



Development of a Mouse-Adapted MERS Coronavirus

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Abstract

First identified in 2012, Middle East respiratory syndrome coronavirus (MERS-CoV) is a novel virus that can cause acute respiratory distress syndrome (ARDS), multiorgan failure, and death, with a case fatality rate of ~35%. An animal model that supports MERS-CoV infection and causes severe lung disease is useful to study pathogenesis and evaluate therapies and vaccines. The murine dipeptidyl peptidase 4 (Dpp4) protein is not a functional receptor for MERS-CoV; thus, mice are resistant to MERS-CoV infection. We generated human DPP4 knock-in (hDPP4 KI) mice by replacing exons 10–12 at the mouse *Dpp4* locus with exons 10–12 from the human DPP4 gene. The resultant human DPP4 KI mice are permissive to MERS-CoV (HCoV-EMC/2012 strain) infection but develop no disease. To generate a mouse model with associated morbidity and mortality from respiratory disease, we serially passaged HCoV-EMC/2012 strain in the lungs of young hDPP4 KI mice. After 30 *in vivo* passages, an adapted virus clone was isolated and designated MERS_{MA}6.1.2. This virus clone produced significantly higher titers than the parental clone in the lungs of hDPP4 KI mice and caused diffuse lung injury and a fatal respiratory infection. In this chapter, we will describe in detail the procedures used to mouse adapt MERS-CoV by serial passage of the virus in lungs. We also describe the methods used to isolate virus clones and characterize virus infection.

Key words Mouse model, Mouse-adapted virus, Serial passage, Virus clone isolation

1 Introduction

Middle East respiratory syndrome (MERS) is a fatal respiratory illness that first appeared on the Saudi Arabian Peninsula in mid-2012. It is caused by a novel betacoronavirus, MERS coronavirus (MERS-CoV) [1]. Shortly after the identification of the virus, its receptor dipeptidyl peptidase 4 (DPP4) was discovered [2]. As of September 2019, the World Health Organization (WHO) has reported 2468 laboratory-confirmed cases of MERS in 27 countries, including 851 associated deaths (fatality rate: ~35%). MERS-CoV does not currently have pandemic potential [3–5]. However, MERS-CoV is still epidemic in the Middle East and remains a cause for significant concern due to the potential spread by global travel as demonstrated by the outbreak in South Korea in 2015 [6–8].

To date, there are only two published reports on autopsy findings from subjects who died from MERS [9, 10] and our understanding of MERS-CoV pathogenesis in humans is still limited. Animal models are useful for the study of viral diseases and play an important role in the investigation of pathogenesis and evaluation of antiviral therapies and vaccines. An ideal animal model should be permissive to the viral infection and develop disease and pathology with similarities to that observed in humans. MERS-CoV infection has been evaluated in two nonhuman primate (NHP) models, the rhesus macaque and common marmoset [11–15]. Both species are susceptible to MERS-CoV infection. However, MERS-CoV caused only a transient lower respiratory tract infection without mortality in rhesus macaques [11–13]. In common marmosets, the consequences of MERS-CoV infection are controversial. Falzarano et al. reported the common marmoset reproduced several features of MERS-CoV infection in humans including progressive severe pneumonia [15], while another group observed only mild to moderate nonlethal respiratory disease following MERS-CoV infection [14]. Thus, the common marmoset is potentially a model to study pathogenesis and evaluate antiviral therapies and vaccines. However, NHPs are expensive, their availability limited, and their use may raise ethical concerns. In contrast, small animal models provide advantages over NHPs, including reduced cost, availability in large numbers, ease of handling, and species-specific reagents, especially for the studies of highly pathogenic viruses like MERS-CoV in the Biosafety Level 3 (BSL-3) laboratory. Unfortunately, common small laboratory animals like mice [16, 17], ferrets [18], guinea pigs [19], and hamsters [20] are not susceptible to MERS-CoV infection because their homologous DPP4 cannot be bound and utilized by MERS-CoV as a host receptor for entry [21, 22]. Haagmans et al. detected MERS-CoV RNA in the respiratory tract of New Zealand white rabbits following inoculation but found no clinical signs of disease [23].

Several strategies have been used to overcome this receptor incompatibility and develop mouse models of MERS-CoV infection. In 2014, we developed the first mouse model of MERS-CoV infection [24]. We delivered a recombinant adenovirus 5 encoding human DPP4 (hDPP4) to the lungs of mice. Transient expression of hDPP4 by adenovirus transduction made the mice temporarily permissive for MERS-CoV infection, but animals developed only mild lung disease. Generation of transgenic mice expressing a virus receptor is a common strategy to make mice permissive to infection. Several groups in addition to ours developed mice with transgenic expression of hDPP4 using different promoters [25–28]. The disease severity following MERS-CoV infection in transgenic mice correlated with the cellular distribution and expression level of hDPP4. In transgenic mice expressing hDPP4 driven by the cytokeratin 18 promoter [26] or a ubiquitous promoter [25, 27, 28],

MERS-CoV replicates and causes respiratory disease and mortality. However, the lethality was found to be secondary to overwhelming central nervous system (CNS) disease or multiorgan damage. This was also observed in mice transgenic for human ACE2 driven by the cytokeratin 18 promoter and infected with SARS-CoV [29]. In transgenic mice expressing hDPP4 under the human surfactant protein C (SPC) promoter, which restricts expression to bronchiolar and alveolar epithelia, MERS-CoV infection caused only mild disease [26]. Thus, these transgenic mice do not reproduce a severe lung disease phenotype that resembles MERS.

Alternative strategies for the creation of mouse models of MERS-CoV infection are generation of DPP4 humanized mice and adaptation of the virus to the animals. Pascal et al. reported a model in which all of the mouse *Dpp4* exons had been humanized and also generated humanized monoclonal antibodies against the MERS-CoV S protein using a novel strategy [30]. MERS-CoV infection in this model caused pulmonary edema, vascular cuffing, and alveolar septal thickening with an associated ~20% weight loss, necessitating euthanasia [31]. Another MERS mouse model was engineered by changing two amino acids in the mouse *Dpp4* locus using CRISPR-Cas9 technology [32]. This model supported MERS-CoV replication without severe disease. Similarly, our human DPP4 knock-in mouse model supported MERS-CoV replication but did not lead to a severe lung disease phenotype [33]. Two mouse-adapted (MA) strains of MERS-CoV were subsequently developed independently by serial passage of the HCoV-EMC/2012 strain [1] in the lungs of the two humanized mouse models [32, 33]. The resultant MERS-15 and MERS_{MA}6.1.2 mouse-adapted MERS-CoV strains replicated to high titers in the lungs of the CRISPR-Cas9 genetically engineered mouse model and the hDPP4 knock-in mouse model, respectively. The respiratory disease that developed in both mouse models and the associated mortality shared similarities with severe cases of MERS [32, 33]. Mouse adaptation was also successfully used to generate several SARS-CoV strains capable of modeling severe SARS-CoV lung disease in mice [34–36]. Thus, the adaption of the virus to enhance virulence in the mouse is a very useful approach to generate mouse models for coronavirus-associated lung disease.

2 Materials

These materials can be altered to fit the requirements for other viruses of interest.

1. HCoV-EMC/2012 strain.
2. hDPP4 knock-in mouse (C57BL/6 strain with mouse *Dpp4* exons 10–12 replaced with the human codons).

3. Insulated foam box filled with ice.
4. 2.0 mL sterile plastic screw-top tubes with O-ring caps.
5. Sterile DMEM (serum free).
6. 1 mL insulin syringe fitted with a 28 gauge (g) \times 1/2 inch needle
7. Ketamine/xylazine (87.5 mg/kg ketamine/12.5 mg/kg xylazine).
8. Pipettes and filtered pipette tips: 10 μ L, 20 μ L, 200 μ L, 1 mL.
9. 1000 mL polypropylene beaker
10. A scale with sensitivity in the 10–50 g range for weighing mice.
11. A flat-bottomed container with sides that fits on the weighing platform of the scale and can prevent mice from escaping (e.g., a 400 mL disposable beaker).
12. 30 cm long metal straight forceps to transfer mice from the cage
13. Transparent vacuum desiccator.
14. Polystyrene foam surface (e.g., a polystyrene box top).
15. Disposable absorbent bench underpads.
16. Precision glide needles 25 g \times 5/8 inch.
17. Spray bottle filled with 70% ethanol.
18. Spray bottle filled with Virex Plus.
19. Surgical tools (e.g., scissors, straight forceps, curved forceps, single edge razor blades, curved serrated forceps).
20. 1 \times Dulbecco's phosphate buffered saline (DPBS)
21. 10 mL syringes.
22. Small polystyrene weighing dish.
23. Paper towels.
24. 50 mL disposable tissue grinder.
25. TRIzol reagent.
26. Vero81 cells.
27. Huh7 cells.
28. Sterile 6-well plates and 12-well plates.
29. 37 °C incubator containing 5% CO₂.
30. D10: 1 \times DMEM, 10% FBS, and 1% penicillin/streptomycin (PS).
31. D2: 1 \times DMEM, 2% FBS, and 1% PS.
32. Overlay media: 2 \times DMEM, 4% FBS, and 2% PS.
33. 2% Low melting point agarose in ddH₂O.
34. 10 mL stripettes.
35. Electronic pipette controller.
36. 1 ml graduated transfer pipettes.

37. Water bath.
38. 15 mL sterile conical tube.
39. Dry ice.

3 Methods

All procedures are performed under BSL-3 laboratory conditions and must follow the standard operating protocol of a BSL-3 facility and regulatory agencies.

All manipulations of infectious specimens, samples, and mice must be performed within a biosafety cabinet or within a contained device such as a centrifuge.

All tissue culture media and waste must be bleached and autoclaved prior to disposal.

All instruments must be disinfected with Virex plus or 10% bleach.

3.1 *Intranasal Infection*

1. Rapidly thaw aliquots of virus (*see Note 1*). Place the virus stock aliquots on ice immediately after thawed.
2. Dilute the stock virus in 2.0 mL sterile screw-top tubes with O-ring cap with ice-cold serum-free DMEM to desired inoculum and keep the diluted virus on ice during mouse infection (*see Note 2*).
3. Anesthetize mice by intraperitoneal injection of ketamine/xylazine (87.5 mg/kg ketamine/12.5 mg/kg xylazine) (*see Note 3*).
4. Hold the mouse in a vertical position with the nose pointed upward (*see Note 4*). Carefully pipette 50 μ L of diluted virus onto the nostrils drop by drop, carefully matching the rate at which the mouse inhales (*see Note 5*).
5. Place inoculated mouse in a 1000 mL polypropylene beaker. Repeat until all mice are infected. Transfer mice back to their cage and monitor until they regain consciousness (*see Note 6*).

3.2 *Evaluation of Virulence by Weight Loss and Survival*

1. Check mice and monitor weight every day throughout the duration of virus challenge (*see Note 7*). To evaluate weight, lift the mouse by the tail using the long forceps and place the mouse into the container on the scale.
2. Record weight when the scale reading is stable.
3. Transfer the mouse into a 1000 mL polypropylene beaker. Repeat until all mice are weighed. Transfer mice from beaker back to the cage.

4. Calculate the percentage of weight loss normalized to the starting weight. Euthanize mouse if the weight loss is $\geq 30\%$ of starting weight (*see Note 8*).

3.3 Harvest Virus from the Lungs and Other Organs

1. At indicated days post infection, mice should be anesthetized by intraperitoneal injection of ketamine/xylazine.
2. Once the right plane of anesthesia is attained immediately, place the mouse ventral side up on a polystyrene foam surface covered with absorbent bench underpad (*see Note 9*). Immobilize the mouse by pinning limbs to the foam with 25 g \times 5/8 inch needles.
3. Wet the ventral side of the mouse with 70% ethanol. Pinch the fur/skin near the urethral opening with forceps and pull slightly upward. Make a midline incision through the fur/skin from urethral opening up to the mandible with surgical scissors. Using forceps, peel the fur/skin transversely to both sides along the incision to separate fur/skin from underlying muscles.
4. Lift abdominal muscle and incise through midline up to the base of the thorax. Then make transverse incisions to open the abdominal cavity.
5. Insert surgical scissors under the sternum and cut the diaphragm following the costal arch. Remove the rib cage using scissors and forceps, exposing the lungs and heart.
6. Fill a 10 mL syringe with cold sterile DPBS using a 25 g \times 5/8 inch needle. Insert the needle into the apex of the left ventricle and make a small incision in the right atrium. Slowly perfuse ≥ 5 mL cold sterile DPBS into the left ventricle. Next, insert the needle into the apex of the right ventricle and perfuse ≥ 5 mL cold sterile DPBS (*see Note 10*).
7. Remove the lungs and heart from the thoracic cavity. Remove the liver, kidney, spleen, and small intestine. Place organs in a small polystyrene weighing dish. Remove remaining connective tissue.
8. To harvest the brain, turn the mouse over and wet the fur of the head with 70% ethanol. Grasp the ears with forceps and cut off the skin and fur to expose the skull. Remove remaining skin at the base of the neck to further expose the skull. Immobilize the mouse and make an incision along the sagittal suture of the skull using a single edge razor blade (*see Note 11*). Wedge one prong of the curved serrated forceps into the now opened sagittal suture. Slowly pry up the skull, grasp the piece of skull with forceps and peel outward to remove. Repeat on the other side. Use curved forceps to lift the brain from the skull. Place brain tissue in the small polystyrene weighing dish.

9. Carefully place each organ into a 50 mL disposable tissue grinder filled with 2 mL sterile cold DPBS for homogenization or into 2 mL of TRIzol for RNA extraction (*see Note 12*).
10. Grind the lung tissue and transfer the homogenates or RNA samples into 2.0 mL sterile screw-top tubes with O-ring cap (*see Note 13*). Store samples at -80°C . Thaw and spin down the cell debris in lung homogenates before use.

3.4 Adaptation of the Virus to Mouse by Serial Passage in the Lung

1. To mouse adapt the virus, infect two mice intranasally with 10^5 pfu/mouse HCoV-EMC/2012 strain (*see Note 14*).
2. Two days after infection, prepare lung homogenates from the two mice.
3. Combine 100 μL of lung homogenate from each mouse in a 2.0 mL sterile screw-top tube with O-ring cap on ice. Then inoculate two new mice intranasally with 50 μL /mouse of the mixed lung tissue homogenates.
4. Repeat this process (**steps 2–3**). The virulence of the virus should be evaluated in groups of mice by weight loss and survival after every 5–10 *in vivo* passages.

3.5 Isolation of Viral Clones

After the virulence of the virus has been significantly enhanced, single plaques of the adapted virus should be purified and evaluated.

1. Plate Vero81 cells in D10 media in 6-well plates one day before infection.
2. Rapidly thaw lung homogenates from the selected passage. Mix the lung homogenates from two mice in a 2.0 mL sterile screw-top tube with O-ring cap on ice.
3. Serially dilute the mixed lung homogenates tenfold in 2.0 mL sterile screw-top tubes with O-ring caps, using ice-cold serum-free DMEM (*see Note 15*). Keep the dilutions on ice.
4. Remove the medium from each well and add diluted samples (in a volume of 400 μL) to each well.
5. Place the plates in the 37°C incubator for 1 h and rotate gently every 15 min.
6. Melt 2% low melting point agarose and maintain in a 65°C water bath.
7. Mix overlay media and 2% low melting point agarose at a volume ratio of 1:1. Rotate the tube several times to fully mix. Overlay cells with 1.5 mL of mixed media using 10 mL stripettes.
8. Let the plates sit in the hood for ~ 5 min at RT or until the agarose overlay turns solid. Add 0.5 mL D2 medium on the top of solidified agarose.

9. Place the plates in the 37 °C incubator.
10. After 3 days, plaques should be visible. Remove the liquid on the top of the agarose. Circle the visible plaques on the underside of the plates using a permanent marker (*see Note 16*).
11. Vertically penetrate the agarose and pipette the circled plaque several times with a 1 mL graduated transfer pipette. The agarose above the plaque will be pulled into the pipette.
12. Transfer the agarose above the plaque into a 15 mL conical tube filled with 500 µL DMEM by pipetting up and down several times.
13. Transfer the 500 µL DMEM containing the agarose into a 2.0 mL sterile screw-top tube with O-ring cap.
14. Repeat the procedure and pick six single plaques. Store the tubes at –80 °C.
15. Thaw the tubes containing the isolated plaques on ice.
16. Propagate viruses from the isolated plaques in Huh7 cells (*see Note 17*). To do this, plate Huh7 cells in D10 media in a 6-well plate one day before infection. Remove medium and add 500 µL of DMEM containing the agarose into the well. Place in the 37 °C incubator for 1 h. Remove the 500 µL DMEM and add 2 mL D2 media.
17. Place the plates in the 37 °C incubator.
18. At 1 day post infection, freeze cells by putting the plate on dry ice and then thawing the cell. Harvest cell lysate and store at –80 °C.
19. Titrate the serially passaged viruses by plaque assay on Vero81 cells. Briefly, Vero81 cells are plated in a 12-well plate. On the next day, thaw the virus and serially dilute the virus by tenfold for at least three dilutions with serum-free medium. Remove medium from the wells (Vero81 plate) and add 250 µL of diluted virus into each well of the plate. Incubate the plate at 37 °C incubator for 1 h and with gentle rotations of the plate every 15 min to prevent drying of cells. In the meantime, melt 2% low melting point agarose and maintain it in a 65 °C water bath. After 1 h of incubation, remove the 250 µL inoculum and overlay cells with 1 mL of 1:1 mix of 2× DMEM and 2% low melting point agarose. After the agar overlay turns solid, add two drops of 2% FBS DMEM medium on the top of the solid agar by 10 mL stripettes. The plate should be incubated at 37 °C and 5% CO₂ for about 3 days. After 3 days, fix the wells by adding 25% formaldehyde to completely fill the wells for 20 min. Then aspirate the liquid from each well and remove the agarose. Stain the wells with 0.1% crystal violet to delineate plaques. Remove the crystal violet, rinse wells with DPBS, and count plaques in the hood.

20. Evaluate the virulence of the plaque-isolated virus by monitoring weight loss and survival in mice after intranasal infection with 10^5 pfu/mouse as mentioned above.
21. Characterize virus distribution by titrating virus load in various organ homogenates and virus genomic RNA abundance in tissues using qRT-PCR.
22. Characterize lung histology after infection.
23. Use next-generation sequencing to identify changes in the MERS-CoV genome sequence following serial passage [33].

4 Notes

1. To rapidly thaw the frozen virus, immerse the vial in a plastic beaker filled with water at room temperature within the bio-safety cabinet. The rate of thawing will depend on the volume (approximately 2 min or until ice crystals melt).
2. Always keep freshly thawed virus aliquots or dilutions on ice. This makes the virus more stable and leads to more reproducible results.
3. Check that mice are fully anesthetized by observing the pedal reflex. If mice are not fully anesthetized, they may move excessively or sneeze when virus is applied.
4. While holding mice, make sure no pressure is applied over the throat area to avoid interference with respiration.
5. Do not place the pipette tip inside the nostril. If a mouse does not inhale the droplet, stop pipetting, and wait until the mouse inhales what is already applied.
6. Monitor the health of mice before returning to the cage.
7. Record weights from the day of infection until recovery.
8. Animal euthanasia should follow the institution's Animal Care and Use guidelines. There may be differences in institutional requirements regarding when euthanasia is required based on weight loss.
9. Use the lid of an insulated foam shipping box. Cut absorbent bench underpad (42×58 cm) into small pieces that fit the lid.
10. Carefully keep the tip of the needle in the lumen of the ventricle. Be sure to puncture the right atrium as this will help to drain blood during the perfusion. The lung will turn white after perfusion.
11. Make sure that the incision does not exceed the thickness of the skull; avoid cutting into the brain tissue.
12. The individual organs can be divided into pieces and transferred to grinders with PBS or TRIzol separately.

13. Lung homogenates are immediately transferred to tubes on ice. RNA samples are transferred to a tube and incubated for at least 15 min at room temperature per our BSL-3 specific standard operating procedures.
14. At 2 days post infection with 10^5 pfu/mouse, HCoV-EMC/2012 strain replicates in the lung of the hDPP4 KI mice to a titer of around 4×10^6 pfu/mL, which equals 2×10^5 pfu in 50 μ L. We chose the 10^5 pfu/mouse inoculum to begin because we wanted to use a similar dose range during in vivo serial passage while skipping the titration step.
15. Titrate the homogenates of the passage of interest and select a virus dilution that produces ≤ 10 plaques in a well. This allows identification of individual clear single plaques.
16. Only circle the unambiguous clear single plaques.
17. Compared to Vero 81 cells, we found that the MERS-CoV RNA genome is more stable when propagated in Huh7 cells (less likely to introduce genomic deletions, insertions, or point mutations).

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