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Risk & Benefit Analysis of Gain of Function Research

Supplemental Material – Animal Models for Coronaviruses and Influenza Viruses

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1 Introduction

Animal models are essential for the study of viral pathogenesis and for the development of medical countermeasures. A variety of animal models have been developed for coronaviruses and influenza viruses. Individual animal models are suited for use with particular virus strains and for conducting particular types of studies (e.g. transmission versus pathogenesis studies, for influenza model systems), thus the features of an animal model constrain the types of experimental approaches for which it can be used. Additionally, how well the animal model mimics human infection determines the degree to which the results translate to human populations, which influences both the risks and potential benefits of the research. This supplemental report provides an overview of the available animal models for coronaviruses and influenza viruses, which provides a foundation for the evaluation of risks and benefits of research conducted using animal models.

2 Animal Models of Severe Acute Respiratory Syndrome Coronavirus (SARS-CoV) and Middle East Respiratory Syndrome Coronavirus (MERS-CoV)

2.1 Background

2.1.1 Use of Animal Models in Research

Animal models are essential to understanding the pathology of viral disease and developing vaccines and medical countermeasures *in vivo*. Live animal models are necessary to compliment cell culture studies because cell culture systems cannot recapitulate the complexity of the human innate and adaptive immune responses to infection.[1] As a result, cell culture systems provide limited insight into the symptomology and host immune response during infection, relative to the use of animal models. In addition, the lack of an interactive immune system in cell culture models constrains their utility for understanding how potential medical countermeasures (MCMs) may affect and be affected by the immune response and how that relates to SARS-CoV or MERS-CoV infection in a host.[2] Animals also allow researchers to examine pathogenesis and MCM efficacy in depth by designing investigations that cannot be done in humans due to ethical concerns. Thus, animal models critically complement cell culture systems and human studies to provide in-depth insight into disease pathogenesis as well as MCM safety and efficacy.

Appropriate animal models are critical for the development of new vaccines and therapeutics. To demonstrate the efficacy of a vaccine or therapeutic, the model must show the ability of the countermeasure to prevent pathology associated with infection following a challenge.[3] In addition, under the FDA's Animal Efficacy Rule, vaccines and therapeutics against rare, emerging, or virulent agents such as SARS-CoV can achieve regulatory approval provided efficacy is demonstrated in multiple animal models that display clinical illness representative of human disease.[4,5] (Whether the Animal Rule applies

1 (2015b) Interviews with coronavirus researchers.
2 (2015b) Interviews with coronavirus researchers.
3 (2015b) Interviews with coronavirus researchers.
4 Sutton TC, Subbarao K (2015) Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology* 479-480C**:** 247-258
5 FDA. Product Development Under the Animal Rule: Guidance for Industry. http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm399217.pdf. Last Update October 2015. Accessed November 23, 2015.

to the development of MCMs targeting MERS-CoVs is uncertain, as the number and distribution of MERS cases in the Kingdom of Saudi Arabia may enable the conduct of clinical trials, which is preferable. This issue will be addressed on a case-by-case basis if sponsors seek approval of a MERS-CoV vaccine or therapeutic under the Animal Rule.)[6] The conditions that must be met in order for the FDA to deem animal study data effective include a well-understood pathophysiology of the disease and its prevention or substantial reduction by the proposed drug, demonstration in more than one animal species, including a non-rodent, with an immune response similar to humans that accurately predicts human response, and an animal study endpoint indicative of the desired benefit in humans. While there is no requirement for the use of a specific species, the sponsor must provide scientific justification that the animal used to study countermeasures exhibits key characteristics of the human disease when exposed to the challenge agent.[7] In sum, development of a pathogenesis model that adequately mirrors the route of infection, severity, clinical signs, and levels of mortality and morbidity seen in humans is critical for advancing countermeasure development and for satisfying the FDA Animal Rule.

2.1.2 Replication and Pathogenesis Models

Replication models are those that support viral replication but do not mimic human disease while pathogenesis models are those that support viral replication and emulate the clinical course and pathological features of human disease. Both replication models and pathogenesis models play important roles in the study of MERS-CoV and SARS-CoV. In replication models, infectious virus may be recovered from various organs and there may be evidence of tissue pathology, but clinical symptoms are either absent or vastly different than those seen in humans. If viral replication can be confirmed, replication models can be used in transmission studies and to demonstrate that countermeasures can inhibit viral replication. However, replication models have limited utility for the investigation of symptomology or immune responses to viral infection.

For those reasons, pathogenesis models are a necessary compliment to replication models. To be useful for the study of mechanisms underlying pathogenesis, the animal model must reflect key aspects of the disease.[8] The ideal pathogenesis model is one that mimics human disease by mirroring the route of infection, severity, clinical signs, disparate effect on corresponding demographic groups, and comparable levels of mortality and morbidity. The presence and distribution of viral receptors as well as virus titers should be similar to that in humans. Two strategies have been used to develop pathogenesis models using laboratory animal hosts that do not naturally support infection and/or do not recapitulate human pathology upon infection: (1) adapting a virus to the animal, and (2) sensitizing the host to infection through expression of human virus entry receptors. In either case, immunological reagents such as antibodies, antigens, proteins, and plasmids that can be used in investigational assays must be available and economical small animal models are preferred. Mice are highly desirable for this purpose because of the wide body of knowledge concerning mouse biology, particularly genetics and immunology.

Human viruses are studied using replication and pathogenesis animal models either because the virus does not exhibit species tropism or because tropism limitations are overcome via genetic manipulation of the host or virus.[9] Naturally susceptible laboratory species may serve as pathogenesis models if they display the proper symptoms, or as replication models if they do not. If suitable laboratory animals are not naturally susceptible to infection, animal models can be developed by adapting a wild type virus to the

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6 (2015m) Personal communication from FDA representative.

7 http://www.fda.gov/downloads/drugs/guidancecomplianceregulatoryinformation/guidances/ucm399217.pdf

8 (2015c) Interview with vaccinology expert.

9 Virgin S (2007) Pathogenesis of Viral Infection. In *Fields Virology*, David M. Knipe PMH (ed), Vol. 1, 5 edn, 11, pp 342-343. Philadelphia: Lippincott Villiams & Wilkins.

host through passaging or by adapting the host to the virus by expressing human viral receptors or other host restriction factors.

Critically, creating a pathogenesis model often requires more than simply introducing the human virus entry receptor to a host to permit virus replication. Virus titers in mice that are symptomatic or asymptomatic may be similar, demonstrating that viral replication is not the only determinant of pathogenesis. Pathology also arises from deleterious host immune responses to the infection. Thus, model systems in which the virus induces a response from the host immune system are essential for the study of pathogenesis, which may require adapting a virus to the host.[10] Pathogenesis should be fully characterized in the model because disease mechanisms of an adapted virus or in a transgenic animal may differ from those in the natural host. When using small mammals to study respiratory disease it is especially important to consider that some animals are physically incapable of replicating human macropathology regardless of infection.[11] For example, mice do not sneeze or cough, and so cannot perfectly replicate human disease.[12] Models may require higher doses or specific routes of infection to display symptoms. This should be taken into consideration while creating models because the more remote the dose and infection route are from human cases, the less relevant the results may be for human infection.[13]

Researchers have pursued multiple approaches for developing model systems to study Severe Acute Respiratory Syndrome coronavirus (SARS-CoV) and Middle East Respiratory Syndrome coronavirus (MERS-CoV), including the use of naturally susceptible hosts (alt-GoF), transgenic animals (alt-GoF), and animal-adapted viruses (GoF). Model coronaviruses (e.g. mouse hepatitis coronavirus (MHV)) and human autopsy data represent additional alternatives to the use of animal-adapted viruses. This report reviews the state of model systems for SARS-CoV and MERS-CoV in each category and analyzes their relative benefits and limitations.

2.2 CoV Animal Models Created Using Gain of Function Approaches

2.2.1 Adaptation of Viruses to Hosts

2.2.1.1 Use of gain of function approaches to develop pathogenesis models for SARS-CoV and MERS-CoV

Serial passaging of virus in mice, a gain of function approach because it alters host tropism and enhances the virulence of the virus in mice, is a commonly used method for developing pathogenesis models. Viruses that do not naturally replicate in a host can be serially passaged to select for new variants that replicate more efficiently and more closely recapitulate human pathogenesis. Serial passaging has been used to adapt SARS-CoV to mice, and work to develop a mouse-adapted MERS-CoV strain is in progress.

Roberts and colleagues serially passaged wild-type SARS-CoV in the respiratory tracts of young BALB/c mice, resulting in a lethal virus that causes physical disease and mortality. The new strain, called MA15, is associated with high viral titers in the respiratory tract as well as histopathologic findings indicative of severe pulmonary disease. Several nucleotide substitutions were identified in the MA15 genome that confer increased infectivity and lethality to mice. Mice aged 6 to 8 weeks, 4 months, and 13 months were all susceptible with severe morbidity or death occurring within days, thus providing a model to study the

[10] (2015b) Interviews with coronavirus researchers.
[11] McCray PB, Jr. *et al* (2007) Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. *Journal of virology* 81**:** 813-821.
[12] (2015b) Interviews with coronavirus researchers.
[13] Ibid

age-dependent effects of SARS-CoV. Infection of mice with the MA15 virus provides a pathogenesis model that is small, accessible, and easily produced in large quantities. Although mice infected with MA15 recapitulate many elements of SARS-CoV disease observed in human cases, this model does not exhibit some features of human SARS-CoV infections, such as diffuse alveolar damage.[14]
Day and colleagues used a similar approach to adapt SARS-CoV to young mice. The SARS-CoV-Urbani strain was serially passaged in mice 25 times to create an adapted strain that causes a lethal pulmonary syndrome in 5 to 6 week old mice, v2163. The v2163 strain acquired nine mutations during passaging and largely mimics human disease, but lung pathology lacked hyaline membrane formation (a membrane composed of mostly proteins and dead cells that lines the alveoli and deceases oxygen transfer) seen in humans. In contrast to MA15, which causes severe disease in mice aged 2 to 13 months, the lethality of v2163 in younger mice is advantageous for rapid evaluation of potential medical countermeasures.[15]
Developing animal models for MERS-CoV has been complicated by the lack of permissive species. The host receptor DPP4 plays an integral role in viral entry and host restriction, and mice exhibit naturally low levels of DPP4 expression. As a result, researchers have been unable to productively infect mice without altering the DPP4 receptor (discussed in the "transgenic animal" section below).[16] Work to develop a mouse-adapted strain of MERS-CoV using transgenic mice expressing permissive receptors is ongoing.

2.2.1.2 Benefits and Limitations of Using Animal-Adapted Strains

The mouse-adapted strains of SARS-CoV better mimic human disease pathogenesis than other animal model systems (discussed below), and mouse-adapted strains of MERS-CoV are expected to serve as the best pathogenesis model for the study of MERS-CoV as well. For this reason, coronavirus researchers prefer the use of mouse-adapted strains for most studies involving SARS-CoV and MERS-CoV, including studies to elucidate basic mechanisms of disease pathogenesis as well as experiments testing the safety and efficacy of candidate MCMs.[17,18] Mice serve as an attractive model system because of the large number of reagents available and the ability to produce mouse lines with controlled genotypes and phenotypes, which allow researchers to tease out host factors that are involved in diverse responses to infection.[19] Mouse-adapted strains give researchers the flexibility to utilize different strains of mice with targeted deficiencies (i.e. under-expression of a certain immune system component) to probe the host immune response or outbred mice to investigate how differences in host genetic backgrounds impact infection. In particular, knockout mice can be used to demonstrate that certain factors are necessary for viral clearance or for generating immunity following vaccination. In addition, adapted strains can be used to test whether MCMs can prevent or reduce the pathology associated with human disease, which provides a robust system for screening the efficacy of MCM candidates and thus is important for advancing countermeasure development. The adapted strains of SARS-CoV have been used in vaccine development, representing a significant advance towards satisfying the FDA Animal Rule.[20]

An additional benefit associated with the use of mouse-adapted viruses is that these strains are less pathogenic to humans, thereby providing greater safety in the laboratory. Passaging a virus in a new species most often leads to altered or switched host tropism, not enhanced tropism in the naturally

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14 Roberts A *et al* (2007) A mouse-adapted SARS-coronavirus causes disease and mortality in BALB/c mice. *PLoS pathogens* 3**:** e5.

15 Day CW *et al* (2009) A new mouse-adapted strain of SARS-CoV as a lethal model for evaluating antiviral agents in vitro and in vivo. *Virology* 395**:** 210-222.

16 (2015b) Interviews with coronavirus researchers.

17 Ibid

18 Ibid

19 Frieman M *et al* (2012) Molecular determinants of severe acute respiratory syndrome coronavirus pathogenesis and virulence in young and aged mouse models of human disease. *Journal of virology* 86**:** 884-897.

20 Sutton TC, Subbarao K (2015) Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology* 479-480C**:** 247-258.

susceptible hosts.[21] Mutations that increase virulence in one host often reduce virulence in other hosts due to changes in receptor binding affinity. The six mutations that arose during the creation of MA15 enhance binding to the mouse ACE2 receptor and lead to loss of the virus's ability to bind to human ACE2 receptors; as a result, mouse-adapted strains are deficient in replication relative to wild-type SARS-CoV in primary human airway epithelia (HAE) cells.[22] Other mutations may also contributed to host switching and decrease binding or replication in human cells. For example, a mutation in a glycoprotein increased virus yields in mice but decreased yields in human cells.[23]

The main limitation associated with the use of adapted changes is that adaptive changes that arise during passaging may affect pathogenesis mechanisms, potentially complicating comparisons between mice and humans.[24] For that reason, understanding how adaptation mechanisms alter the phenotypes under study is critical for the correct interpretation of results.[25]

2.2.1.3 Summary of adaptation models

Multiple SARS-CoV strains have been adapted to mice through serial passaging to create mouse models that replicate many key features of human disease, and efforts to develop mouse-adapted MERS-CoV strains are ongoing. Due to the fact that mouse-adapted strains recapitulate key features of human disease pathogenesis, these strains are important tools for the study of viral pathogenesis and for testing the safety and efficacy of MCM candidates. However, adaptation may alter the biology of the virus, limiting the relevance of results to human disease.

2.3 CoV Animal Models Created Using Alternative Approaches

2.3.1 Use of Naturally Susceptible Hosts

An alternative to the use of viruses that have been adapted to laboratory animals is the use of naturally susceptible hosts, including wild type hosts and hosts with targeted genetic deficiencies. However, laboratory animals that are naturally susceptible to infection with SARS-CoV and MERS-CoV have been found to support viral replication but remain asymptomatic or develop symptoms dissimilar to those in humans. That is, naturally susceptible hosts function primarily as replication models, not pathogenesis models.[26] Replication models can be used in vaccine and therapeutic development to demonstrate diminished replication, an important proof of concept for medical countermeasures.[27] Additionally, identifying a natural replication model is often the first step in creating a pathogenesis model. However, replication models provide limited insight into how viruses interact with host systems to produce deleterious health effects.

2.3.1.1 Use of animals that are naturally susceptible to SARS-CoV infection

For SARS-CoV, most laboratory animals, including mice, hamsters, ferrets, and non-human primates, can be productively infected but few display overt clinical disease. SARS-CoV uses the human angiotensin-converting enzyme-2 (ACE2) as the host receptor, which is present in an assortment of species. SARS-CoV has been shown to infect rhesus macaques, cynomolgus macaques, common marmosets, and African

21 (2015b) Interviews with coronavirus researchers.
22 Ibid
23 Ibid
24 Frieman M *et al* (2012) Molecular determinants of severe acute respiratory syndrome coronavirus pathogenesis and virulence in young and aged mouse models of human disease. *Journal of virology* 86**:** 884-897.
25 (2015b) Interviews with coronavirus researchers.
26 (2015b) Interviews with coronavirus researchers.
27 Ibid

green monkeys. Clinical symptoms, viral replication, and pathology vary between species, but all experience pneumonitis similar to that seen in the majority of human infections. Common marmosets experience multi-organ involvement with fever, diarrhea and hepatitis that closely mimic the hepatic and gastrointestinal pathology seen in some human cases. However, no non-human primate (NHP) species consistently reproduces severe clinical disease and mortality was not observed in any species.[28,29] Several strains of inbred mice support viral replication, including BALB/c mice. Young mice fail to display clinical signs of disease or develop pulmonary pathology and infection is cleared rapidly. Aged mice develop severe disease, mimicking the age-dependent effects of SARS-CoV on humans. This model is useful for the study of age-dependent susceptibility, but is not an ideal pathogenesis model for studying medical countermeasures.[30] Age-associated changes in the immune system and other organ systems may affect responses to vaccines or anti-virals, meaning that aged mice do not represent the broader population of young or adult mice.

Golden Syrian hamsters are highly permissive to SARS-Co and support viral replication to high titers in the lungs with clearance after seven to ten days. Replication is accompanied by histopathological changes in the lungs and infection elicits a neutralizing antibody response. However, hamsters do not display any outward clinical signs or mortality. Exercise wheels were used to show a slight drop in activity, but no additional symptoms have been observed. Ferrets also support SARS-CoV infection with varying degrees of clinical illness. A number of studies have demonstrated that infected ferrets exhibit fever and sneezing and have high virus titers in the upper respiratory tract, but SARS-CoV-induced mortality is absent.[31] Taken together, naturally susceptible hosts of SARS-CoV represent replication models.

2.3.1.2 Use of animals that are naturally susceptible to MERS-CoV infection

Most laboratory animals are not permissive to MERS-CoV infection. MERS-CoV attaches to target cells through binding of its Spike membrane glycoprotein (S protein) to the host glycoprotein dipeptidyl peptidase 4 (DPP4). Species lacking DPP4 are naturally non-permissive, and some hosts with high levels of DPP4 expression are refractory to infection due to differences between the amino acid sequences of the animal and human DPP4 proteins. Specifically, significant amino acid variation within the binding site for the S protein prevents virus attachment and renders cells non-permissive.[32] Host restriction prevents common laboratory animals from serving as disease models. Mice are naturally non-permissive due to low levels DPP4 expression. Hamsters, guinea pigs, and ferrets express high levels of DPP4 and have been evaluated as models, but are also non-permissive. Rabbits support some level of viral replication, but remain asymptomatic, thus serving as a replication model.[33]

Rhesus macaques and common marmosets have been evaluated as models of MERS-CoV infection. Both species support replication of MERS- CoV infection; however, the extent and severity of disease vary. In rhesus macaques, observed clinical signs were mild to moderate. Studies by De Wit and Yao determined that rhesus macaques develop transient lower respiratory tract infections, produce neutralizing antibodies, and show high levels of viral replication following a combination of intratracheal, ocular, oral, and

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28 Gretebeck LM, Subbarao K (2015) Animal models for SARS and MERS coronaviruses. *Current opinion in virology* 13**:** 123-129.

29 Sutton TC, Subbarao K (2015) Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology* 479-480C**:** 247-258.

30 Ibid

31 Sutton TC, Subbarao K (2015) Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology* 479-480C**:** 247-258.

32 van Doremalen N *et al* (2014) Host species restriction of Middle East respiratory syndrome coronavirus through its receptor, dipeptidyl peptidase 4. *Journal of virology* 88**:** 9220-9232.

33 Sutton TC, Subbarao K (2015) Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. Virology 479-480C: 247-258..

intranasal inoculation.[34,35] An increase in body temperature, reduced appetite, increased respiratory rate, and cough were also reported, yet none of the animals required euthanasia. Viral RNA was detected in the respiratory tract but not in non-respiratory tissue samples.[36] These results show that infected macaques replicate the pulmonary symptoms but not the renal failure observed in humans.

In contrast to rhesus macaques, common marmosets developed severe signs of respiratory disease. Falzarano and colleagues tested the suitability of marmosets as a model by inoculating animals via combined intratracheal, intranasal, oral and ocular routes. Marmosets developed severe pneumonia and extensive lung lesions with several animals requiring euthanasia.[37] In additional studies common marmosets have shown multi-organ involvement with fever, diarrhea and hepatitis. Viral RNA has been detected in blood samples, as well as in the respiratory tract, lymph nodes, gastrointestinal tract, kidney, heart, liver, spleen, and brain, indicating systemic dissemination of the virus.[38]

The observed disease in marmosets reproduces the documented disease progression in severe MERS-CoV cases more closely than rhesus macaques, which appear to model the mild to moderate cases. That is, the marmoset is a severe disease model, whereas the rhesus macaque serves as a transient disease model.[39] Taken together, non-human primate models represent an intermediate between a true pathogenesis model and a replication model. Non-human primates closely recapitulate a variety of aspects of human disease, but no single species represents the whole spectrum of human pathogenesis. Nonetheless, evaluating CoV MCMs in non-human primates is desirable before proceeding to human trials because researchers have no clinical experience with human coronavirus MCMs and non-human primates most closely replicate the human immune system.[40]

2.3.1.3 Use of mice with targeted deficiencies

The use of knockout mice with targeted deficiencies is a common strategy for the development of pathogenesis models, as such hosts often allow the virus to replicate more easily and provide initial passages during the process of adaptation or attenuation.[41] Several strains of knockout mice with targeted deletions of genes have been evaluated following SARS-CoV infection. Beige mice that lack natural killer (NK) cell function, CD1-/- mice that lack NK-T cells, and RAG1-/- mice that lack T and B lymphocytes do not display clinical disease when infected with SARS-CoV.[42] The only knockout mouse strain that exhibits notable clinical disease is STAT1-/- mice, which support prolonged viral replication in the lungs and develop more severe and longer-lasting pneumonia than wild type mice.[43] Mice with

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34 de Wit E *et al* (2013) Middle East respiratory syndrome coronavirus (MERS-CoV) causes transient lower respiratory tract infection in rhesus macaques. *Proceedings of the National Academy of Sciences of the United States of America* 110**:** 16598-16603.

35 Yao Y *et al* (2014) An animal model of MERS produced by infection of rhesus macaques with MERS coronavirus. *J Infect Dis* 209**:** 236-242.

36 van Doremalen N, Munster VJ (2015) Animal models of Middle East respiratory syndrome coronavirus infection. *Antiviral research* 122**:** 28-38.

37 Falzarano D *et al* (2014) Infection with MERS-CoV causes lethal pneumonia in the common marmoset. *PLoS pathogens* 10**:** e1004250.

38 van Doremalen N, Munster VJ (2015) Animal models of Middle East respiratory syndrome coronavirus infection. *Antiviral research* 122**:** 28-38.

39 Ibid.

40 Gretebeck LM, Subbarao K (2015) Animal models for SARS and MERS coronaviruses. *Current opinion in virology* 13**:** 123-129.

41 (2015b) Interviews with coronavirus researchers.

42 Sutton TC, Subbarao K (2015) Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology* 479-480C**:** 247-258

43 Gralinski LE, Baric RS (2015) Molecular pathology of emerging coronavirus infections. *The Journal of pathology* 235**:** 185-195.

targeted deficiencies are not permissive to MERS-CoV due to restriction at the host receptor binding site.[44]

2.3.1.4 Benefits and Limitations of Using Naturally Susceptible Hosts

Although the use of naturally susceptible hosts enables the study of wild type coronavirus strains, a key benefit relative to the use of animal-adapted strains, naturally susceptible hosts of SARS-CoV and MERS-CoV have significant limitations as animal model systems.

Animals that are naturally susceptible to SARS Co-V and MERS-CoV infection support viral replication but do not recapitulate human disease pathogenesis, which limits their utility for basic science studies investigating how viruses interact with host systems to cause disease as well as for MCM development. If appropriate immune correlates can be established, replication models can be used to study the host immune response to infection following immunization with vaccine candidates. The ability to identify immunological pathways and factors associated with protection may allow researchers to draw inferences to humans, thereby generating hypotheses about which immune system components must be activated by an effective vaccine and/or predicting the clinical efficacy of the vaccine candidate.[45] However, limited information can be generated using this approach, and all hypotheses should be confirmed in pathogenesis models. [46]

Replication models may provide easy metrics to demonstrate vaccine or drug efficacy, but the sole use of replication models could lead to the development and release of subpar or dangerous countermeasures.[47] MCMs may cause unintended side effects or interactions, which are unpredictable and may not be observed in asymptomatic animal models.[48] Therefore, conducting safety testing in a pathogenesis model is critical, as was demonstrated with a SARS-CoV vaccine candidate. The candidate vaccine was successful in non-human primate replication models but produced severe adverse effects when tested in mouse pathogenesis models. After vaccinated mice were challenged with live SARS-CoV virus, the mice displayed an immunopathologic Th2-type response, which is predictive of a harmful response to the vaccine in humans.[49] If the vaccine had been allowed to go to clinical trials without being tested in a pathogenesis model, clinical trial participants may have been harmed.[50]

Marmosets and macaques are likely to play an important role in the development of countermeasures for SARS-CoV and MERS-CoV, but opportunities for NHP research are limited and expensive, costing approximately $75,000 for a small trial involving 16 animals.[51] Only a few laboratories within the United States are approved to work with NHPs at the biosafety levels required for MERS-CoV and SARS-CoV, and the research capacities of those that are approved are limited by the number of available cages, supplies, and trained personnel. Together the costs and laboratory limitations restrict the number of primate studies that can be conducted, meaning very few countermeasures can be tested. These factors also make it difficult to conduct studies in large enough sample sizes for statistical evaluation and to draw robust conclusions.[52]

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44 Sutton TC, Subbarao K (2015) Development of animal models against emerging coronaviruses: From SARS to MERS coronavirus. *Virology* 479-480C**:** 247-258

45 (2015c) Interview with vaccinology expert.

46 (2015b) Interviews with coronavirus researchers.

47 Ibid

48 Ibid

49 Tseng CT *et al* (2012) Immunization with SARS coronavirus vaccines leads to pulmonary immunopathology on challenge with the SARS virus. *PloS one* 7**:** e35421.

50 (2015b) Interviews with coronavirus researchers.

51 (2015b) Interviews with coronavirus researchers.

52 Gretebeck LM, Subbarao K (2015) Animal models for SARS and MERS coronaviruses. *Current opinion in virology* 13**:** 123-129.

Finally, mice with targeted immune deficiencies are not suitable for testing the efficacy or safety of MCMs because many do not have complete immune systems and therefore cannot react to a vaccine or therapy in the same manner as a wild type animal. Furthermore, if the deficient gene product plays an important role in the host response to viral infection in wild type hosts, the relevance of insights about pathogenesis mechanisms to humans will be limited.

2.3.1.5 Summary of naturally susceptible hosts

SARS-CoV is capable of productively infecting mice, hamsters, ferrets, and several species of non-human primates, though not all species exhibit clinical signs or mortality. MERS-CoV exhibits a greater degree of host species restriction due to differences in the expression level and amino acid sequence of the human DPP4 receptor relative to that of common laboratory animals. Replication of MERS-CoV is limited to some NHP species, and rabbits are the only small mammals that have been found to be permissive to infection. Naturally susceptible species largely act as replication models – that is, these models are either asymptomatic or exhibit symptoms that are dissimilar to those observed in human disease. As a result, these models provide limited insights into pathogenesis or disease progression. Though they can be used to show the ability of a candidate MCM to block or reduce viral replication during infection, naturally susceptible hosts are not sufficient for demonstrating all possible effects of a vaccine or antiviral.

2.3.2 Transgenic Models: Adapting the Host to the Virus

2.3.2.1 Examples of transgenic approaches

Use of transgenic animals expressing the human virus entry receptor represents another alternative to the use of animal-adapted viruses, for animal hosts that are not naturally permissive to infection and/or do not recapitulate human disease pathology upon infection. Transgenic approaches have been used to develop mouse models for SARS-CoV and MERS-CoV.

Tseng and colleagues developed a transgenic mouse model for SARS-CoV by expressing human ACE2 (hACE2), the functional receptor for SARS-CoV, under a global promoter.[53] Expression of hACE2 antigen was detected in the lungs, kidneys, liver, heart, skeletal muscle, spleen, and other tissues. Transgenic mice supported more robust viral growth than non-transgenic mice upon infection with wild type virus, and manifested respiratory and generalized illness, tissue pathology, and inflammatory cytokine responses. Transgenic mice uniformly developed clinical illness and died within eight days. Transgenes can also be regulated by a host promoter to restrict expression to specific tissues or cell types. Yang and colleagues introduced the human ACE2 gene into the mouse genome under control of the endogenous mouse ACE2 promoter.[54] The expression pattern of hACE2 under the mouse promoter more closely mimicked the native ACE2 distribution in mice than that seen using a universal promoter, which better recapitulated cell- and tissue-specific permissiveness to infection. Transgenic mice showed severe pathologic changes after infection with SARS-CoV, but did not experience mortality.[55] McCray and colleagues used a different approach to create transgenic mice in which the expression of human ACE2 was targeted to epithelial cells.[56] In these mice, human ACE2 was expressed in airway epithelial cells as

[53] Tseng CT *et al* (2007) Severe acute respiratory syndrome coronavirus infection of mice transgenic for the human Angiotensin-converting enzyme 2 virus receptor. *Journal of virology* 81**:** 1162-1173.

[54] Yang XH *et al* (2007) Mice transgenic for human angiotensin-converting enzyme 2 provide a model for SARS coronavirus infection. *Comparative medicine* 57**:** 450-459.

[55] Ibid

[56] McCray PB, Jr. *et al* (2007) Lethal infection of K18-hACE2 mice infected with severe acute respiratory syndrome coronavirus. *Journal of virology* 81**:** 813-821.

well as in epithelia of other internal organs, but not alveolar epithelia cells. The transgenic mice inoculated intranasally with SARS-CoV lost weight, became lethargic, showed labored breathing, and became moribund. Expression of hACE2 was sufficient to convert the normally mild SARS-CoV infection into a rapidly fatal disease.

Similar approaches have been taken to create transgenic mouse models for MERS-CoV. Agrawal and colleagues created transgenic mice globally expressing human DPP4, which were highly susceptible to infection and suffered significant morbidity and mortality.[57] Mice experienced weight loss and rapid elicitation of acute inflammatory responses followed by death within days. However, although acute renal failure has been reported to occur in some MERS-CoV patients, no viral RNA was detected in the liver or kidneys.

Gene knock-in technology can also be used to sensitize mice to MERS-CoV infection.[58] Pascal and colleagues used VelociGene technology to replace mouse DPP4 with the human ortholog, creating mice that express human DPP4 in place of mouse DPP4. The knocked-in gene is under the regulation of the native mouse promoter to preserve correct physiological expression and tissue distribution. The resulting mice showed high levels of MERS-CoV replication in lungs, which was consistent with radiographic findings from human cases, but did not show clinical signs of disease or mortality. Additionally, mice exhibited inflammation and viral RNA in the brain, but there is no evidence of MERS-CoV replication in the human brain during infection.

A variant of this approach involves introducing mutations in the mouse DPP4 receptor to render it permissive to the wildtype MERS-CoV.[59] This technique has not yet been used to create animal models, thus whether this approach will generate models that better recapitulate human disease pathogenesis than existing transgenic animals is unknown.

Adenovirus vectors are an additional tool for introducing and expressing a gene of interest in an animal model. Zhao and colleagues developed a mouse model for MERS-CoV by transducing mice with a recombinant, non-replicating adenovirus vector expressing the human host cell receptor DPP4.[60] Infection with the adenovirus vector led to expression of hDPP4 on all lung cells, not only those natively expressing DPP4. Mice developed inflammatory cell infiltration, failed to gain weight or lost weight, and cleared the virus six to eight days post infection. While this model does not replicate human pathogenesis, adenovirus vectors are polytropic and could be used to create animal models in other species. Adenovirus transfection is faster than creating traditional transgenic animals since no cross-breeding is necessary. The adenovirus technique has unique advantages for asking questions about host immune responses since any mouse line with targeted deficiencies can be sensitized to infection, whereas humanized transgenic mice must be crossed with other strains to knock out immune functions.[61] However, the use of transduction methods to create transgenic animal models has several drawbacks. While the adenovirus can be somewhat localized by inoculation method, expression may not match natural patterns. Another drawback is that individual transduction-based models may vary due to differences in the infection efficacy of the transducing vector, complicating synthesis of results. Finally, researchers must also be careful not to

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57 Agrawal AS *et al* (2015) Generation of a transgenic mouse model of Middle East respiratory syndrome coronavirus infection and disease. *Journal of virology* 89**:** 3659-3670.

58 Pascal KE *et al* (2015) Pre- and postexposure efficacy of fully human antibodies against Spike protein in a novel humanized mouse model of MERS-CoV infection. *Proceedings of the National Academy of Sciences of the United States of America* 112**:** 8738-8743.

59 Cockrell AS *et al* (2014) Mouse dipeptidyl peptidase 4 is not a functional receptor for Middle East respiratory syndrome coronavirus infection. *Journal of virology* 88**:** 5195-5199.

60 Zhao J *et al* (2014) Rapid generation of a mouse model for Middle East respiratory syndrome. *Proceedings of the National Academy of Sciences of the United States of America* 111**:** 4970-4975.

61 (2015b) Interviews with coronavirus researchers.

activate aspects of the host immune system with the adenovirus, which renders it difficult to determine which immune responses result from the MERS-CoV infection and which from the initial adenovirus.

2.3.2.2 Benefits and Limitations of Transgenic Approaches

Transgenic models for SARS-CoV and MERS-CoV mimic the expression patterns of human virus receptors to varying degrees and thus differ in their ability to recapitulate human disease pathogenesis. However, all transgenic models provide defined endpoints, including pathological changes and death, which allow for the definitive analysis of antiviral and vaccine efficacy.[62] The ability to use wild type CoV strains is a key benefit of using transgenic animals, both because results are more likely to be relevant for human disease and because a variety of wild type isolates can be used to tease out how genetic differences between viruses alter disease pathogenesis. Additionally, the ability to establish that a therapy knocks down virus titers in a system with human receptors is valuable for countermeasure development.[63] Finally, for MERS-CoV, a transgenic approach can be used as a starting point for adaptation of the virus to mice.[64] Viruses that do not productively infect animals cannot be adapted; therefore enabling virus replication in mice through expression of the human DPP4 receptor is the first step toward developing a fully mouse-adapted strain.

However, transgenic models have significant limitations for the study of pathogenesis and for MCM development. As shown by efforts to develop transgenic mouse models for SARS-CoV and MERS-CoV, simply expressing the right receptor does not guarantee that the resulting model will be a pathogenesis model. First, expression of a transgene may not be sufficient for viral entry and replication. Cell culture analysis showed that not all cells expressing hACE2 in Tseng's mice were permissive to SARS-CoV infection.[65] The reason for this is not known, but this result demonstrates that hACE2 expression alone may not be sufficient for maintaining effective viral replication. An additional limitation is that transgene expression may not mimic human gene expression, limiting the relevance of results using transgenic animals to human disease. Universal promoters may lead to over-expression, which is not physiologically relevant, while differences between the host promoter and the human promoter could cause dissimilar expression levels and tissue distribution. If cells that do not natively express the viral receptor are made susceptible, the resulting infection of additional cell types may alter pathogenesis. For example, transgenic mice with more widespread expression of ACE2 develop lethal neurological disease rarely seen in human SARS-CoV patients.[66] If transgene expression and tropism does not mimic humans, the model may not be useful for investigating tissue tropism and organ-to-organ spread. The more disparate transgene expression is from that observed in humans, the lesser the utility of the model. Stable transgenic animals also cannot be used to investigate immune system components and host genetic differences in the same manner as adapted strains.

2.3.2.3 Summary of transgenic models

Transgenic models allow the study of wild-type SARS-CoV and MERS-CoV in animals expressing human receptors. The approaches used to create transgenic mice include stable expression of human viral receptors under the control of global promoters, host promoters, cell-type specific promoters, as well as inducing expression using adenovirus vectors. Each technique results in slightly different gene expression

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62 Tseng CT *et al* (2007) Severe acute respiratory syndrome coronavirus infection of mice transgenic for the human Angiotensin-converting enzyme 2 virus receptor. *Journal of virology* 81**:** 1162-1173.

63 (2015b) Interviews with coronavirus researchers.

64 Ibid

65 Tseng CT *et al* (2007) Severe acute respiratory syndrome coronavirus infection of mice transgenic for the human Angiotensin-converting enzyme 2 virus receptor. *Journal of virology* 81**:** 1162-1173.

66 Frieman M *et al* (2012) Molecular determinants of severe acute respiratory syndrome coronavirus pathogenesis and virulence in young and aged mouse models of human disease. *Journal of virology* 86**:** 884-897.

and reproduces human disease symptoms to a different degree, limiting the utility of these systems for studying pathogenesis and for testing MCMs.

2.3.3 Coronavirus Naturally Pathogenic to Laboratory Animals: Mouse Hepatitis Virus

The coronavirus mouse hepatitis virus (MHV) has been used as a model to generate basic knowledge about coronavirus biology but cannot serve as a substitute for MERS-CoV or SARS-CoV for pathogenesis studies or MCM development studies. Adult mouse infections of MHV are usually asymptomatic. While infant mice exhibit pathology during infection, the symptoms and disease course do not mimic those of MERS-CoV or SARS-CoV.[67] MHV has been useful for the study of mechanisms universal to coronaviruses, which has led to the discovery of generalizable information about coronavirus polymerases, proteases, and other nonstructural proteins.[68] However, coronaviruses do not share the core machinery often targeted by antivirals or vaccines, and studies have shown that inhibitors that successfully target one coronavirus do not work for the other .[69,70] Thus, the efficacy and safety of all countermeasures tested in the context of MHV infection must be confirmed using SARS-CoV or MERS-CoV. In addition, due to the unique features of SARS-CoV and MERS-CoV, pathogenesis, transmissibility, and the effects of SARS-CoV and MERS-CoV in humans cannot be investigated using MHV.[71]

2.3.4 Human Autopsy Data

Human autopsy data can be an alternative source of pathogenesis information. SARS associated lung pathology was described from examination of post-mortem tissue samples; however, pathologic changes associated with MERS have not been reported due to a lack of autopsy data.[72] Autopsies are not often performed in Middle Eastern cultures, and data has not yet been shared from the most recent outbreak in the Republic of Korea.[73] In the absence of autopsy data from MERS-CoV-associated fatal cases, the description of MERS-CoV pathogenesis in humans is limited to clinical data such as radiographs.[74] Human autopsy data can provide direct insight into human disease pathogenesis, a key benefit of this approach. However, autopsy data is devoid of time series information, making it difficult to determine the order in which pathogenic effects occurred. Diversity in genetic backgrounds, life histories, and chronic conditions must all be taken into account and can complicate the identification of pathology caused by viral infection versus comorbidities. Those who die from infection are not necessarily representative of all infected individuals. MERS-CoV has increased morality rates in the elderly and those with pre-existing health conditions, so autopsy information from these individuals may not fully represent pathology seen in younger, otherwise healthy persons.

2.4 Conclusion: Benefits of Gain of Function Approaches for Animal Model Development

Model systems are essential for understanding the pathology of viral disease and for developing vaccines and therapeutics. Mouse-adapted strains of SARS represent the only model system that recapitulates

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67 Baker DG (1998) Natural pathogens of laboratory mice, rats, and rabbits and their effects on research. *Clinical microbiology reviews* 11**:** 231-266.

68 (2015b) Interviews with coronavirus researchers.

69 Ibid

70 Ibid

71 Ibid

72 Gretebeck LM, Subbarao K (2015) Animal models for SARS and MERS coronaviruses. *Current opinion in virology* 13**:** 123-129.

73 (2015b) Interviews with coronavirus researchers.

74 van Doremalen N, Munster VJ (2015) Animal models of Middle East respiratory syndrome coronavirus infection. *Antiviral research* 122**:** 28-38

disease pathogenesis observed during human infections of SARS-CoV. As existing animal models for MERS-CoV do not replicate human disease pathology, mouse-adapted strains of MERS are expected to serve as the sole pathogenesis model for the study of MERS-CoV infection as well. As such, animal-adapted strains provide significant advantages over alternative model systems for the study of disease pathogenesis and for advanced MCM development. Adapted strains provide a greater range of information about pathogenesis, including the role of tissue tropism, than transgenic animals because the host viral receptors retain native expression patterns. Most naturally susceptible hosts are asymptomatic or display dissimilar symptoms to humans and thus cannot be used to study disease pathogenesis. While human autopsy data are uniquely capable of providing insight into human disease pathology, limited autopsy data are available, and the static nature of the data and the presence of co-morbidities in many SARS-CoV/MERS-CoV patients complicate interpretation of the data.

The use of animal-adapted strains of CoVs is also critical for advanced MCM development. Though transgenic animals and naturally susceptible hosts can be used to demonstrate that MCMs diminish viral replication, an important proof of concept for early stage MCMs, animal-adapted strains that replicate human disease pathology provide a much more robust system for demonstrating the safety and efficacy of MCM candidates. In addition, because adapted strains provoke a response from the host immune system, use of these strains can reveal MCM side effects or adverse reactions that are not seen in asymptomatic models.

3 Animal Models for Influenza Viruses

3.1 Introduction

Animal models are essential for the study of disease pathogenesis and transmission. Although seasonal influenza viruses can be studied using human challenge studies, ethical considerations limit the number and types of studies that can be carried out. A variety of animal model systems have been developed for the study of influenza viruses, including mice, ferrets, guinea pigs, swine, non-human primates, and domestic canines and felines. Each model has strengths and weaknesses for the study of pathogenesis and/or transmission, arising from differences in susceptibility and symptomology relative to humans. This report reviews the animal model systems that have been used for GoF studies and evaluates the strengths and weaknesses of each. The features of human disease are first summarized, then each animal model is discussed in turn.

3.2 Summary of Human Disease

Influenza viruses infect humans by both airborne and direct contact or indirect contact (e.g. fomite) transmission. Droplet transmission occurs when droplets produced by coughing or sneezing travel a short distance and come into contact with another individual's conjunctiva, mouth or nasal mucosa. Airborne transmission entails the production of infectious droplet nuclei, smaller than true droplets, that remain suspended in the air and can be inhaled by an individual.[75] Uncertainty remains as to the relative importance of airborne, droplet, and contact-based spread.[76] Observational studies of influenza outbreaks suggest roles for both contact and airborne transmission, but contact modes are thought to require higher doses of virus for effective transmission.[77] Susceptibility and tissue tropism is determined by the distribution of virus specific receptors throughout the respiratory and gastrointestinal tracts. In humans,

[75] Bridges CB *et al* (2003) Transmission of influenza: implications for control in health care settings. *Clinical infectious diseases : an official publication of the Infectious Diseases Society of America* 37**:** 1094-1101.
[76] Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320.
[77] Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

the primary targets for the virus are epithelial cells of the upper and lower respiratory tracts. Influenza hemagglutinin (HA) binds to sialic acid residues on glycoproteins of the respiratory epithelial cell surface. Human influenza viruses preferentially bind to sialic acids in an α-2,6 configuration, while avian strains preferentially bind to sialic acids displaying an α-2,3 linkage.[78] Sialic acid in the 2,6 configuration is predominantly expressed in the human trachea, which contributes to the characteristic upper respiratory tract infection of seasonal human strains.[79] In contrast, sialic acid in the α-2,3 configuration is located in the lower respiratory tract.[80] Transmission is dependent on the relative amount and length of viral shedding. Many experimental human studies have described the course of influenza virus infection in placebo-treated and untreated volunteers challenged with wild-type influenza virus.[81] In a recent meta-analysis of volunteer challenge studies, viral shedding was found to begin within the first day after inoculation, peak on day 2, and cease by day 8 or 9 post-infection.[82]

Uncomplicated influenza is an acute respiratory disease largely confined to the upper respiratory tract and is characterized by the acute onset of symptoms within one to two days after infection. Systemic symptoms include fever, chills, headache, myalgia, lethargy, and anorexia, while respiratory symptoms include dry cough, nasal congestion and discharge, and sore throat. Cough and sore throat may persist for several days after systemic symptoms subside.[83] The frequency of symptomatic infection is approximately 66%. Fever is observed in 37% of H1N1 cases, 40% of H3N2 cases, and 7 % of influenza B infections. The total symptoms scores increased on day 1 and peaked on day 3. Systemic symptoms peaked on day 2.[84] Influenza virus replicates in epithelial cells throughout the respiratory system, with virus being recoverable from both the upper and lower respiratory tracts of people naturally or experimentally infected. Non-fatal influenza viral infections predominantly involve the upper respiratory tract and trachea, but fatal cases of influenza usually show evidence of pneumonia.[85]

Primary influenza viral pneumonia begins as uncomplicated influenza disease in the upper respiratory tract, but in cases of severe disease illness rapidly progresses with symptoms of lower respiratory tract disease.[86] Examination of fatal cases of seasonal influenza revealed extensive diffuse alveolar damage with variable degrees of pulmonary hemorrhage and necrotizing bronchiolitis.[87] There were no signs of direct virus-induced injury in any organ other than the lungs.[88] In seasonal influenza, primary viral pneumonia rarely occurs and usually affects older patients and those with cardiovascular co-morbidities. Pandemic strains, such as the 2009 H1N1 pandemic virus, may shift the epidemiology of influenza disease by disproportionately affecting younger populations and causing greater rates of lower respiratory tract disease and hospitalization.[89] Secondary bacterial infections are common in cases of severe disease, which increases mortality.

In contrast to seasonal strains, infections with highly pathogenic avian strains such as H5N1 can progress to lower respiratory disease with acute viral pneumonia or acute respiratory distress syndrome (ARDS)

78 Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320.
79 Margine I, Krammer F (2014) Animal models for influenza viruses: implications for universal vaccine development. Pathogens (Basel, Switzerland) 3: 845-874.
80 Shinya K *et al* (2006) Avian flu: influenza virus receptors in the human airway. *Nature* 440**:** 435-436.
81 Carrat F et al (2008) Time lines of infection and disease in human influenza: a review of volunteer challenge studies. American journal of epidemiology 167: 775-785
82 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.
83 Ibid
84 Carrat F et al (2008) Time lines of infection and disease in human influenza: a review of volunteer challenge studies. American journal of epidemiology 167: 775-785
85 Taubenberger JK, Morens DM (2008) The Pathology of Influenza Virus Infections. Annual review of pathology 3: 499-522
86 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.
87 Mauad T et al (2010) Lung pathology in fatal novel human influenza A (H1N1) infection. American journal of respiratory and critical care medicine 181: 72-79.
88 Ibid
89 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

due to their increased affinity to cells of the lower respiratory tract where sialic acids in the α-2,3 configuration are present. Highly pathogenic H5N1 strains have also been found to trigger a cytokine storm with elevated levels of tumor necrosis factor TNF-alpha and interferon IFN-beta. Pathological examinations of tissues from fatal human cases revealed diffuse alveolar damage, interstitial fibrosis, bronchiolitis, hemorrhages, and abundant presence of macrophages in the lungs.[90] The 2009 pandemic strain of H1N1 can also target the lower respiratory tract, resulting in severe respiratory distress syndrome.[91]

3.3 Animal Models of Influenza Pathogenesis and Transmission

3.3.1 Mouse

Mice are the most commonly used model of influenza. Mice are not naturally permissive to most human influenza strains because their airways are dominated by sialic acid α-2,3 linkages and lack sialic acid α-2,6 linkages.[92] Often, viruses must first be passaged in murine tissues to adapt the virus to replicate more efficiently in mice. The majority of studies use one of a few mouse-adapted laboratory strains such as A/Puerto Rico/8/34 ("PR8") or A/WSN/1933 ("WSN"). These strains (as well as PR8 reassortant strains) are used both because the strains and course of infection are well-characterized and as a risk mitigation measure, as PR8 strains cannot infect humans.[93] However, a number of pandemic and highly pathogenic strains, including the 1918 H1N1 pandemic strain, HPAI H5N1, and the 2009 H1N1 pandemic strain, do not require adaptation to replicate efficiently in mice.[94] The degree of susceptibility depends not only on the virus strain, but also on the mouse genetic background. BALB/C and C57BL/6 inbred mice are susceptible to the mouse-adapted laboratory strains A/Puerto Rico/8/34 or AWSN/1933, whereas outbred mice usually resistant. DBA/2J and A/J inbred mice, a model which was developed in 2009, are more susceptible to non-adapted strains, relative to BALBC and C57BL/6 mice.[95]

Mice are a poor model of transmission, as transmission efficiency varies depending on the virus strain and mouse strain used and is inefficient and inconsistent in mouse/virus pairs that exhibit limited transmission.[96] However, mice are commonly used in pathogenicity studies. Signs of disease differ from human infections and are often dose dependent. Lethal doses result in huddling, ruffled fur, lethargy, anorexia, weight loss, labored breathing, and death. Viral replication and resulting tissue damage are concentrated in the lower respiratory tract rather than the upper airways. Unlike humans, mice do not display fever, sneezing, or coughing, but do display hypothermia. Infection with WSN and PR8 results in severe pneumonia and mortality in BALB/C and C57BL/6 mice. Some HPAI strains spread to the brain, kidney, thymus, health, liver, and spleen.

Mice have several strengths as pathogenesis models for the study of influenza virus infection. The genetics of established mice lines are well characterized and standardized, and inbred mice show homogenous and reproducible responses to infection. Additionally, a large number of genetically modified mouse strains and immunological reagents are available, which can be used to tease apart the

90 Margine I, Krammer F (2014) Animal models for influenza viruses: implications for universal vaccine development. Pathogens (Basel, Switzerland) 3: 845-874.

91 Shieh W-J et al (2010) 2009 Pandemic Influenza A (H1N1) : Pathology and Pathogenesis of 100 Fatal Cases in the United States. The American Journal of Pathology 177: 166-175.

92 Margine I, Krammer F (2014) Animal models for influenza viruses: implications for universal vaccine development. Pathogens (Basel, Switzerland) 3: 845-874.

93 PR8 reassortant strains used as a risk mitigation measure include the HA and/or NA genes from a pathogenic strain (human seasonal or animal strain) and the remaining six or seven genes from PR8.

94 Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320.

95 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

96 Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320

roles of various immune components and molecular pathways. Finally, large numbers can easily be bred and used in experimental design, allowing statistically robust data to be obtained.[97]

3.3.2 Ferret

Ferrets are valuable models of influenza and are the only small mammal model equally well suited to studying both pathogenesis and transmission. Ferrets are susceptible to a variety of non-adapted human influenza viruses including seasonal strains, pandemic strains, and influenza B strains. This may be reflective of the similar distribution of α -2,6 sialic acid receptors within the ferret respiratory tract relative to humans.[98] Ferrets can also be infected with influenza A viruses isolated from birds and swine, due to expression of α -2,3 sialic acid receptors in lower respiratory tissues. Ferrets models are highly applicable for transmission studies as influenza is able to transmit through direct contact, respiratory droplets, and aerosols.[99] Ferrets display an exquisite sneeze reflex, which adds to their value for studying transmission though respiratory droplets.[100] Ferrets can also be used to study influenza pathogenesis. Ferrets display upper respiratory infection patterns and clinical symptoms similar to humans with fever, nasal discharge, and sneezing as prominent symptoms. Seasonal influenza infection rarely progresses to pneumonia though some pandemic and highly pathogenic strains can lead to more extensive pulmonary damage. Highly pathogenic avian influenza strains can also spread to extrapulmonary organs causing diarrhea and neurological damage.[101] Some pandemic strains such as the reconstructed 1918 strain and 2009 H1N1 manifest as upper respiratory infections as opposed to the lower respiratory tract infections seen in humans.[102] Notably, mouse-adapted strains of PR8, which are commonly used as a risk mitigation measure, replicate poorly in ferrets do not produce fever or other signs of infection, although ferret-adapted PR8 strains (less commonly used) produce an extensive infection.[103]

Despite their strengths as models for pathogenesis and transmission, the ferret model system has several technical limitations. First, ferrets are genetically outbred and thus may show variability in host responses. The availability of reagents for detailed phenotyping and studying the immune system is limited, and genetically modified knock-in or knock-out lines are lacking.[104] Due to the larger size and increased husbandry requirements of ferrets, the use of large groups may be cost prohibitive. The use of fewer animals can lower the statistical analyses that can be performed, sometimes limiting the conclusions drawn.

3.3.3 Guinea Pig

Guinea pigs are susceptible to a wide range of non-adapted human isolates including seasonal strains, pandemic 2009 strains, 1918 pandemic strains, and highly pathogenic strains of avian influenza H5N1. Laboratory strains of PR8 exhibit low virus titers and are non-transmissible in guinea pigs, but can be adapted through serial passaging to become more transmissible.[105] Both sialic acid α-2,3 and α-2,6

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97 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

98 Belser JA et al (2011) The ferret as a model organism to study influenza A virus infection. Disease models & mechanisms 4: 575-579.

99 Ibid

100 Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320.

101 Margine I, Krammer F (2014) Animal models for influenza viruses: implications for universal vaccine development. Pathogens (Basel, Switzerland) 3: 845-874

102 Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320.

103 Liu C (1955) Studies of influenza infection in ferrets by means of fluorescein-labelled antibody. I. The pathogenesis and diagnosis of the disease. *The Journal of experimental medicine* 101**:** 665-676.

104 Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320.

105 Lowen AC *et al* (2014) Transmission in the guinea pig model. *Current topics in microbiology and immunology* 385**:** 157-183.

receptors are widely represented in the nasal tract and the trachea, while α-2,3 receptors are the main receptor in the lung. Human influenza transmits efficiently through direct contact or aerosols, though there are differences in transmissibility between strains, while transmission through fomites is inefficient.[106,107] Influenza B also transmits well in guinea pigs, providing one of the only models of influenza B transmission. Guinea pigs have limited utility for pathogenesis studies. Seasonal strains replicate to high titers in nasal passageways and are confined to the upper respiratory tract, similar to human infection. However, guinea pigs lack clinical disease manifestations with no fever, weight loss, lethargy, coughing, or sneezing, though there is some increased production of mucus in nasal passages. The virus does not disseminate to other organs, including the heart, liver, spleen, kidney, brain or colon.[108] Guinea pig strain does not appear to affect susceptibility or outcome, as inbred strains display similar viral titers during infection as outbred Hartley guinea pigs. The guinea pig model system has several technical strengths. Guinea pigs are commercially available, small in size, and easy to handle and house, allowing higher numbers of experimental animals in a study.[109] However, few reagents are available for detailed immunophenotyping of guinea pigs.[110]

3.3.4 Non-human Primate

Non-human primates used in influenza research include rhesus macaques, pig-tailed macaques, cynomolgus macaques, squirrel monkeys, and African green monkeys. The main advantage of studies using non-human primates is the ability to examine immune reactions in species closely related to humans. Because of this, non-human primates are used to study pathogenesis and medical countermeasures, but are not used in transmission studies[111]. Non-human primates are naturally susceptible to human isolates due to the similarities of their respiratory tract anatomy and sialic acid receptor distribution. Despite these similarities, non-human primates do not consistently develop symptoms upon infection with seasonal strains.[112] The virus attaches more strongly to cells from the lower respiratory tract, which differs from the attachment pattern seen in humans.[113] Some studies have found virus in heart, spleen, and brain tissues, but extrapulmonary dissemination is inconsistent and varies between animals.[114] Nasal discharge, coughing, sneezing, fever, and ARDS are displayed only upon infection with highly pathogenic strains.

The common use of primates in scientific studies means that reagents for investigating molecular and immune responses are widely available. However, non-human primates have several drawbacks as model systems for the study of influenza. Host responses can vary due to the genetically outbred nature of non-human primate populations. Additionally, animals obtained from different sources display highly variable responses to infection. Non-human primate trials are complicated by prohibitively high costs, low availability of animals, the need for experienced personnel, and moral issues.[115] Obtaining statistically robust results from non-human primate studies can be difficult due to these limitations.

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106 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

107 Mubareka S et al (2009) Transmission of influenza virus via aerosols and fomites in the guinea pig model. The Journal of infectious diseases 199: 858-865.

108 Sun Y et al (2010) Guinea pig model for evaluating the potential public health risk of swine and avian influenza viruses. PloS one 5: e15537.

109 Radigan KA et al (2015) Modeling human influenza infection in the laboratory. Infection and drug resistance 8: 311-320.

110 Schafer H, Burger R (2012) Tools for cellular immunology and vaccine research the in the guinea pig: monoclonal antibodies to cell surface antigens and cell lines. *Vaccine* 30**:** 5804-5811.

111 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

112 Margine I, Krammer F (2014) Animal models for influenza viruses: implications for universal vaccine development. Pathogens (Basel, Switzerland) 3: 845-874.

113 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

114 Rimmelzwaan GF et al (2003) A primate model to study the pathogenesis of influenza A (H5N1) virus infection. Avian diseases 47: 931-933.

115 Bouvier NM, Lowen AC (2010) Animal Models for Influenza Virus Pathogenesis and Transmission. Viruses 2: 1530-1563.

3.3.5 Swine

Pigs have been used to study aerosol transmission of some strains, but their main use of pigs is in the development of swine vaccines for swine influenza strains.[116] Pigs are susceptible to avian and human strains due to the expression of both α-2,3 and α-2,6 sialic acid linkages on epithelial cells. Symptom occurrence and intensity vary depending on the strain and can include fever, loss of appetite, labored breathing, nasal discharge, occasional coughing, and sneezing. Replication occurs in the epithelium of the entire respiratory tract and virus does not disseminate to extrapulmonary organs. Histologic lesions may occur as tracheobronchitis and bronchointersitial pneumonia with infiltration of alveolar septa by large macrophages. Although some molecular biology and immunology reagents are available, swine model systems have several limitations. Swine are genetically outbred, which can lead to host response variability.[117] Additionally, obtaining animals with no prior exposure to influenza is essential, as preexisting antibodies may confound study results.[118] Finally, pigs are large and have problematic husbandry and waste disposal requirements that complicate swine studies.

3.3.6 Domestic Canine and Feline

Canines and felines are not considered standard models of human influenza virus, but are of interest due to their susceptibility to circulating influenza strains and potential role as intermediate species during avian influenza virus adaptation to mammals. These species may be more useful as sentinels for human disease than as model systems.[119] Canines are susceptible to infection by some strains including avian H5N1 but are not capable of transmitting the virus to other mammals. Symptoms include conjunctivitis and fever resolved with no other adverse events. Felines are susceptible to some avian and human strains of influenza. After infection with H5N1, animals showed fever, conjunctivitis, lethargy, and labored breathing. Virus was found in the respiratory and digestive tracts, liver, kidney, brain, and lymph nodes. Some species-specific reagents are available, but few experimental studies have been conducted on domestic canines and felines.

3.4 Comparison of Benefits and Limitations of Animal Models for Influenza

3.4.1 Benefits and Limitations for Transmission Studies

Human influenza can spread through airborne (aerosol or droplet), direct contact, or indirect contact (i.e. fomite) transmission. Model systems that exhibit transmission through one or all transmission pathways are the most valuable for studying transmissibility. Viral shedding through sneezing, coughing, and excreting nasal mucus is important to viral transmission; thus species such as ferrets, non-human primates, and guinea pigs that display these symptoms more often exhibit animal-to-animal transmission. Guinea pigs can be used to study aerosol transmission and direct contact transmission but may be less applicable for the study of transmission through fomites. Guinea pigs are also the only model of influenza B transmission.

Ferrets display efficient transmission through both airborne and contact transmission routes and are widely used in transmission studies. Ferret transmission experiments can contribute distinctive insights into the pandemic potential of novel strains since there is a quantitative link between estimates of transmission efficiency among ferrets and efficiency among humans. Estimates of ferret secondary attack

[116] Margine I, Krammer F (2014) Animal models for influenza viruses: implications for universal vaccine development. Pathogens (Basel, Switzerland) 3: 845-874.
[117] Ibid
[118] Barnard DL (2009) Animal models for the study of influenza pathogenesis and therapy. Antiviral research 82: A110-122.
[119] Ibid

rate (SAR) are correlated with variations in human SAR estimates at the subtype level. Specifically, mean ferret respiratory droplet SAR explained 66% of the variation in mean human SAR across subtypes, suggesting that respiratory droplet experiments in ferrets are valuable for estimating human-to-human transmissibility. Ferrets are used to evaluate the transmissibility of circulating animal influenza viruses and thus are valuable for identifying viruses of concern for epidemic spread in humans. However, small sample sizes and biological uncertainties prevent definitive conclusions about human transmissibility.[120] PR8 can be used in transmission studies in ferrets and guinea pigs, but the strain must first be adapted to the host as mouse-adapted strains transmit poorly in these species. (Of note, this adaptation would be considered a GoF approach.) In this way, PR8 can also be used to study mutations that give rise to increased transmission.

3.4.2 Benefits and Limitations for Pathogenesis Studies

The value of an animal model for the study of human pathogenesis is largely determined by the degree to which the animal replicates human symptoms and susceptibility to disease. Receptor distribution is thought to play a large role in the susceptibility of various animals to seasonal and pandemic strains of influenza. Animals such as ferrets, guinea pigs, and non-human primates that share a similar distribution of α-2,3 and α-2,6 sialic acid receptors to humans more closely mimic susceptibility and can often be directly infected with human isolates. Animals such as mice that do not have similar receptor distribution and display resistance to infection by many influenza viruses, especially human seasonal strains, often require the use of adapted strains. Therefore, the mouse model is less useful for the study of currently circulating seasonal strains, though mice are naturally susceptible to a number of highly pathogenic avian influenza strains. PR8 infection and pathogenesis are well characterized in mice, ferrets, and guinea pigs. Because PR8 is well understood and easily manipulated, it can be used as a backbone to introduce mutations that may alter pathogenicity or other viral characteristics in these animals, as a risk mediation strategy for performing a GoF approach.

Animals that reproduce human symptoms of influenza provide the most utility for the study of disease pathogenesis. Animals such as guinea pigs that do not show symptoms are useful for the study of viral replication, but not pathogenesis. Species such as mice that are symptomatic and mimic some symptoms of human disease are more widely utilized for pathogenesis studies. Ferrets and non-human primates are especially valuable because they display important hallmarks of human disease such as fever and sneezing that mice do not.

Ferrets are unique among animal models because they are the only species equally applicable to the study of transmissibility and pathogenesis. Studying both aspects of influenza in one model allows scientists to more closely replicate the full course of human disease from initial infection and onset of symptoms to transmission and infection of another.

For both pathogenesis and transmission studies, genetic diversity impacts the translatability of findings to other outbred groups. Animals with standardized inbred lines and well-characterized genomes and transcriptomes display more predictable responses and allow results to be replicated more easily. Mice and guinea pigs are available as both inbred and outbred lines. No inbred lines of ferrets or non-human primates are currently available, which means that host genetic variability must be accounted for when attempting to replicate studies or generalize results to other groups. Size and husbandry requirements are also important considerations because obtaining the large numbers of animals necessary for statistically robust studies may be cost and resource limited. Despite their limited susceptibility to influenza stains, mice are nevertheless preferred because their small size and low costs allow studies to include many

[120] Buhnerkempe MG et al (2015) Mapping influenza transmission in the ferret model to transmission in humans. eLife 4.

specimens. Guinea pigs are also small and cost effective. Ferrets are larger and more costly, which often limits the number of ferrets included in a study.