Video Article

Intratracheal Inoculation of Fischer 344 Rats with *Francisella tularensis*

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Abstract

Pulmonary infection with the bacterium *Francisella tularensis* can lead to the serious and potentially fatal disease, tularemia, in humans. Due to the current lack of an approved tularemia vaccine for humans, research is focused on vaccine development utilizing appropriate animal models. The Fischer 344 rat has emerged as a model that reflects human susceptibility to *F. tularensis* infection, and thus is an attractive model for tularemia vaccine development. Intratracheal inoculation of the Fischer 344 rat with *F. tularensis* mimics pulmonary exposure in humans. The successful delivery into the rat trachea is critical for pulmonary delivery. A laryngoscope with illumination is used to properly intubate the tracheae of anesthetized rats; the correct placement within the trachea is determined by a simple device to detect breathing. Following intubation, the *F. tularensis* culture is delivered in a measured dose via syringe. This technique standardizes pulmonary delivery of *F. tularensis* within the rat trachea to evaluate vaccine efficacy.

Video Link

The video component of this article can be found at https://www.jove.com/video/56123/

Introduction

F. tularensis (Ft) causes the human disease, tularemia. When the bacteria are acquired through the pulmonary route, this leads to pneumonic tularemia, which has high morbidity and mortality[1]. *F. tularensis* is considered a biothreat agent due to the danger associated with aerosolized forms, and there is currently no vaccine approved for human use in the U.S. An intensive effort is currently underway to develop vaccines and therapeutic measures against pneumonic tularemia, to protect the human population against the illicit use of this bacterial biothreat.

Much of the tularemia research has focused on the mouse model, due to the extreme sensitivity of mice to *F. tularensis* infection, and the prevalence of reagents. However, mice have proven to be a difficult model for vaccine development, due to the difficulty of demonstrating vaccine efficacy in this model[2]. Recently, the Fischer 344 rat has been developed as a model for tularemia vaccine development[3]. The sensitivity of the Fischer 344 rat to various *F. tularensis* subspecies mimics human sensitivity[4], and rats can be protected against *F. tularensis* pulmonary challenge by vaccination with a live vaccine strain known to protect humans[5,6,7]. Because the Fischer 344 rat models some features of *F. tularensis* infection of humans, it may be an extremely useful model for the development of a vaccine that protects against pulmonary *F. tularensis* exposure.

An effective vaccine needs to protect humans against pulmonary exposure to *F. tularensis*. The most likely pulmonary exposure from weaponized *F. tularensis* would be aerosolized bacteria inhaled into the lungs[8]. However, aerosol generation of *F. tularensis* is both dangerous and cumbersome, and requires specialized equipment and containment. An alternate route of pulmonary exposure in the rat that is perhaps more adaptable for multiple laboratories lacking specialized equipment is via intratracheal inoculation[6]. This technique utilizes a laryngoscope for the correct placement of a catheter within the trachea of an anesthetized rat. Placement within the trachea, rather than the esophagus, is verified by a simple device that visualizes airflow from the lungs. *F. tularensis* is subsequently delivered into the lungs through the catheter by administration with a syringe, followed by the introduction of air into the catheter to ensure pulmonary delivery of the bacteria. In contrast, Jemski[5] previously reported that *F. tularensis* inoculated into Fischer 344 rats via the intranasal route could not be cultured from the lungs until 3 days post-inoculation, indicating that intranasal inoculation in rats does not result in direct delivery of bacteria into the lungs.

Select agent forms of *F. tularensis* (*F. tularensis* subsp. *tularensis*, *F. tularensis* subsp. *holarctica*) require Biosafety Level 3 (BSL3) containment procedures, which would prevent videography. However, *F. novicida* (Fn) is exempt from select agent status due to its avirulence in healthy humans, and can be utilized safely under Biosafety Level 2 (BSL2) conditions[9,10]. Moreover, Fn serves as the basis for live attenuated vaccines that can protect against *F. tularensis* pulmonary exposure when delivered via intratracheal inoculation[11,12,13]. The technique presented here allows for the study of infections that occur through the pulmonary route utilizing rats as a model for humans. This technique can be performed without the need for specialized aerosol-generating equipment. Fn was used for the techniques filmed here.

Protocol

This work was performed in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the National Institutes of Health. Animal protocols involving rodents were approved by the University of Texas at San Antonio Institutional Animal Care and Use Committee (IACUC) under protocol MU009(RA).

1. Prepare Catheter, Trachea Indicator, and *F. tularensis* Inoculum

1. **Prepare Catheter (20 G x 2 in)**
    1. Cut the 20 G x 2 in needle and grind the tip of the needle to a blunt and smooth finish with a rotary tool.
    NOTE: The length of the blunted needle should allow the metal tip to rescind approximately 3 mm into the catheter sleeve when fully seated onto the blunted needle. The blunted needle gives the catheter structural rigidity to allow placement of the catheter in the trachea, and the absence of the blunted needle tip protruding from the catheter sleeve prevents injury to the trachea.
    2. Clean the blunted needles and catheters using 70% ethanol and sterilize under UV light for 15 min.

2. **Prepare Trachea Indicator**
    1. Trim a 1,000 µL pipette tip to allow the tip to sit into a catheter opening and form an air-tight seal.
    2. Remove a filter from a 200 µL aerosol barrier pipette tip and place inside the previously trimmed 1,000 µL pipette tip.
    3. Ensure the filter can move freely inside the 1,000 µL pipette tip when the tip is pointed tip-down.

3. ***F. tularensis* Inoculum**
    1. Grow *F. tularensis* strain overnight on appropriate agar plate at 37 °C. Scrape approximately 100 µL of the bacterial lawn from the agar plate with a sterile inoculating loop and use to inoculate a 500 mL Erlenmeyer flask containing 250 mL of appropriate liquid growth medium (for Fn: tryptic soy broth (TSB) supplemented with 0.1% L-Cysteine hydrochloride monohydrate) and incubate shaking overnight at 37 °C.
    NOTE: *F. novicida* used in the video was grown on a tryptic soy agar (TSA) plate supplemented with 0.1% L-Cysteine hydrochloride monohydrate.
    2. Centrifuge the overnight grown liquid culture at 4,221 x g at room temperature for 10 min and remove the supernatant without disrupting the bacteria pellet.
    CAUTION: Please follow institution's biosafety recommendations to discard supernatant.
    3. Gently re-suspend the bacterial pellet with 25 mL of appropriate liquid medium (Fn: TSB supplemented with 0.1% L-Cysteine hydrochloride monohydrate) containing 10% glycerol, using a 25 mL pipette.
    4. Using a 1 mL pipette, aliquot the resuspended bacterial culture into 500 µL aliquots in vials, freeze in a dry ice/ethanol bath, and store at -80 °C.
    5. To determine the titer of the frozen culture vials, remove two frozen vials stored at -80 °C, thaw on ice, and perform serial dilutions in Phosphate-buffered Saline (PBS) followed by plating on appropriate growth medium (Fn: TSA containing 0.1% L-Cysteine hydrochloride). Plates are incubated 24 - 48 h at 37 °C, and colony forming units (CFU) are enumerated to calculate the number of bacterial cells within the frozen culture vials (average of two vials).
    6. Prepare the bacterial inoculum by thawing a frozen vial on ice, and then dilute the culture with PBS to the final concentration of 10^7 CFU/100 µL. The inoculum should be at a concentration that will yield the desired CFU in 100 µL (the final concentration of glycerol within the inoculum cannot be more than 3%, to prevent asphyxiation of the rat). Frozen culture vials stored at -80 °C can be used for up to six months after preparation.

2. Rat Anesthesia

1. Connect the anesthesia chamber to the properly operating isoflurane vaporizer of an anesthesia machine.
2. Connect the gas scavenging tube on the anesthesia chamber to the gas scavenging system.
3. Open the oxygen flow on the anesthesia machine to 4 L/min.
4. Set the isoflurane vaporizer to 5%.
5. Place the rat in the anesthesia chamber.
NOTE: We typically utilize rats between 8 - 10 weeks old (130 - 180 g); younger rats are difficult to inoculate via this technique due to the small size of the mouth cavity.
6. When the induction of anesthesia has taken place, maintain the rat at 5% isoflurane, 2 L/min oxygen to effect. This takes about 3 - 10 min.
7. Determine the depth of anesthesia by the quality and rate of respiration and heartbeat, and reaction to reflex stimulation as in the toe pinch test. Ideal depth of anesthesia is indicated by 1 - 1.5 s count between each breath.

3. Intratracheal Inoculation

1. Remove the rat from the anesthesia chamber and place the rat dorsally on the rodent intubation stand. Attach the front teeth of rat to the holder to keep rat in place.
NOTE: If the rat starts to awaken during the procedure, the catheter can be removed and the rat returned to the anesthesia chamber to reach a deeper plane of anesthesia.
2. Use dominant hand to move the tongue to same side as support hand with broad point dressing thumb forceps.
3. Use support hand and secure the tongue with the laryngoscope against the rat's lower jaw and visualize the trachea and esophagus of the animal. The trachea will open and close as the rat breathes.

4. Insert the catheter containing a 20-gauge blunted-needle, prepared in step 1.1, into the trachea. There may be slight resistance, and insertion into trachea may be "bumpy" due to the catheter rubbing the tracheal cartilages. Due to moisture or shallow respiration, the trachea can be covered by the epiglottis which prevents visualization of the trachea. Gently touching the edge of the epiglottis cartilage will cause the cartilage flap to open and uncover the trachea.
5. Remove the blunted-needle from the catheter while ensuring the catheter remains in the trachea.
6. Allow a few seconds to pass for rat to be able to breathe around catheter inserted into trachea.
7. Firmly seat the trachea indicator onto the catheter opening. Movement of the aerosol barrier will indicate the catheter is inserted correctly into the trachea.
8. Ensure that the rat is laying on the intubation stand such that the chest of the animal is facing perpendicular to the plane of the intubation stand.
9. Remove the trachea indicator and deliver 100 µL (10^7 CFU) of the inoculum containing *F. tularensis* using a 200 µL pipette tip, firmly seating the tip against the catheter opening.
10. Attach a 1 mL slip tip tuberculin syringe filled with air and deliver 300 µL of air to ensure inoculum reaches the lungs of the rat.
11. Remove the catheter from the trachea and remove the rat from the surgical platform.
12. Allow the rat to awaken and return to the cage. Make sure that the breathing has returned to normal.
13. Determine the CFU in the *F. tularensis* inoculum as described in step 1.3.5 to confirm the CFU delivered in the trachea.
14. Repeat steps 3.1 - 3.9 on unvaccinated (naïve) animals with 100 µL PBS in place of the inoculum.

Representative Results

The humoral response to intratracheal inoculation of *F. tularensis* in the rat can be determined by enzyme-linked immunosorbent assay (ELISA) against UV-inactivated bacteria, as described previously[11]. Total Immunoglobulin G (IgG) response of Fischer 344 rats to inactivated whole cell bacteria was assessed post-intratracheal inoculation with an attenuated strain of Fn (10^7 CFU inoculum) at day 14 and day 28 (**Figure 1**). Mock-vaccinated rats received PBS intratracheally. An increase in serum antibody titers against Fn post-inoculation relative to naïve mock-vaccinated animals indicates the intratracheal vaccination efficacy. Low serum reactivity may indicate incorrect intratracheal placement.

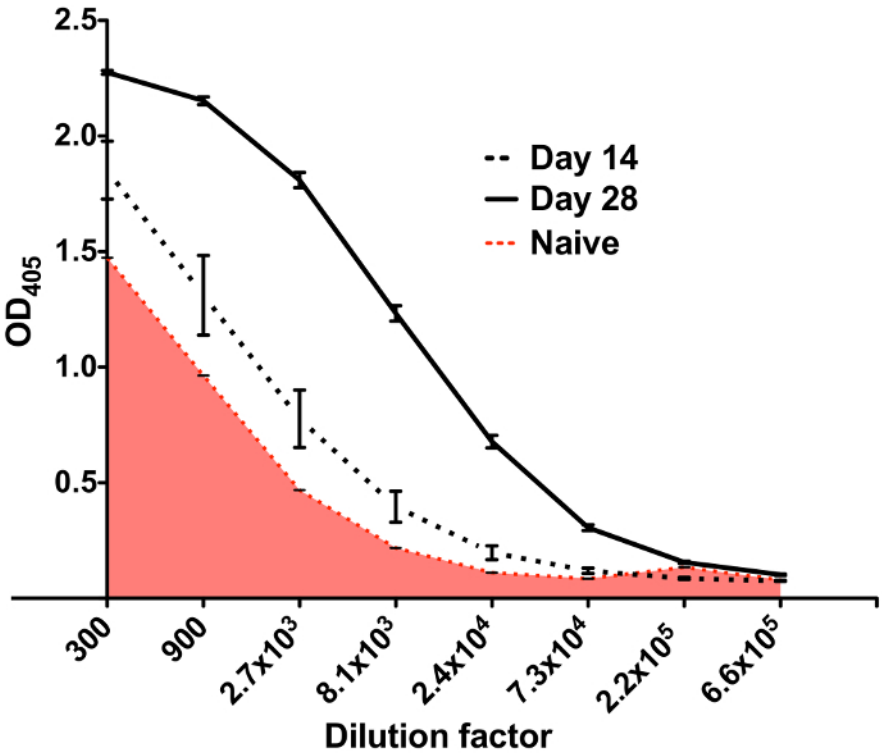

Figure 1: Total IgG Responses to Live Attenuated Fn Intratracheal Inoculation in F344 Rats. Sera from F344 rats (n = 5) inoculated intratracheally with a live attenuated Fn strain (10^7 CFU) were analyzed for total IgG levels reactive with whole cell Fn at day 14 and day 28 post-inoculation. Mock-vaccinated (naïve) rats (n = 5) were inoculated intratracheally with PBS. Red area denotes reactivity of naïve sera (F344 rats mock-vaccinated with PBS). The error bars represent the SEM. Please click here to view a larger version of this figure.

Discussion

The Fischer 344 rat is becoming an important model for tularemia vaccine development[3]. Exposure to *F. tularensis* through the pulmonary route is critical for demonstrating efficacy against weaponized forms of *F. tularensis*, because these are delivered as aerosols. Intratracheal inoculation of the rat facilitates exposure of the rat lungs to *F. tularensis* without the need for large, expensive, and complicated aerosol generating

equipment. All experiments utilizing select agent forms of *F. tularensis* additionally require BSL3 containment, which typically occurs in severely restricted space. Thus, this technique minimizes the amount of additional equipment that needs to be housed within that work environment.

Because Fn was the *F. tularensis* strain utilized for videography, all techniques were performed under BSL2 containment. Adaptation of this technique to the BSL3 environment includes all procedures performed within a biosafety cabinet by personnel wearing biosafety gear (full hood, powered air purifying respirator (PAPR), protective cover all with hood, double gloves, booties), and these adaptations reduce mobility, dexterity, and visibility. The trachea indicator is an important component that allows the confirmation that the catheter was correctly placed within the trachea, considering it is often difficult to otherwise make this determination when working under BSL3 conditions.

There are anatomically correct rat "simulators" that have a trachea and esophagus, and these are useful to perfect the technique prior to working with live animals. However, working with the simulator is not identical to working with a live rat. One means to determine if this technique is performed correctly in the live animal is to utilize Trypan blue dye as the inoculum on an anesthetized rat, and after the procedure immediately euthanize the animal. Dissection of the lung tissue and stomach will reveal if the dye was delivered into the lungs and not the esophagus. A rat that has been vaccinated with Fn/Ft by this technique can also be euthanized shortly after inoculation and the lung tissue plated to determine actual deposition within the lung.

Correct intratracheal inoculation will be important for evaluation of tularemia vaccine efficacy in the Fischer 344 rat, but it may also be useful for other vaccine and/or therapeutic applications in rats as well, including biodefense. Thus, this technique may be adaptable to a variety of pulmonary applications utilizing the rat model. While delivery by an aerosol generating device may be more similar to a biothreat scenario, the intratracheal inoculation of *F. tularensis* represents a relatively simple, cost-effective alternative for tularemia vaccine development.

Disclosures

The authors have nothing to disclose

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References

1. Ellis, J., Oyston, P. C. F., Green, M., Titball, R. Tularemia. *Clin Microbiol Rev.* **15**, 631-646 (2002).
2. Lyons, C. R., Wu, T. H. Animal models of Francisella tularensis infection. *Ann N Y Acad Sci.* **1105** (2007).
3. Hutt, J. A., Lovchik, J. A., Dekonenko, A., Hahn, A. C., Wu, T. H. The Natural History of Pneumonic Tularemia in Female Fischer 344 Rats after Inhalational Exposure to Aerosolized Francisella tularensis subspecies tularensis Strain Schu S4. *Am J Pathol.* **187**, 252-267 (2017).
4. Ray, H. J., *et al.* The Fischer 344 rat reflects human susceptibility to Francisella pulmonary challenge and provides a new platform for virulence and protection studies. *PloS one.* **5**, e9952 (2010).
5. Jemski, J. V. Respiratory tularemia: comparison of selected routes of vaccination in Fischer 344 rats. *Infect Immun.* **34**, 766-772 (1981).
6. Wu, T. H., *et al.* Vaccination of Fischer 344 rats against pulmonary infections by Francisella tularensis type A strains. *Vaccine.* **27**, 4684-4693 (2009).
7. Mara-Koosham, G., Hutt, J. A., Lyons, C. R., Wu, T. H. Antibodies contribute to effective vaccination against respiratory infection by type A Francisella tularensis strains. *Infect Immun.* **79**, 1770-1778 (2011).
8. Oyston, P. C., Sjostedt, A., Titball, R. W. Tularaemia: bioterrorism defence renews interest in Francisella tularensis. *Nat Rev Microbiol.* **2**, 967-978 (2004).
9. Kingry, L. C., Petersen, J. M. Comparative review of Francisella tularensis and Francisella novicida. *Front Cell Infect Microbiol.* **4**, 35 (2014).
10. Rohmer, L., *et al.* Comparison of Francisella tularensis genomes reveals evolutionary events associated with the emergence of human pathogenic strains. *Genome Biol.* **8**, R102 (2007).
11. Chu, P., *et al.* Live attenuated Francisella novicida vaccine protects against Francisella tularensis pulmonary challenge in rats and non-human primates. *PLoS Pathog.* **10**, e1004439 (2014).
12. Signarovitz, A. L., *et al.* Mucosal immunization with live attenuated Francisella novicida U112DiglB protects against pulmonary F. tularensis SCHU S$ in the Fischer 344 rat model. *PloS one.* **7**, e47639 (2012).
13. Cunningham, A. L., *et al.* Enhancement of vaccine efficacy by expression of a TLR5 ligand in the defined live attenuated Francisella tularensis subsp. novicida strain U112DiglB::fljB. *Vaccine.* **32**, 5234-5240 (2014).

