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Influenza-mediated Lung Infection Models

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Summary

Laboratory rodent influenza infection models have been and continue to be a critical tool for understanding virus-host interactions during infection. The incidence of seasonal influenza infections coupled with the need for novel therapeutics and universal vaccines highlight the need to uncover novel mechanisms of pathogenesis and protection. Mouse models are extremely useful for the evaluation of influenza vaccines and provide an invaluable tool to probe the immune response. This chapter describes the technique of intranasal inoculation of male C57BL/6J mice with an H1N1 strain of influenza (A/Puerto Rico/8/1934) and methods for assessing the optimum dose for infection, viral titers in lung tissue, and severity of disease.

Keywords

Intranasal inoculation; Influenza; H1N1; Body weight loss; Foci forming units; Lung damage

1. Introduction

Influenza infection is a significant cause of morbidity and mortality worldwide; the World Health Organization estimates that there are 3–5 million severe influenza cases every year, causing 250,000–500,000 deaths globally [1–3]. Influenza virus infections, both A and B, are seasonal and even though efficacious vaccines are available, factors such as viral genetic drift and mismatched vaccine strain selections can negatively impact the effectiveness of the vaccine and available anti-viral therapeutics. Additionally, individuals with known risk factors (elderly, obesity, hypertension, asthma, and heart disease) are at higher risk for severe disease including influenza viral pneumonia [4–6]. Therefore, laboratory studies focused on improving our understanding of the pathogenesis, immunobiology, and genetic drift of influenza viruses are critical for the development of next generation/universal vaccines [7].

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²⁵It is recommended to develop a pathology grading scale to determine the extent and intensity of influenza induced pathology to correlate with other endpoints. If not working with a board-certified pathologist, representative lung pathology grading schemes can be found in the literature. However, the most accurate grading of lung pathology will be done by a blinded, board-certified pathologist.

The murine influenza infection model has been fundamental to understanding the virus life cycle and replication as well as the host defense response. The utility of this laboratory model has led to better understanding of the viral replication kinetics, spread, and tropism as well as insight into the immune responses required to clear the virus. Additionally, rodent models of populations with known risk factors have allowed us to better understand the differing infection and immune response that may increase morbidity and mortality associated with an influenza infection [8]. However, many mechanisms of host-virus interactions remain unclear and further studies are needed to develop better vaccines and therapeutics.

Murine models of influenza infection have both advantages and disadvantages. Using mice provides a well characterized animal model with multiple options for differing genetics and phenotypes to understand vaccination response and characterize the immune response to influenza infection. Mouse breeding and reproduction is relatively quick and produces large litters thereby facilitating laboratory influenza studies of sufficient group sizes to achieve high statistical power. However, it should be acknowledged that there are differences in the mouse lung physiology and anatomy that may affect the extrapolation of data to human influenza infection. For instance, mice are obligate nose breathers whereas humans breathe utilizing the nose and the mouth [9]. Additionally, the branching of airways in the mouse is more simplistic than in the human lung, making the inhalation of the virus and its subsequent deposition different [10,11]. Influenza infection in mice requires a mouse adapted strain of influenza given that mice do not normally acquire and spread seasonal influenza strains [12]. Lastly, the morbidity of influenza lung infection in mice presents as weight loss and thus does not precisely model the respiratory syndrome seen in humans [12]. These differences between murine and human influenza infection should be considered when attempting to extrapolate data from laboratory infection studies.

Most mouse strains used for laboratory influenza infections are inbred thus creating numerous distinct models each of which is comprised of genetically identical individuals. However, it has been reported that the genetic background of the mouse can influence the susceptibility and immune response to influenza [13]. For instance, the A/J and DBA/J strains are more susceptible to influenza-induced pathology where as C57Bl/6 and BALB/c mice are considered more resistant to influenza induced pathology but exhibit significant weight loss post-infection and are therefore routinely used for lethal challenge research studies [13]. The variation in susceptibility amongst inbred strains of mice has been studied across multiple subtypes of influenza (H1N1, H3N2, avian and swine strains). It is thought that the variation in susceptibility is a result of genetic mutations in *Mx1*, *Stat1*, *Pkr*, *Ifnar1*, and/or *Ncr1* that have been shown to influence the anti-viral response [14–18]. Additionally, wild caught mice are resistant to influenza infection based partially on *Mx*- or *Mx*+ allele frequency [19–22]. Therefore, most strains of influenza used in mouse models require “mouse adaptation” to create a virus that will reproducibly infect and replicate in the murine respiratory tract and induce a weight loss phenotype.

Despite the differences in murine and human influenza infections, this model of viral infections is important and necessary to study and identify novel mechanisms of virus/host interactions and evaluate the protective efficacy of antiviral therapeutics and experimental

vaccine formulations. This chapter will describe methods for studying a mouse-adapted influenza virus infection in mice. We have selected the infection of C57BL/6J male mice, a commonly used commercially available inbred strain, with an H1N1 strain of influenza (A/Puerto Rico/8/1934; PR8), as an example. However, it should be noted that these methodologies can be used for the study of other influenza viruses capable of infecting various strains of mice.

2. Materials

2.1 Infection

1. Sterile, pharmaceutical grade, endotoxin-free, physiologic (0.9%) saline or 1X phosphate-buffered saline (PBS)
2. Isoflurane
3. Isoflurane vaporizer
4. Isoflurane induction chamber (see Note 1)
5. Sterile 200 μ L pipet tips
6. Single channel 200 μ L pipette
7. Sterile 1000 μ L pipet tips
8. Single channel 1000 μ L pipette

2.2 Monitoring weight loss after infection

1. Scale accurate in the range of 10 to 50 g with at least a 0.01 g resolution.
2. Washable/autoclavable and shatter resistant flat bottom container (e.g., plastic 1000 mL beaker).
3. Mode of identification of individual experimental animals. Examples are tail markings or tattoos, ear tags, ear punches and injectable transponders (see Note 2).

2.3 Quantification of lung viral titers using influenza virus focus forming assay (FFA)

1. Sterile capped tubes (assorted)
2. Tube racks

¹The size of the isoflurane induction chamber should be sufficient to contain the animal with a normal posture but small enough to reduce “dead space.” Additionally, ensure that the ports/tubing leading to the chamber are open to make sure the anesthetic vapor reaches the chamber. Isoflurane vaporizer and induction chamber should be used in combination with a carbon filter and/or Class II BSC or to minimize exposure of the user.

²Mice should be tracked individually since weight loss/infectivity can be variable. The most affordable method is either tail marking using a “permanent” marker (requires periodic remarking every 2–3 days) or ear punching. Ear punching does not require anesthesia in rodents when performed by a skilled individual. Ear punching can generally be performed at approximately 15–17 days of age, after the ear has “thinned.” The ear punch procedure should be performed using clean gloves and a sanitized ear-punch. Manually restrain the animal and place the punch device on the pinna of the ear (external ear) in a location where you want to mark the animal for identification. Press firmly to punch with a commercially available ear punch tool to create a circular hole or semicircular notch through the ear. Bleeding after ear punching is uncommon and the animal can be released directly into the cage. Injectable transponders are implanted subcutaneously.

3. Sterile pipet tips and serological pipets (assorted)
4. Pipetting devices
5. Tissue Homogenizer
6. Sterile 12-well flat-bottom tissue-culture-treated plate
7. Class II biological safety cabinet
8. 37°C cell culture incubator with 5% CO₂
9. 34–35°C cell culture incubator with 5% CO₂ (see Note 3)
10. 37°C water bath (see Note 4)
11. Centrifuge
12. Vortex
13. Timer
14. Inverted microscope
15. White light box
16. Hemocytometer or other cell counting device
17. Freezers, –20 and –80°C
18. Refrigerator, 4°C
19. Liquid nitrogen storage
20. Madin-Darby canine kidney (MDCK) cells (see Note 5)
21. MDCK cell culture maintenance medium: Minimum Essential Medium supplemented with Earl's salts and L-glutamine (MEM), 10% fetal bovine serum (FBS; non-heat-inactivated), 100 U/mL penicillin-100 µg/mL streptomycin, 1 mM sodium pyruvate, and 1X non-essential amino acids
22. 0.05% Trypsin-EDTA
23. Sterile PBS (1X) for cell culture
24. 1 mg/mL TPCK-trypsin (TPCK: L -(tosylamido-2-phenyl) ethyl chloromethyl ketone) in 1 mM HCl (pH 3), 20 mM CaCl₂
25. Virus diluent: Dulbecco's Modified Eagle Medium (DMEM), 100 U/mL penicillin-100 µg/mL streptomycin, 1% bovine serum albumin (BSA; Fraction V), and 25 mM HEPES; supplemented with 1.0 µg/mL TPCK-trypsin immediately prior to use (see Note 6)

³. Influenza viruses such as PR8 often grow optimally at a slightly cooler temperature (34–35°C) than the temperature recommended for MDCK cell growth (37°C).

⁴. Media, PBS and trypsin should be pre-warmed to 37°C before putting on MDCK cells to avoid cold shocking the cells.

⁵. There are several sub-types of MDCK cells. MDCK (NBL-2) (ATCC® CCL-34) cells are recommended for use in the FFA procedure described here.

⁶. If fungal contamination is anticipated/possible supplement with 0.25 µg/mL Amphotericin B.

26. FFA overlay: DMEM, 100 U/mL penicillin-100 µg/mL streptomycin, 0.1% BSA (Fraction V), 3.7 g/L sodium bicarbonate, 0.01% w/v DEAE-dextran, and 1.2% Avicel® RC-591 NF microcrystalline cellulose (MCC) w/v; supplemented with 1.0 µg/mL TPCK-trypsin immediately prior to use (see Note 6)
27. 10% neutral buffered formalin (NBF)
28. Wash buffer: PBS 1X with 0.1% Tween® 20
29. Permeabilization buffer: 1X PBS with 10 mM glycine and 0.5% Triton™ X-100
30. Avidin stock solution: 10 mg/mL in 1X PBS and 5 mM sodium azide
31. Biotin stock solution: 10 mg/mL in DMSO
32. Antibody diluent: 1X PBS, 1% BSA (Fraction V), 0.1% Tween® 20, and 1% (w/v) non-fat dry milk powder
33. Anti-influenza A virus NP antibody clones A1 and A3; biotin conjugated and pre-mixed 1:1
34. Streptavidin conjugated to horseradish peroxidase (HRP)
35. Immunohistochemical (IHC) staining reagent for peroxidase-labeled conjugate detection (see Note 7)

2.4 Identification of Lung Pathology after Influenza Infection

1. Surgical tools (e.g., scissors, scalpel)
2. 3 mL Syringe
3. Thin catheter or a 19 gauge blunt needle, able to fit in mouse trachea
4. Surgical sutures, dental floss, or small string
5. 10% NBF solution
6. Paraffin
7. Device for sectioning tissues to 5 µm thickness such as a cryostat.
8. Positively charged glass slides
9. Hematoxylin and eosin stains

3. Methods

3.1 Preparation of Virus Inoculum

1. Thaw a stock ampule of virus on ice. Virus stock should be thawed just before use for optimal infectivity. The use of repeatedly freeze/thawed virus aliquots is not recommended.

⁷·KPL TrueBlue™ Substrate (Sera Care, Milford, MA) is recommend however, other vendors may have comparable products.

2. Dilute virus sample with sterile saline or PBS (usually two or three dilutions may be necessary differing by 10-fold in the range of 1:100 to 1:50,000) (see Note 8) to make the dose of virus per mouse about a 50 μ L volume (See Note 9).
3. Virus and diluted virus should be kept on ice or at 4°C throughout preparation and before administration. Concentration of virus given to mice will depend on study and preparation of stock virus (see Note 10).

3.2 Intranasal Inoculation of Virus

1. Fill the isoflurane vaporizer with isoflurane to fill line (do not fill while vaporizer is on; do not over fill the vaporizer).
2. Turn on oxygen tank maintaining 0.5–1 liter/minute flow of oxygen.
3. Place the mouse in the induction chamber
4. Turn on isoflurane vaporizer at 4–5% (See Note 11).
5. Watch mouse until it is deeply anaesthetized (confirmed by the absence of reflexes on the footpad) (see Note 12).
6. Mix virus inoculum well by pipetting up and down
7. The mouse is held by its scruff and the inoculum is gradually released into the nostrils (see Note 13) with the help of a pipette. This method of dosing is shown in Figure 1. The rate of release should be gradual to allow the mouse to inhale the inoculum without forming bubbles. When performed correctly a rapid increase in the breathing rate should be observed.
8. After dosing, the mouse should be placed back in its cage on its back to ensure no bedding blocks nasal passages and to prevent residual virus inoculum from exiting the front of the nostrils. For prolonged anesthesia the cage should have a mild heat source to prevent hypothermia, care must be taken not to over-heat the animals.
9. Visually monitor the mouse until it is moving normally about the cage.

⁸·Pipetting small volumes can increase error. Making serial dilutions to achieve the desired dose/concentration of virus increases experiment-to-experiment reproducibility.

⁹·40–50 μ L is the recommended inoculum dose. Smaller volumes may become stuck in the nasal cavity and expelled or may not evenly distribute in the lung and thus increase variability.

¹⁰·There are multiple sources that may cause lab-to-lab variation in PR8 mouse infection models. The virus is available from multiple commercial sources and stocks can be generated via growth in different culture systems (chicken eggs, MDCK cells, etc). The level of expertise of the laboratory, the source/strain of the mice, age of the mice, propagation method for the virus, etc. may all contribute to variation. Therefore, it is recommended that an MLD (minimal dose that is lethal to a certain percentage (e.g., 50%) of animals) is performed prior to the initiation of experimental studies to identify the specific dosages to be used that fits the experimental goals. Figure 4 shows an example of a survival study performed to identify an MLD₅₀ dose of PR8 in C57BL/6J male mice.

¹¹·The use of a calibrated vaporizer provides the most consistent and safe delivery of inhalant anesthesia. If a standard vaporizer is not available, isoflurane may be suspended in glycerol/paraffin/mineral oil and administered on cotton or gauze in an induction chamber and/or nose cone. Use of isoflurane without a precision vaporizer has a very small margin of safety. Animals can easily become overdosed if not constantly monitored.

¹²·If animals are not anesthetized to a sufficient level and wake up too quickly they may expel the inoculum instead of breathing it in fully. This can create variation in infection rates.

¹³·The technician should try to evenly distribute the inoculum between the two nostrils.

3.3 Evaluation of Weight Loss after Infection

1. Monitor weight loss and recovery throughout the duration of the model to assess morbidity associated with infection. Due to the daily flux in mouse weights, define a 2-hour window each day when the mice will be weighed. To evaluate weight, zero or tare the scale with the container in place before use (see Note 14). Gently pick up the mouse by the base of the tail and transfer the animal to the container on the scale. Once a steady reading can be made, which is generally when the mouse has stopped moving around, record the weight. Ensure that a baseline weight measurement has been taken immediately prior to infection (see Note 15) for each mouse. This initial weight is necessary for the calculation of the daily percent weight loss.
2. Calculate the daily percent weight change for each animal over a 14 day study duration. Determine the difference between the pre- and post-infection weights and divide this difference by the pre-infection weight to obtain percent body weight change. Alternatively, divide post-infection body weight by baseline (pre-infection) body weight for each mouse to obtain the percent of original body weight. Example weight loss curves over time of mice given differing doses of PR8 are shown in Figure 2.

3.4 Quantification of Viral Titer in Lung Tissue by FFA

1. Excise the lung tissue into a pre-weighed, labeled tube.
2. Re-weigh the tube and subtract the empty tube weight to calculate the weight of the lung tissue.
3. Homogenize the lung tissue in 1–2 ml of virus diluent and store on ice or 4°C (see Notes 16–17).
4. The day before assay, trypsinize the MDCK cells, count and dilute cell number to desired seeding density (see Note 18) with MDCK cell culture maintenance medium. Add 1.0mL of diluted cells to each well of a 12-well assay plate. Incubate overnight (16–24 hours) at 37°C, 5% CO₂.
5. Prepare a set of six tubes for each lung sample in which to perform 10-fold serial dilutions by adding 450 µL of virus diluent per tube.
6. Perform 10-fold serial dilutions for each sample. Briefly, add 50 µL of homogenized lung to the first tube and pulse vortex. Repeat this process by

¹⁴The scale with weight container in place should be checked periodically to ensure a zero reading when empty, re-zero as necessary.

¹⁵Because of daily flux in weight the baseline weight can be calculated as the average of multiple measurements taken up to 2–3 days prior to infection.

¹⁶FFA does not need to be performed immediately after necropsy. Samples can be batched up, frozen and stored at –80°C for later analysis. Snap freezing tissues using liquid nitrogen is recommended.

¹⁷If lung tissue is not homogenized completely, pieces of connective tissue can clog pipet tips resulting in inaccurate pipetting and/or cause MDCK cytotoxicity thereby negatively impacting FFA performance and reproducibility. Therefore, clarifying lung homogenates by centrifugation (10,000 × g) at 4°C for 5 minutes is recommended.

¹⁸An MDCK cell monolayer confluence of 75–85% is recommended, to achieve this cell density seed cells at 1.66×10⁵ cells/mL – 2.08×10⁵ cells/mL between 16 and 24 hours prior to the assay respectively. In house evaluation of seeding density to achieve desired confluence may be necessary.

serially transferring 50 μL of 10^{-1} to 10^{-2} ; 10^{-2} to 10^{-3} ; etc. to create the remaining dilutions making sure to change pipet tips and vortex after each dilution.

7. Remove culture medium from the assay plate wells and wash the MDCK monolayers once with 1X PBS (do not allow cells to dry).
8. Remove the PBS wash and add 100 μL of lung homogenate serial dilutions to the plate. Each dilution should be plated in duplicate wells. One 12-well plate allows for the analysis of 6 serial 10-fold dilutions of a single sample plated in duplicate.
9. Incubate the assay plates at 34–35°C, 5% CO_2 for 1 hour. Rock assay plates every 10–15 minutes to ensure even distribution of inoculum and to prevent drying of the cell monolayer.
10. Add 1 mL of 1.2% Avicel MCC-based FFA overlay [23] per well. It is not necessary to remove inoculum prior to addition of overlay unless inoculum cytotoxicity is anticipated.
11. Incubate the assay plates at 34–35°C, 5% CO_2 for 2 days (see Note 19).
12. Fix plates by adding 1 mL of 10% NBF directly to the overlay of each well and incubating for 30–60 minutes at room temperature (see Note 20).
13. Remove FFA overlay/NBF and wash plates 3X with wash buffer (0.5 mL per well per wash) (see Note 21).
14. Remove wash buffer, add 0.5 mL permeabilization buffer per well and incubate 15–20 minutes at room temperature.
15. Remove permeabilization buffer and wash plates 3X with wash buffer (0.5 mL per well per wash).
16. Dilute avidin stock solution 1:10,000 in 1X PBS. Remove wash buffer from plate, add 200 μL diluted avidin blocking solution per well and incubate 15 minutes at room temperature with periodic rocking.
17. Remove avidin blocking solution and wash plates 3X with wash buffer (0.5 mL per well per wash).
18. Dilute biotin blocking stock solution 1:1,000 in 1X PBS. Remove wash buffer from plate, add 200 μL diluted biotin blocking solution per well and incubate 15 minutes at room temperature with periodic rocking.

¹⁹ Assay incubation times will vary from 2–3 days depending on influenza virus strain-specific growth kinetics in MDCK cells. Whenever a new virus is being evaluated some optimization of incubation time may be required.

²⁰ Plates may also be fixed with –20°C 80% acetone in 1X PBS. If using this methodology; remove overlay, wash monolayers twice with 1X PBS, add 2.0 mL pre-chilled acetone fixative per well, incubate for 10–12 minutes at room temperature, remove acetone, allow plates to briefly dry (1–5 minutes; excess drying can increase background), wash three times, and proceed to avidin solution incubation. Two step fixation/permeabilization using NBF and Triton-X generally results in improved IHC staining.

²¹ Plates may be stored up to one-week post-fixation at 4°C in 1X PBS with 1% BSA and 0.1% Tween 20 provided sufficient buffer is used to prevent plates from drying out during storage.

19. Remove biotin solution and wash plates 3X with wash buffer (0.5 mL per well per wash).
20. Dilute biotin labeled anti-influenza A virus NP clones A1 and A3 1:1,000 (or tested optimal concentration) in antibody diluent. Remove wash buffer from plate, add 200 µL diluted A1 and A3 solution per well and incubate 1 hour at room temperature with periodic rocking every 10–15 minutes (see Note 22).
21. Remove A1 and A3 solution and wash plates 3X with wash buffer (0.5 mL per well per wash).
22. Dilute streptavidin-HRP 1:1,000 (or tested optimal concentration) in antibody diluent. Remove wash buffer from plate, add 200 µL diluted streptavidin-HRP solution per well and incubate 30 minutes at room temperature with periodic rocking every 10–15 minutes.
23. Remove streptavidin-HRP solution and wash plates 5X with wash buffer (0.5 mL per well per wash).
24. Develop plate using IHC staining reagent for peroxidase-labeled conjugate detection in accordance with manufacturer protocols (see Note 23).
25. Quench developing reaction in accordance with staining reagent manufacturer protocol.
26. Dry plates in the dark overnight or according to staining reagent manufacturer's protocol and manually enumerate foci using a white light box for increased contrast. The titer for a given dilution series should be scored using the well with the highest number of distinct and countable foci. The foci forming units (FFU) titer for a given sample is calculated as follow:

$$\frac{\text{Sum of foci per well at same dilution}}{\text{Number of wells scored}} \times \text{The dilution factor} \div 0.1 \text{ mL} \\ = \text{FFU/mL}$$

For lung homogenates the calculated FFU/mL and measured tissue weight should be used to express titers as FFU/g tissue weight to normalize for differences in the size/weights of lung samples assayed.

3.5 Lung Histology

1. Euthanize mouse on selected day after infection based on experimental design/endpoints. Figure 3 show examples of lung histology on days 3 and 7 after infection, respectively (see Notes 24).

²²When titrating samples of mouse origin, such as lung homogenates, it is necessary to use biotin-tagged anti-influenza antibodies and to sequentially incubate plates with avidin and biotin blocking solutions to decrease assay background. However, this protocol can be adapted for the analysis of influenza samples of non-mouse origin. If titrating non-mouse samples, avidin and biotin blocking is not necessary and anti-influenza A virus NP clones A1 and A3 that are not biotin labeled should be used in conjunction with an anti-mouse HRP conjugated secondary antibody.

²³For KPL TrueBlue™ Substrate (SeraCare Life Sciences) is recommended use 200 µL per well and quench with tap water once staining becomes sufficiently intense but before background begins to develop.

2. Surgically expose and isolate the trachea taking care to keep it intact. Taking care not to puncture or cut the lungs, open the chest cavity to expose the lungs.
3. Gently inflate the lungs with 10% NBF. This technique can be done via pushing the fluid through a syringe and an attached thin catheter inserted in a small hole in the trachea. Tie off the trachea with surgical sutures to ensure the liquid does not leave lungs. Monitor inflation of lungs as to not overfill resulting in artifactual damage.
4. Surgically remove the whole lungs.
5. Immerse the infused tissue in 10% NBF for a minimum of 48 hours.
6. Fix, paraffin embed, section (Figure 3, tissues were sectioned at 5 μ m), and mount the sections on positively charged glass slides.
7. Stain with hematoxylin and eosin (H&E).
8. Evaluate lung histology.
9. Correlate the histologic evaluations with weight loss (see Note 24).

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²⁴. At day 3 post-infection there is minimal pathology with some inflammation/apoptosis of the epithelium of the larger airways and some immune cells present in tissue whereas at day 7 post-infection extensive perivascular and peribronchiolar infiltrates as well as alveolar congestion are observed.

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Figure 1.
Picture illustrating the intranasal inoculation of virus to an anesthetized mouse.

Figure 2.

Percentage of weight loss of male C57BL/6 mice after saline or PR8 infection (either 1275 or 1700 FFU). Mice were euthanized if they lost more than 20% of their original body weight.

Figure 3.
Lung histology of C57BL/6 mouse after saline or PR8 infection (day 3 or 7 post infection (p.i.)). H&E stain shown at 200x magnification.

Figure 4.

Percentage of survival of male C57BL/6 mice after saline or PR8 infection (either 1275, 1700, or 2000 FFU). Mice were euthanized if they lost more than 20% of their original body weight per institutional animal use guidelines.