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Assessing *Acinetobacter baumannii* virulence and persistence in a murine model of lung infection

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Abstract

Acinetobacter baumannii is a Gram-negative opportunistic pathogen and a leading cause of ventilator-associated pneumonia. Murine models of *A. baumannii* lung infection allow researchers to experimentally assess *A. baumannii* virulence and host response. Intranasal administration of *A. baumannii* models acute lung infection. This chapter describes the methods to test *A. baumannii* virulence in a murine model of lung infection, including assessing the competitive index of a bacterial mutant and the associated inflammatory responses.

Keywords

Lung infection; pneumonia; murine model; *Acinetobacter baumannii*; inflammation

1. Introduction

Acinetobacter baumannii is an important opportunistic pathogen that commonly infects critically ill patients in hospital settings. While this aerobic, Gram-negative coccobacillus was once largely considered an innocuous environmental organism, *A. baumannii* and related species have emerged in recent years as important threats to global public health. Driving its clinical significance is the rapid acquisition of multi-drug resistance phenotypes, which have arisen at alarming rates [1,2]. This has led the World Health Organization to prioritize multi-drug resistant *A. baumannii* among a group of bacterial pathogens that pose the greatest threat to human health [3].

A. baumannii causes a range of infections, including pneumonia, bacteremia, skin and soft tissue infections, as well as urinary tract infections [4,5]. In hospital settings, these *A. baumannii* infections frequently occur when normal anatomical barriers are breached, such as in the case of ventilator-associated pneumonias, central line-associated bloodstream infections, surgical wound infections, and catheter-associated urinary tract infections. In particular, ventilator-associated pneumonias are among the most frequent causes of hospital-

acquired *A. baumannii* infections, where mortality rates approach 75% [6]. While less frequent, cases of community-acquired *A. baumannii* pneumonia have also been reported, with mortality rates as high as 64% [7]. Taken together, the challenges presented by this emerging pathogen underline the importance of studying its biology and pathogenesis in animal infection models.

Over the last decade there has been significant effort to characterize the mechanisms by which *A. baumannii* thrives in the host environment. This work has identified a number of virulence factors and systems required for optimal virulence, including protein secretion machinery [8–11], micronutrient acquisition and homeostasis systems [12–17], and proteins involved in the assembly and modification of outer membrane and capsular polysaccharide structures [18–20]. Additionally, a number of genome-wide transposon mutagenesis screens have identified *A. baumannii* genes required for infection in eukaryotic hosts, including those involved in micronutrient acquisition and capsule formation, amino acid and purine metabolism, biofilm formation, and oxidative and osmotic stress responses [21–23].

Studies examining the pathogenesis of *A. baumannii* have employed a number of eukaryotic host model organisms, including mouse, rat, guinea pig, rabbit, zebrafish, and insect larvae infection models [24–29]. Because of the low virulence of most *A. baumannii* strains in mice, some murine models of infection with this pathogen utilize mechanisms to suppress the host immune response by rendering mice neutropenic with antibody or cyclophosphamide treatments [27,30–33]. While these model systems often result in more lethal infections, they confound the ability to study interactions between *A. baumannii* and the innate immune system. By contrast, the use of immunocompetent murine infection models has driven the discovery of several components of the host immune response to *A. baumannii* that are important for protection against this pathogen. These include detection by pattern recognition receptors [34–37], production of reactive oxygen species [38], and the release of antimicrobial proteins by immune cells [12].

Here, we describe a murine model of acute *A. baumannii* pneumonia in an immunocompetent host that can be utilized to characterize both bacterial factors important for *A. baumannii* pneumonia, as well as the host immune response to this pathogen.

2. Materials

2.1 Lysogeny broth (LB)

LB is used to culture *Acinetobacter* spp. Agar is added when solid medium is required. Mix the following in ddH₂O and autoclave:

1. Tryptone, 10 g/L
2. Yeast extract, 5 g/L
3. Sodium chloride, 10 g/L
4. (*optional*) agar, 15 g/L

2.2 Phosphate buffered saline (PBS)

Endotoxin-free PBS for Avertin stock can be purchased. To generate sterile PBS for bacterial suspensions and serially dilutions, mix the following in 800 mL ddH₂O:

1. 8 g sodium chloride
2. 0.2 g potassium chloride
3. 1.44 g sodium phosphate dibasic
4. 0.24 g potassium phosphate monobasic

Adjust the pH to 7.4 with HCl. Add ddH₂O to 1 L total volume and autoclave to sterilize.

2.3 Neutral buffered formalin

Neutral buffered formalin solution (10%) is used to fix lung tissue prior to paraffin embedding and sectioning. It can be purchased or generated by adding the following to 100 mL ddH₂O:

1. 4 g sodium dihydrogen orthophosphate (monohydrate)
2. 6.5 g disodium hydrogen orthophosphate (anhydrous)
3. 100 mL formaline (40% aqueous solution of formaldehyde)
4. 800 mL ddH₂O for use

2.4 Diluted optimal cutting temperature (OCT) compound (25%)

Optimal cutting temperature compound is diluted to allow tracheal instillation for lungs to be frozen and sectioned. It can be purchased or generated by thoroughly mixing the following:

1. 75 mL ddH₂O
2. 25 mL optimal cutting temperature (OCT) compound

2.4 Mice

Specific-pathogen free age and sex-matched 6–12 week old C57BL/6, BALB/c mice (The Jackson Laboratory) or Swiss Webster mice (Charles River Laboratories). Mice should be allowed food and water *ad libitum*. All protocols and experimentation require approval from an institutional animal care and use committee.

2.5 Anesthesia and euthanasia

Avertin anesthesia:

1. Avertin (2,2,2-tribromomethanol)
2. *tert*-Amyl alcohol (2-Methyl-2-butanol)
3. Sterile, endotoxin-free PBS

Avertin should always be protected from light [39]. Generate a 100% stock by dissolving 1 g Avertin (2,2,2-tribromomethanol) in 1 mL *tert*-Amyl alcohol (2-Methyl-2-butanol) by

vigorous vortexing. The 100% stock can be stored at 4°C for approximately 1 month. Generate a 1.25% concentrated stock by diluting into sterile, endotoxin-free PBS pre-warmed to 37–42 degrees. Ensure full dissolution of the stock by vigorous vortexing and/or rocking at room temperature. The working solution should be kept in the dark at 4°C if it is not to be used immediately.

Euthanasia: CO₂ inhalation or ketamine/xylazine anesthesia with exsanguination is used for euthanasia. Ketamine/xylazine stock should be prepared in sterile PBS (or 0.9% saline) at 20 mg/mL ketamine and 2 mg/mL xylazine. For non-survival surgery, ketamine/xylazine should be administered at 150 mg/kg ketamine and 15 mg/kg xylazine, with additional as needed to maintain a surgical plane of anesthesia. Alternatively, overdose with ketamine/xylazine or pentobarbital can be used as approved by the institutional animal care and use committee. All drugs are administered intraperitoneally using a 0.5 mL-1 mL insulin syringe with an attached 28 G needle.

2.6 Bacterial suspension

1. LB
2. Sterile PBS
3. Sterile 96-well plate
4. Sterile petri plates with agar media containing appropriate antibiotics
5. Disposable 1-cm cuvettes or alternative method to measure optical density at 600 nm

2.7 Intranasal application

The following items should be prepared prior to the anesthesia procedure.

1. Isothermal pads pre-warmed to 37°C
2. 1 Cotton swabs
3. Puralube ointment
4. P50-P200 pipettor and tips

2.8 Measurement of bacterial load

1. 70% ethanol spray bottle
2. Styrofoam block (*e.g.* from conical tube holders)
3. 21 G needles
4. Forceps and scissors
5. 50-mL conical tubes filled with ethanol and sterile water to sterilize surgical tools

6. Sterile Bullet Blender lysis tubes and Bullet Blender Tissue Homogenizer (Next Advance Inc., Troy, NY); alternatively, sterile Whirl-pak bags (*e.g.* Nasco, Fort Atkinson, WI; Fisher B00736WA) and plastic or metal rolling pin
7. Sterile PBS (for serial dilutions of bacteria)
8. Sterile 96-well plates
9. P10-P300 multi-channel pipettor
10. Petri dishes with agar media containing appropriate antibiotics

2.9 Digestion of lungs for flow cytometry

11. 1.5-mL tubes
12. Dulbecco's Modified Eagle Medium (DMEM)
13. Fetal bovine serum (FBS)
14. Type I collagenase
15. DNase
16. 2X digestion medium: DMEM + 10% FBS containing 2 mg/mL collagenase type I and 0.2 mg/mL DNase, filter sterilized
17. 70- μ m filters
18. 50-mL conical tubes
19. Red blood cell lysis buffer

2.10 Tracheal instillation for bronchoalveolar lavage fluid collection and preparation of formalin-fixed paraffin embedded or frozen lung tissue for histopathology

1. 70% ethanol
2. Styrofoam block (*e.g.* from conical tube holders)
3. 21 G needles
4. Fine-tipped angled forceps and fine-tipped sharp scissors
5. 50-mL conical tubes filled with ethanol and sterile water to sterilize surgical tools
6. 21 G blunt needle or tracheostomy tool
7. Suture threads cut to 2" lengths and one cut to 5"
8. *Bronchoalveolar lavage fluid (BALF)*: Sterile PBS, sterile 1 mL syringes
9. *Formalin fixed and paraffin embedded lung tissue*: neutral buffered formalin solution (10%), 50-mL conical tubes containing 10 mL neutral buffered formalin
10. *Frozen lung tissue*: 25% optimal cutting temperature compound (OCT) in ddH₂O, dry ice, small weigh boats (~4.5 cm)

3. Methods

3.1 Preparation of *Acinetobacter* spp. for acute lung infection of mice

1. Streak *Acinetobacter* sp. strain from -80°C stock to LB agar with appropriate antibiotics for single colonies and incubate overnight at 37°C .
2. Inoculate 3 mL LB using 1–2 colonies *Acinetobacter* sp. and incubate with shaking 37°C at 180 rpm for 12–16 h. If antibiotics must be used to maintain selection, appropriate control strains should be generated to allow identical growth conditions.
3. Inoculate 10 mL LB with 10 μL overnight culture in an appropriate tube (*e.g.* 50-mL conical tube with cap sealed loosely) and incubate at 37°C with shaking at 180 rpm until mid-logarithmic growth phase (*see* example in Fig. 1; *see* **Note 1**). To achieve high density suspensions (*i.e.* 1×10^9 colony forming units), inoculate 50 mL LB with 50 μL overnight culture in a 250-mL Erlenmeyer flask and incubate at 37°C with shaking at 180 rpm.
4. Centrifuge at $5,900 \times g$ in a fixed-rotor centrifuge for 6 min at 4°C , discard the supernatant and resuspend in an equal volume of ice-cold sterile PBS. Repeat centrifugation and resuspension.
5. Centrifuge at $5,900 \times g$ in a fixed-rotor centrifuge for 6 min at 4°C , discard the supernatant and resuspend in 1 mL ice-cold PBS. Keep bacterial suspensions on ice.
6. Measure the culture optical density at 600 nm (OD_{600}). Quantify bacteria against a standard curve for the number of colony forming units (CFU)/ OD_{600} (*see* **Note 2**).
7. Dilute the bacteria to the desired density in ice-cold sterile PBS and keep bacterial suspensions on ice. For a competitive infection, mix wild-type and mutant bacteria at 1:1 ratio following generation of individual strain suspensions at the appropriate density. Bacterial suspensions prepared in this way can be similarly used for systemic infections (*see* **Note 3**).
8. Following infection, transfer 10 μL of each inoculum to the top row of a 96-well plate containing 90 μL ice-cold sterile PBS in each well (*i.e.* to generate dilutions 10^{-1} – 10^{-8}). Using a multi-channel pipettor, plate 10 μL serial dilutions to LB agar plates. Use dried LB agar plates to maintain separation of the plated serial dilution aliquots. If the experiment is to calculate the competitive index of two strains, plate to media containing the appropriate antibiotics to differentiate between the strains (*i.e.* wild-type and mutant grow on LB, while only the mutant grows on LB containing appropriate antibiotics) to confirm the plating scheme.
9. Incubate plates overnight at 37°C and count total bacterial input to the lungs.

3.2 Mice infection via intranasal application

1. Neutropenia can be induced 24 h prior to anesthesia if desired (see **Note 4**). For Avertin anesthesia, all chemicals and stock solutions should be kept protected from light. Prepare an Avertin stock as described above, and administer 125–250 mg/kg to mice by intraperitoneal injection. Mice should be unresponsive to toe pinching, but breathing regularly.
2. Apply lubricating ointment to the mice eyes using a sterile cotton swab.
3. Holding the mouse gently by the scruff, position the animal upright using a forefinger to maintain a straight trachea (Fig. 2). Confirm the mouse is breathing normally.
4. Cover a 37°C heated isothermal pad with a paper towel and position it in the cage at an angle.
5. Using a P50-P200 pipettor, apply 30–50 µL bacterial suspension to the nostril as the animal inhales, breathing in the bacterial suspension (see **Note 5**).
6. Hold the mouse upright for 1 min before placing the mouse prone on the heated isothermal pad with the head elevated. Successful intranasal administration will lead to a change in breathing rate that should normalize before placing the mouse on the heated pad. If the animals are not sufficiently anesthetized, they may snort or cough up the inoculum, which reduces the likelihood of successful infection.
7. Monitor the animal until fully awake and remove the heated isothermal pad.

3.3 Measurement of lung bacterial loads and immune response

1. Acute lung infection can cause mice to temporarily show signs of illness including loss of coat quality. Mice should be observed for clinical signs of illness including responsiveness. Any moribund animals should be euthanized humanely in accordance with an approved animal care protocol. Acute infection of wild-type mice can be observed for up to 48 h post infection (hpi), at which point bacterial burdens diminish (Figure 3) [40].
1. Euthanize mice by CO₂ inhalation and record body weight.
2. Sterilize the Styrofoam board surgical surface by spraying down with 70% ethanol. Pin the mouse to the board using 21 G needles. Disinfect the mouse coat with 70% ethanol. Sterilize the surgical tools by keeping in 50-mL conical filled with ethanol, then dipping in sterile water prior to use. Expose the peritoneum by a vertical excision with blunt scissors and removal of the ventral skin. Expose the thoracic cavity by cutting through the peritoneum and diaphragm. Excise the lungs from the animal and place in a Bullet Blender lysis tube filled with 0.7 mL ice cold PBS or Whirl-Pak bag. If desired, measure the lung mass by weighing the lysis tube with and without the lung. If the samples will be used for protein analysis, add protease inhibitors to the PBS and sterilize by filtration prior to filling the tubes.

3. Sterilizing tools in between each step, harvest additional organs such as the liver and spleen to assess bacterial dissemination. Spleens are placed in Bullet Blender lysis tubes with 0.7 mL PBS or Whirl-pak bags. Livers can be divided into two Bullet Blender lysis tubes with 0.5 mL PBS or placed in Whirl-pak bags.
4. *Homogenize tissues using the Bullet Blender:* Homogenize tissues in Bullet Blender lysis tubes on power 10 for 5 min at 4°C. Homogenize lungs for two cycles.
5. *Homogenize tissues using Whirl-pak bags (alternate method):* Add 1 mL ice-cold PBS to each bag and seal, removing air bubbles. Tape 2–5 bags with tissues to the benchtop at the sealing end. Using a metal or plastic rolling pin, roll over tissues 70 times to homogenize or 100 times for lungs, rolling away from the body. Visually inspect for uniform homogenization. Collect the homogenized tissues with a 2-mL pipet and transfer to an 2-mL tube on ice.
6. Transfer 100 μ L of each homogenate to the top row of a 96-well plate. Serially dilute the 10 μ L tissue homogenates into 90 μ L ice-cold sterile PBS (*i.e.* to generate dilutions 10^0 - 10^{-7}) using a multi-channel pipettor. Using a multi-channel pipettor, plate 10 μ L of the serial dilutions to dried LB agar plates. For spleens and any organs with low bacterial burdens, also plate 100 μ L organ homogenate to lower the limit of detection. If the experiment is to calculate the competitive index of two strains, plate to media containing the appropriate antibiotics to differentiate between the strains (*i.e.* wild-type and mutant grow on LB, while only the mutant grows on LB containing appropriate antibiotics).
7. Incubate plates overnight at 37°C and count total bacterial output in the lungs and other organs. For any organs with no detectable bacterial burdens, set at the limit of detection for statistical purposes.
8. *Competitive infection:* The number of the mutant strain CFU is determined by serial plating to selective medium. To determine the number of wild-type bacteria in each organ, subtract the mutant CFU counted from selective medium from the total CFU counted from LB plates. The competitive index is the ratio of mutant/wild-type CFU after normalizing for input, *i.e.* (mutant output CFU/ mutant input CFU)/(wild-type output CFU/wild-type input CFU).
9. *Cytokine analysis:* Centrifuge the remaining homogenate at $21,000 \times g$ for 5 min and transfer the supernatant to a fresh tube to use for enzyme-linked immunosorbent assay (ELISA) or multi-analyte profiling (Luminex Corp., Austin, TX). Homogenates or supernatants can be stored at -80°C prior to analysis. For improved precision, homogenate supernatants can be normalized for total protein content.
10. *Digestion of lungs for flow cytometry:* Place excised lungs in 1.5-mL tubes containing 1 mL ice-cold DMEM + 10% FBS and keep on ice. Using fine-tipped scissors, chop the lungs into small pieces. Transfer lung pieces to a 15-mL conical tube, collecting any remaining lung pieces from the 1.5-mL tube with an

additional 1.5 mL medium. Add 2.5 mL 2X digestion medium, and incubate at 37°C with shaking for 15–60 min. Set a 70-µm filter on a 50-mL conical and decant the digested lung onto the filter. Push the tissue through the filter with the back of a 3-mL syringe plunger to generate single cell suspensions. Centrifuge at 1200 rpm for 5 min at 4°C in a swinging-bucket rotor and discard supernatant. Resuspend the pellet in 5 mL 1X RBC lysis buffer and incubate for 5 min on ice. Stop the lysis with 25 mL DMEM or PBS. Decant the single cell suspension through a new 70-µm filter into a new 50-mL conical tube. Centrifuge again, discarding supernatant. Resuspend cells in 1–10 mL DMEM + 10% FBS and count cells on a hemocytometer, avoiding remaining RBCs. The single cell suspensions can be stained and analyzed by flow cytometry using standard procedures.

3.4 Tracheal instillation for BALF collection or lung tissue pathology

1. Euthanize mice and record body weight. Do not perform cervical dislocation prior to tracheostomy. Due to potential pulmonary hemorrhage from CO₂ inhalation [41], ketamine/xylazine anesthesia and exsanguination or overdose is preferred for these procedures. Anesthetize the mice with ketamine/xylazine for non-survival surgery. Monitor that the animal does not respond to stimuli by toe pinch.
2. Sterilize the Styrofoam board surgical surface by spraying down with 70% ethanol. Pin the mouse to the board using 21 G needles. Disinfect the mouse coat with 70% ethanol. If euthanizing the anesthetized mouse by exsanguination, open the thoracic cavity by midline and exsanguinate through cardiac puncture.
3. *Tracheostomy*: Stabilize the head and trachea of the euthanized mouse by creating a 2" loop of suture thread, looping it around the mouse upper incisors and pinning back the loop with a 21 G needle (Fig. 4). Sterilize the surgical tools by keeping in 50-mL conical tubes filled with ethanol, then dipping in sterile water prior to use. Remove skin from the neck with scissors, then carefully expose the trachea by gently parting the submaxillary glands and muscle tissue with two pairs of tweezers, taking care to not rupture any blood vessels. Using fine-tipped angled tweezers, insert a 2" suture thread under the trachea. Using fine-tipped scissors, make a small crosswise incision in the trachea and insert a 21 G blunt needle. Tie the blunt needle in place with the suture thread.
4. *Bronchoalveolar lavage fluid*: Instill up to 0.8 mL sterile PBS into the lungs using a 1 mL syringe attached to the 21 G blunt needle or tracheostomy tool, then withdraw to obtain BALF. BALF collection can be repeated up to three times. To quantify bacteria in the BALF, serially dilute 10 µL and plate to LB agar as described above. To measure cytokines in the BALF, freeze at –80°C until analysis.
5. *Formalin fixed and paraffin embedded lung tissue*: Inflate lung tissue prior to fixation to improve morphological analysis. Instill up to 0.8 mL 10% neutral buffered formalin in the lungs. Leaving the syringe in place, carefully open the

thoracic cavity if not already open, and cut away tissue to allow removal of the trachea, lungs, and heart. Remove the syringe and needle, grip the trachea to prevent formalin loss, and remove the trachea, lungs, and heart. Place the lungs in a 50-mL conical tube containing 10 mL 10% formalin and fix for 1–4 days. Remove the heart if desired, and transfer fixed tissue to an embedding cassette dorsal side down. Embed in paraffin, cut slides 5 μ m sections, stain slides with hematoxylin and eosin (H&E) and examine with a brightfield microscope according to standard procedures.

6. *Frozen lung tissue:* Instill up to 0.8 mL 25% OCT into the lungs. Cut away the heart, remove the lungs and trachea as described above, and gently wash the lungs in sterile PBS and blot dry. Place the lungs dorsal side down in a small weigh boat and freeze on dry ice. Store frozen tissue at -80°C . Cut sections of desired thickness using a cryosectioner: 5 μ m for histopathology or immunofluorescence, 10 μ m for matrix assisted laser desorption/ionization imaging mass spectrometry to image proteins [12,40].

4. Notes

1. Each *Acinetobacter* species or strain must be tested for growth kinetics under the conditions to be used for infections. To compare strains with different growth rates in one infection experiment, subculture slower growing strains earlier to ensure all strains are in mid-log phase when harvested by centrifugation.
2. The appropriate OD₆₀₀ will need to be determined for each *Acinetobacter* strain or species, as differences in capsule or bacterial shape affect light scattering and therefore the OD₆₀₀ to CFU relationship. For example, an *A. baumannii* 17978 suspension diluted 1/50 in PBS with an OD₆₀₀ of 0.36 in a 1 cm plastic cuvette is 1×10^{10} CFU/mL. Once an appropriate OD₆₀₀ is determined for a particular bacterial density, use the same OD₆₀₀ and alter the dilution factor to obtain bacterial cultures of different densities.
3. For systemic *A. baumannii* infection, inject 100 μ L prepared bacterial suspension intravenously. Bacterial burdens are typically assessed at 24 hpi, as mortality or bacterial clearing are often observed after 24 hours.
4. To deplete neutrophils, intraperitoneally inject mice with 3–15 mg/kg anti-mouse Ly6G clone 1A8 at 24 hours prior to infection. Intraperitoneally inject the same dose of isotype control antibody to a second, control group of mice as described above. Repeat procedure 1 h prior to infection, as well as each day post-infection at 24-hour intervals for the duration of the experiment. Confirm neutropenia by flow cytometric analysis using anti-mouse Gr-1 (clone RB6–8C5) and anti-mouse CD11b (clone M1/70) by standard methods.
5. When working with a new *Acinetobacter* strain or mouse strain, experimentally determine the inoculum by infecting with a range of doses, e.g. 3×10^6 – 1×10^9 CFU.

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Figure 1: *A. baumannii* growth curve.

A. baumannii 17978 was grown in 10 mL LB and bacterial growth was monitored by optical density at 600 nm (OD₆₀₀). The arrow indicates 3.5 h or mid-exponential phase when bacteria are typically used for intranasal inoculation.

Figure 2: Intranasal application of *Acinetobacter* suspension.

Anesthetized mice are held upright for intranasal inoculation with bacterial suspension.

Figure 3:

Timecourse of *A. baumannii* lung infection and dissemination. Seven-week-old Swiss Webster mice were intranasally infected with 10^8 CFU *A. baumannii* and monitored over time in hours post infection (hpi). (A) Weight change. Values from 6 to 216 hpi were statistically decreased ($p < 0.05$) from uninfected animals by two-way ANOVA. (B) Bacterial burdens in the lung. Values from 0 to 72 hpi were statistically different ($p < 0.05$) from uninfected animals by Student's *t* test. (C) Bacterial dissemination to the liver. Values from 18 to 36 hpi were statistically different ($p < 0.05$) from uninfected animals by Student's *t* test. Reproduced from [40] with permission from Wiley.

Figure 4: Tracheal instillation.

Following euthanasia, the mouse is pinned to a foam board and the neck is stabilized by pinning a thread looped around the teeth. The neck is cut open and the tissue gently pulled aside to expose the trachea. A suture thread is inserted below the trachea. The trachea is snipped, and a blunt needle is inserted and the suture thread is tied to secure the blunt needle. For BALF collection, PBS is instilled and withdrawn. For lung fixation, the lung is removed after instillation of the appropriate medium.

Figure 5: Determination of appropriate inoculum.

A. nosocomialis M2 was intranasally inoculated into 9-week old C57BL/6J mice at the doses indicated on the x-axis. At 36 hpi, bacterial burdens were assessed in lungs (A), livers (B), and spleens (C). Due to mortality with 3×10^9 CFU inoculum and low dissemination to the spleen with 3×10^8 CFU, an inoculum of 1×10^9 CFU was chosen [8,9].