. Press the start button on the mass dosing controller.

Note: A mist will start descending from the nebulizer heads once started.

7. Increase the fresh air flow rate to 5 LPM after the 10-min nebulizing time is complete.

Note: This increases the clearance of the dose from the chamber.

   a. Set a 5-min timer to ensure the challenge dose has cleared the chamber or settled out of the air.

8. Remove the chamber's lid and use a grasping tool/ protective sleeve to remove the mice from their compartments and return them to their original cage.

⚠ CRITICAL: Be sure to replace the chamber lid in between mice so that no animals can escape by climbing the dividers in the chamber.

Sanitizing the chamber and nebulizers

Timing: 20 min

This step ensures the chamber is sanitized from all BSL2 level agents before leaving the challenge location.

9. Spray the inside of the chamber down with non-alcohol-based cleaner (e.g., peroxiguard) and wipe up debris with the solution.
10. Remove waffle dividers and floorboard, spraying with a non-alcohol-based cleaner and allow to air dry.
11. Spray the inside of the chamber down with a non-alcohol-based cleaner (peroxiguard) and wipe up any debris.

Note: Be sure to spray and wipe down the underside of the chamber lid as well.

12. Gently remove the nebulizer heads from the chamber lid and place them in a container of warm soapy water.
    a. Gently swish the nebulizer heads around and transfer them to a container with clean distilled water.
    b. Swish nebulizer heads to remove any remaining detergent and move to an autoclave sleeve.
    c. Following the manufacturer's instructions, flash steam sterilize the nebulizer head.
        i. We used the following settings: Max temp 121°C, time at temp 3 min, drying time 2 min.
13. Remove nebulizer heads from autoclave sleeves and allow them to dry on their side before being stored.

Euthanasia and data collection

Timing: 1 h

This section is to provide humane euthanasia at the selected endpoint for the study (1 h post-challenge) and for prompt organ collection and bacterial enumeration.

14. Euthanize mice by IP injection of pentobarbital (Euthasol®) following institutional guidelines.

Note: We use a working concentration of 39 mg/mL and inject 10 μL/g of body weight.

15. Once mice are non-reactive to pinch point reflexes, perform a cardiac puncture and remove 1 mL of blood from the mice.
16. Dissect the mice and remove the trachea and lungs from the thoracic cavity.
    a. Place a 1.5 mL Eppendorf on the nose of the mouse and insert an 18-gauge catheter from below the trachea.
    b. Flush the nasal cavity with 1 mL sterile 1× PBS.
17. Excise and separate the lungs and trachea.
    a. Place the lungs into a GentleMacs® C-tube with 1 mL of sterile 1 × PBS.
    b. Homogenize lung samples in C-tubes with a GentleMacs® Octo Dissociator with setting m_Lung_02_01.
    c. Pipette 75 μL of homogenate from the C-tube into 75 μL of sterile 1× PBS to be used as a direct dilution.
18. Remove the esophagus from the trachea samples and place in a 15 mL culture tube with 1 mL of sterile 1× PBS.
    a. Homogenize trachea samples for 5 s using a Polytron® homogenizer.
19. Serially dilute lung and trachea samples 1:10.
20. Plate samples by pipetting 10 μL of the serial dilutions with 4 technical replicates on BG agar with 15% defibrinated sheep's blood and 100 μL/ mL of Streptomycin.
21. Dry plated samples at room temp (20°C–25°C).
    a. Once dry, invert plates and incubate at 36°C for 72 h.
22. After incubation, remove the plates and count CFUs in a dilution row with a countable range to enumerate bacterial burden.

Note: We use a countable range of 5–60 CFU per dot.

23. Multiply the number of CFUs by the dilution factor for each technical replicate.
    a. Average all four technical replicates to obtain the CFU/ mL in the sample.

EXPECTED OUTCOMES

After the successful completion of the aerosol challenge, the mice should have obtained a sufficient infectious dose of bacteria to establish a respiratory infection in the lung (Figure 3B), trachea (Figure 3C), and nasal lavage (Figure 3D). Bacteria are still detectable for at least 7-days post-challenge in all the tissues and for all the doses investigated (data not shown).

LIMITATIONS

One limitation of this protocol is that the mass dosing chamber can simultaneously hold up to 25 mice. There will be a need for a larger volume of challenge dose and multiple rounds of aerosolization if a challenge is needed for more than 25 mice. Of note, we challenged up to 15 mice simultaneously with reproducible results during our experiments. However, we did not challenge the maximum 25 mice at a time that the manufacturer states will fit in the chamber. We cannot speak to the reproducibility of challenging an upper limit of mice in the chamber. Although we challenged mice with a challenge dose as low as 10^6 CFU/mL at 20 mL volume and could enumerate CFUs 1 h post-challenge, there may be a lower challenge dose concentration limit where not all mice will establish an infection. Further studies will be needed to suggest this limit.

TROUBLESHOOTING

Problem 1

Low OD_{600nm} when preparing the challenge dose in step 13.a. in before you begin.

Potential solution

If the OD is lower than desired, ensure that all bacterial growth is collected and deposited into the SSM media when swabbing the plate.

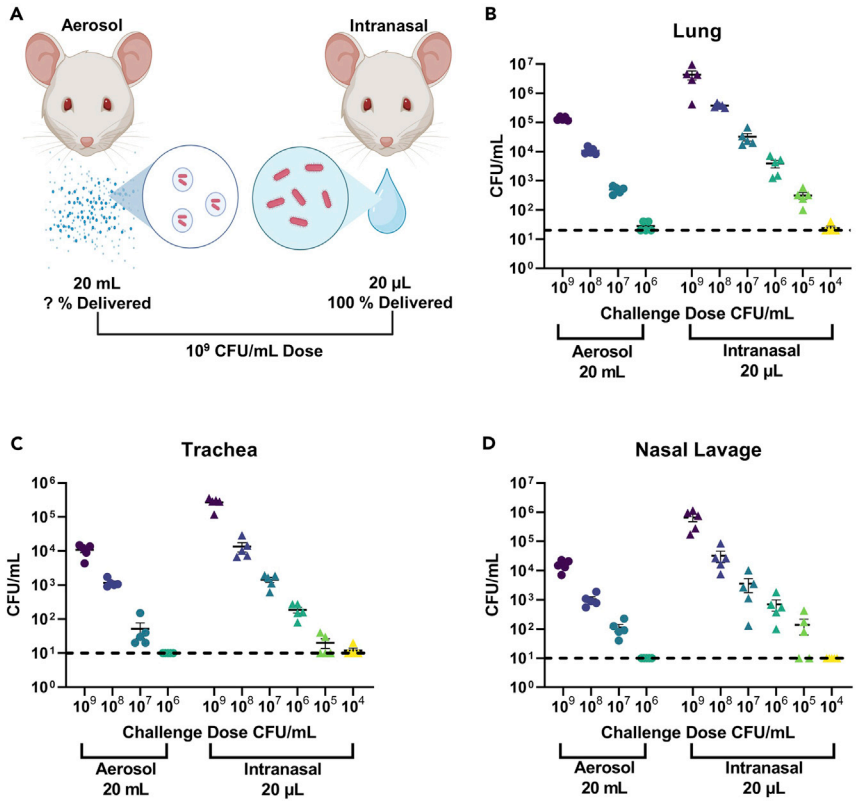

Figure 3. Bacterial quantification 1-h post-challenge

(A–D) *Bordetella pertussis* bacterial burden was quantified from the mice challenged either through aerosol exposure or intranasal instillation (A) in the lung (B), trachea (C), and nasal lavage (D) 1-h post-challenge. The black dashed line indicated the lower limit of detection. The data are represented as mean ± SEM.

- The use of polyester/dacron (not cotton) swabs is required due to the acids within cotton that inhibit *Bordetella spp.* growth.
- New, freshly autoclaved Erlenmeyer flasks should be used to ensure no detergents that limit bacterial growth are present.
- Flasks can be incubated for another 2–6 h if needed to allow for more growth.

Problem 2

No aerosol vapors are emitted through the nebulizer head during challenge in step 6.d.

Potential solution

Thorough cleaning of nebulizer heads to essential to their functionality; however, if the head is clogged, the following steps may be followed.

- Gently remove the nebulizer head from the chamber lid.
- Empty the contents of the nebulizer into a waste container and sit the nebulizer with the top of the head resting on a table.
- Pipette 10–20 µL of dH_2O onto the nebulizer membrane.

Ensure that you do not touch the membrane with the pipette tip.

- Set the controller for a 100% duty cycle and start the program.
- After 30 s, a vapor should rise from the nebulizer membrane.
- Repeat this process 3–5 times.

- Return the nebulizer head into its place and ensure its proper function by pipetting 1 mL dH_2O into the nebulizer head and starting a cycle. Vapor should now be emitting from the nebulizer head.

RESOURCE AVAILABILITY

Lead contact

Further information and requests for resources and reagents should be directed to and will be fulfilled by the lead contact, F. Heath Damron- fdamron@hsc.wvu.edu.

Materials availability

This study did not generate any new reagents, animal/ cell lines, or other such materials.

Data and code availability

This study did not generate or analyze any novel data outside of the bacterial burden per tissue that is shown in Figure 1.

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AUTHOR CONTRIBUTIONS

G.J.B., K.L.W., M.B., and F.H.D. participated in experimental design. All authors participated in the aerosol challenge experiments. G.J.B. and K.L.W. composed the protocol. M.B. and F.H.D. provided expertise and guidance through all aspects of the protocol development. All authors reviewed and provided critical revisions to the protocol.

DECLARATION OF INTERESTS

The authors declare no competing interests.

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