Published in final edited form as:
Microbes Infect. 2011 May ; 13(5): 502–515. doi:10.1016/j.micinf.2011.01.014.

The contribution of animals models to the understanding of the host-range and virulence of influenza A viruses

Christopher D. O'Donnell[a] and **Kanta Subbarao**[a,*]
[a] Laboratory of Infectious Diseases, NIAID, NIH, Bethesda, MD 20892, USA

Abstract

Since ferrets were first used in 1933 during the initial isolation of influenza A viruses, animal models have been critical for influenza research. The following review discusses the contribution of mice, ferrets and non-human primates to the study of influenza virus host-range and pathogenicity.

Keywords

influenza A virus; host range; virulence; pathogenesis; animal models; ferrets; mice; non-human primates

1. Introduction

Of the three types of influenza viruses, A, B, and C, influenza A viruses infect a wide range of hosts and include different subtypes based on the antigenicity of the hemagglutinin (HA) and neuraminidase (NA), while influenza B and C infection is primarily restricted to humans [reviewed in 1 and 2]. Influenza viruses are RNA viruses belonging to the *Orthomyxoviridae* family, and influenza A and B viruses have genomes comprised of 8 negative-sense, single-stranded RNA segments, which encode at least 11 proteins (reviewed in Table 1) [2–3]. The natural hosts of influenza A viruses are waterfowl (order *Anseriformes*) and shorebirds (order *Charadriformes*). Aquatic birds are infected by all 16 HA and 9 NA subtypes of influenza A viruses. Three HA subtypes (H1, H2, H3) and two NA subtypes (N1, N2) have caused widespread, sustained disease in humans in the past century. Each year, widespread outbreaks of seasonal influenza result in an estimated 250,000–500,000 deaths worldwide [1]. Novel influenza subtypes, such as H5, H7, and H9, have been transmitted from wild aquatic birds to domestic poultry and various mammalian species, including pigs, horses, and humans [1].

Direct transmission of influenza viruses with a novel HA gene from animals to humans can cause pandemics, such as the H1N1 pandemic of 1918, which resulted in 20–50 million deaths worldwide, and the 2009 pandemic that caused significant morbidity and mortality in children and young adults [1,4]. Past studies that involved experimental administration of avian influenza viruses (AIV) to humans or human influenza viruses to ducks demonstrated

*Corresponding author. LID, NIAID, NIH 33 North Drive, MSC 3203, Room 3E.13C.1 Bethesda, MD 20892, USA. Phone: + 1 301 451 3839, Fax: + 1 301 480 4749. ksubbarao@niaid.nih.gov.

restricted replication in the heterologous species, presumably due to host range restriction [5,6]. However, more recent natural events have shown that influenza viruses can cross the species barrier; for example, equine influenza virus transmitted to dogs, and AIV transmitted to pigs and humans [1,7,8]. Since 2003, almost 500 cases of human infection with highly pathogenic avian influenza (HPAI) H5N1 viruses have been reported resulting in close to 300 deaths (60% case fatality rate), with most of the infections due to direct transmission from infected poultry to humans. Although HPAI viruses have not transmitted efficiently between humans so far, there is a concern that repeated transmission of AIV from infected birds to humans increases the probability that a virus will emerge that has the molecular changes needed for efficient and sustained virus transmission among humans.

Novel HA and NA glycoproteins can also be introduced into humans as a result of genetic reassortment between avian and human influenza strains, as occurred in the 1957 H2N2 and 1968 H3N2 pandemics [9]. Low pathogenicity avian influenza (LPAI) viruses were the source of these two pandemics [9]. The pandemic 2009 H1N1 virus is a reassortant animal influenza virus that derived genes from North American H3N2 and H1N2 swine viruses and Eurasian avian-like swine viruses [10].

A better understanding of influenza host range and pathogenicity (defined in Table 2) will aid in early recognition of influenza strains with pandemic potential and the development of antiviral strategies. Animal models, which are used as surrogates for evaluating human influenza infection, have contributed to our understanding of host and viral factors involved in the adaptation of influenza viruses to new hosts and their ability to cause disease. The characteristics of the most commonly used animal models are summarized in Table 3. In this review, we discuss the contributions that specific animal models have made to our understanding of influenza host range, pathogenicity and virulence.

2. Influenza host range, pathogenicity, and virulence

2.1 Influenza host range

The ability of an influenza virus to infect a specific host species (host range, defined in Table 2) is determined by the ability of the virus to attach to, replicate in, and release from cells of that specific host. Attachment to sialic acid (SA) receptors is dependent upon the influenza HA, while internal viral proteins and various host factors are critical for viral replication. The NA functions in the release of infectious progeny virus through the removal of SA from the cell surface.

Receptor specificity is a primary determinant of viral attachment and infection of host cells. SA receptor distribution varies between species and among tissues and cell types within the same host [11]. Attachment is initiated by the binding of the HA to SA on the cell surface. Influenza virus binding and infectivity are influenced by the type of galactose linkages (α2,6 or α2,3) present on SA [12]. Human influenza viruses preferentially recognize SAα2,6 receptors, which are expressed on non-ciliated cells and are more prevalent in the upper respiratory tract [11,12,13]. AIV preferentially recognize SAα2,3 receptors, which correlates with the predominance of SAα2,3 receptors on intestinal epithelial cells of birds and cells of the lower respiratory tract in humans. SAα2,3 receptors are also expressed on ciliated cells in the upper respiratory tract of humans [1,11,12,14,15]. The enhanced virulence of HPAI viruses bearing SAα2,3 receptors, causing acute viral pneumonia that progresses to acute respiratory distress syndrome (ARDS) and multiple organ failure, is thought to be in part due to viral tropism for cells of the lower respiratory tract [1,14,15]. How well animal models recapitulate human disease maybe determined by the distribution of receptors and resulting infection in the respiratory tract.

Amino acids at positions 226 and 228 in the receptor binding site (RBS) of the H3 HA play a key role in pathogenicity and host range as these residues determine receptor specificity [12,13]. Gln-226, found specifically in AIV, determines specificity for SAα2,3, while Leu-226 correlates with SAα2,6 specificity in human H2 and H3 influenza viruses [12]. In most human influenza viruses, Leu-226 is associated with Ser-228, while Gln-226 is associated with Gly-228 in AIV [reviewed in 13]. Among H1 viruses, Asp-190 and Glu-225/Asp-225, which have been identified in human and swine isolates, and Glu-190/Gly-225 found in avian isolates, determine preferential binding to SAα2,6 or SAα2,3, respectively [reviewed in 13].

Influenza virus encodes three polymerase proteins PA, PB1, PB2 and the NP protein, which form the ribonucleoprotein (RNP) complex, which is responsible for transcription and viral replication (Table 1). However, since the virus encodes only 11 proteins, it must utilize the host cell's machinery to aid in its replication. High-throughput RNAi screens in human cells has been utilized to identify hundreds of human genes that are required for the influenza replicative cycle [16–19]. Further characterization of these gene products may reveal important host range determinants.

2.2 Influenza pathogenicity and virulence

Pathogenicity, defined in Table 2 as the ability of an influenza virus to cause disease in the host, is linked to host range, as the virus must be able to infect and replicate in host cells before it can cause disease, and this requires the viral and host factors mentioned above. Second, for an influenza virus to be considered pathogenic, infection of the host must have harmful consequences such as clinical illness and possible lethality. At the cellular and tissue level, pathogenic viruses can induce cell death and cytokine production. Virulence is a measure of disease severity and can range from mild to severe. Clinical symptoms and the pathological consequences depend on both the influenza virus strain and the infected host.

Disease caused by influenza viruses may be relatively mild, such as that observed with seasonal human influenza viruses, or severe, like that observed with HPAI or 1918 influenza viruses. A number of factors determine influenza virus virulence, including the amount of virus in the inoculum, in the tissues following viral replication, as well as, the kinetics of viral replication. The tissue tropism of the virus, such as infection limited to the lung or spread to extrapulmonary sites, such as the brain, also affects virulence. Finally, the ability of the host to clear the virus, which relies upon the host innate and adaptive immune response, is also a determinant of virulence.

Several specific viral and host factors are determinants of influenza virus virulence. Viral factors include the multibasic cleavage site in the HA, residues 627 and 701 of the PB2 protein, NA stalk length, and gene constellation [reviewed in 1 and 13,20]. Host factors that affect virulence are SA receptor distribution, interacting proteins such as heat shock proteins (HSPs) and host cell polymerases, and the innate and adaptive immune systems. An interaction between the influenza virus PB2 protein and mitochondrial antiviral signaling protein (MAVS) may play a role in influenza virus virulence [21]. Animal models have been used to study each of these determinants, as well as, other viral and host factors that determine host range and pathogenicity and have led to a better understanding of these aspects of influenza virus infection.

3. Influenza host range

3.1 Mice: influenza host range

Mice are among the most commonly used mammalian models for evaluating influenza infection (Table 3).

3.1.1 Receptor specificity—Commonly used laboratory strains of mice express both SAα2,6 and SAα2,3 receptors in their respiratory tract, making them susceptible to both human and avian influenza viruses. However, the specific distribution of these receptors in mice is still being studied and may vary by mouse strain. For example, a recent study reported expression of both SAα2,6 and SAα2,3 receptors in multiple organs of BALB/c mice, including the trachea, lungs, cerebellum, spleen, liver, and kidney, while studies with C57BL/6J mice reported a lack of SAα2,6 expression in the lungs, but SAα2,3 receptors were demonstrated in ciliated airway and type II alveolar epithelial cells [22,23]. Genetic variations that exist between mouse strains in the many host genes involved in viral replication, and the innate and adaptive immune response, can greatly influence the outcome of the disease [24].

Though it appears likely that commonly used laboratory strains of mice express both receptors, a predominance of SAα2,3 receptors may explain why human influenza A virus subtypes (H1, H2, and H3) generally must be adapted to mice before they replicate efficiently, while several LPAI and HPAI viruses, such as H5N1, H2, H6, and H7 viruses, can infect mice without adaptation [1,14,15,25–27]. Some highly virulent human influenza viruses, such as the reconstructed 1918 influenza virus, can also infect mice without adaptation [28,29]. However, considering that most human influenza viruses are unable to infect mice without prior adaptation despite the presence of SAα2,6 receptors, one can infer that receptor distribution alone does not determine infectivity. A summary of influenza specific host factors that have been identified in mice, as well as different animal models, is presented in Table 4.

3.1.2 Mx1 protein expression—Mx1 belongs to a family of interferon-induced proteins that selectively inhibit influenza virus replication by interfering with mRNA synthesis [30]. The Mx1 protein, a nuclear protein in mice, is both necessary and sufficient to protect mice from influenza virus infection. While the Mx proteins were initially discovered in an inbred mouse strain (A2G) that exhibited a high degree of resistance to influenza A virus infection, most inbred strains of mice carry defective Mx1 alleles and are highly susceptible to mouse-adapted influenza viruses [31]. The human homolog, MxA, is a cytoplasmic protein that inhibits influenza replication at a later step than murine Mx1 [30]. However, unlike murine Mx1, MxA alone cannot prevent influenza virus infection; a functional immune system is necessary for protection in humans [30].

3.1.3 Mouse adaptation mutations—In addition to the host factors mentioned above, viral factors also play an important role in influenza host rang in mice. Human influenza viruses can be mouse adapted through serial passage of the virus in the lungs [reviewed in 1 and 2]. This results in mutations in several influenza gene segments. Analysis of the mutations has been beneficial for identifying viral proteins and specific residues within the proteins that may be crucial for influenza viruses to overcome host range restriction and cross the species barrier to infect new hosts efficiently. Mutations have been identified in the influenza HA, PB1, PB2, NA, and M1 gene segments (Table 5) [32]. A correlation was reported between the replicative capacity and virulence of mouse-adapted viruses; mutant viruses with increased viral yield in vivo showed enhanced virulence and induced severe pathology [32]. Mutations in the HA, NA, and M1 proteins increased viral yield, while combinations of PB1 and PB2 mutations restricted host range and changed the virus to a mouse specific strain [32]. Further studies have identified mutations in the HA gene of mouse adapted H3N2 viruses that clustered within two distinct regions in the HA1 and HA2 subunits. Some of these mutations (at amino acids 162, 210, and 218 in HA1 and 154 in HA2) enhanced replication and virulence in mice [33]. The HA mutations may increase its affinity for SAα2,3 receptors to adapt to the murine host [reviewed in 1 and 2].

Comparison of the H7N7 virus SC35, which exhibits low virulence in mice, with its mouse-adapted variant SC35M, which is highly virulent in mice, led to the identification of 7 amino acid differences in the polymerase and NP genes [34]. Some mutations resulted in a moderate (PB2 701N and 714R, PA 615N, NP 319K) or dramatic (PB1 13P and 678N) enhancement of polymerase activity, while the PB2 333I mutation actually suppressed polymerase activity [34]. These polymerase-enhancing mutations contribute to the increased virulence observed in mice infected with SC35M. Similar polymerase mutations were found in H5N1 HPAI and human isolates, possibly demonstrating convergent evolution of SC35M-like mutations in mammalian and HPAI H5N1 strains [34]. The observations from this study support the idea that mutations in the polymerase complex, and enhanced activity of viral polymerase are critical for influenza virus adaptation to a new host, such as HPAI virus adaptation to mammalian hosts [34].

3.2 Ferrets: influenza host range

Since Smith and colleagues initially isolated an influenza A virus from a ferret in 1933 [35], ferrets have been considered an ideal animal model for influenza research (Table 3). A summary of influenza specific host factors that have been identified in ferrets is presented in Table 4.

3.2.1 Receptor specificity—Ferrets are susceptible to many human and avian influenza viruses. Ferrets share similarities to humans in clinical signs of illness, lung physiology, airway morphology, and cell types present in the respiratory tract [36]. The distribution of SAα2,6 and SAα2,3 receptors in the ferret respiratory tract was reported to resemble that of humans, with SAα2,6 receptors in the upper respiratory tract extending into the lower respiratory tract as far as the bronchioles and SAα2,3 receptors in the lower respiratory tract distal to the respiratory bronchioles [36]. However, in a recent study, a predominance of SAα2,6 receptors was reported throughout the respiratory tract of ferrets, including the lower respiratory tract [37]. Replication and attachment of human H3N2 viruses occur primarily in the upper respiratory tract, while attachment and infection with AIV generally occurs in the lower respiratory tract [14،15،38،39]. Human influenza viruses attach to the surface of ciliated epithelial cells in the airways and to type I pneumocytes in the alveoli of ferrets and primarily cause upper respiratory tract disease and occasionally bronchitis and pneumonia [14،15،36]. AIV attachment to type II pneumocytes in the alveoli and to the bronchioles may explain why AIV disease is more severe in the lungs while the bronchi and trachea are less affected [14،15،36].

Receptor specificity plays a critical role in influenza virus transmission, which is one the primary reasons why ferrets are often used for studying influenza virus transmission [40]. Studies suggest a general correlation between influenza virus transmission and SAα2,6 binding affinity [40]. Human influenza viruses, which preferentially bind SAα2,6 receptors, are transmitted efficiently between ferrets via direct contact or respiratory droplets, while AIV isolates, including H5N1, which bind SAα2,3 receptors, do not transmit efficiently in ferrets [40]. Additionally, 1918 influenza virus variants with SAα2,3 or mixed SAα2,3/SAα2,6 receptor specificity are less transmissible than wild-type 1918 influenza virus, which has SAα2,6 specificity [40]. This may explain why AIV do not transmit efficiently between humans. However, it should be noted that H9N2 viruses with a leucine residue at amino acid position 226 in the HA RBS, which is responsible for human virus-like receptor specificity, can transmit among ferrets by direct contact but not by aerosol transmission [41].

Five amino acid changes were identified in a reassortant viruses carrying the H9N2 surface proteins on a human H3N2 backbone that were required for aerosol transmission, suggesting that only a few specific changes are needed for currently circulating H9N2 AIV, and

possibly other AIV, to become transmissible. Three amino acid changes were found in the surface proteins (HA and NA) while 2 were found in the internal proteins (PB2 and M1) [41]. There is significant concern that prolonged cocirculation of H5N1 viruses and human influenza viruses will generate reassortant H5N1 viruses with the ability to transmit efficiently between humans.

3.2.2 Immune response—Interestingly, a lower induction of lymphocytes was observed in nasal washes as natural transmission proceeded in ferrets compared to the initial direct intranasal infection [42]. Cytotoxic T cells clear the virus from the respiratory tract of ferrets and influenza-induced suppression of cell-mediated immunity is well documented [42]. Diminished lymphocyte induction and transient impairment of cellular immunity have both been associated with influenza infection [42]. If a reduction in the T-cell response to influenza infection is observed after transmission of the virus, the immune response to influenza infection could be significantly impaired, allowing for more efficient infection.

3.3. Non-human primates: influenza host range

Although other animal models are more inexpensive and may be more convenient to use, none are as close to humans in physiology and genome sequence as non-human primates (Table 3). Non-human primates are being investigated as an animal model for influenza since disease in these animals may more closely reflect that seen in humans than small animals. However, from a practical standpoint, this animal model is not as frequently used because of their high cost and difficulty working with a restricted species. Various species of non-human primates, including both Old World (Rhesus and cynomolgus macaques) and New World monkeys (squirrel and cebus), have been used to evaluate a variety of human and avian influenza viruses [reviewed in 39].

3.3.1 Receptor specificity—Unlike ferrets, the pattern of viral attachment in non-human primates differs significantly from that in the human respiratory tract; viral attachment is not observed in the trachea, bronchus, or bronchioles of cynomolgus macaques after H3N2 virus infection (Table 3) [36]. This may explain why cynomolgus macaques do not display clinical signs of disease following infection with human H3N2 viruses. The reduced susceptibility of non-human primates to seasonal human influenza viruses may, in part, be due to differences in receptor distribution between non-human primates and humans.

4. Influenza virulence

4.1 Mice: influenza virulence

Though many influenza viruses do not replicate in mice, there are a number of influenza viruses that do, and these viruses exhibit varying levels of virulence in mice (Table 6). The parameter most commonly used for measuring influenza virus disease (Table 3) in mice is weight loss (a 20–25% loss in body weight generally requires euthanasia). Oxygen saturation (SaO2) in the blood is used to measure viral replication, with a correlation between a decrease in SaO2 with increased viral replication [43]. Measuring viral titers in the respiratory tissues is critical since even modest reductions in virus load can prevent lethality.

4.1.1 Mild/moderate virulence in mice—Influenza viruses such as the 2009 pH1N1, H9N2, and some seasonal influenza viruses exhibit mild to moderate virulence, with viral replication generally restricted to the respiratory tract. These viruses are generally not lethal for mice [29,38]. Studies in mice with the 2009 pH1N1 virus have identified the importance of internal viral proteins in influenza pathogenesis. A mutation in the PB2 protein (T271A), which has previously been shown to enhance polymerase activity in human cells, enhances

viral replication in the mouse lung. This mutation is present in the avian-derived PB2 protein of the 2009 pH1N1 virus and may be associated with high polymerase activity [44]. The 2009 pH1N1 virus possesses a glutamic acid at position 627 in the PB2 protein (627E) [44] though a lysine at this site is associated with influenza host range, virulence and tissue distribution in mice [45,46]. Nearly all AIV encode glutamic acid at this position, while human influenza viruses encode lysine, and the E627K substitution is associated with the ability of AIV to replicate efficiently in mammalian cells [45]. Some HPAI viruses, including H5N1 and H7N7 viruses isolated from fatal cases in humans, have a lysine residue at this position [reviewed in 1,46]. The presence of glutamic acid at position 627 of the 2009 pH1N1 PB2 protein may explain why this virus only exhibits mild virulence in mice. Interestingly, however, substitution of E to K at this residue in the 2009 pH1N1 virus did not confer higher virulence and replication efficiency in mice, suggesting 627K is not required for adaptation of the swine origin 2009 pH1N1 virus to mammals [44].

4.1.2 Severe virulence in mice

4.1.2.1 HPAI and 1918 H1N1 influenza viruses: Influenza viruses that cause severe disease in mice without prior adaptation include HPAI and 1918 human influenza viruses (Table 6) [reviewed in 1,39]. These viruses spread to extrapulmonary tissues and infection can result in high mortality [1,28,38,47]. Lethality of H5N1 viruses in BALB/c mice has been attributed to their ability to spread to multiple organs, including the brain, while infection with non-lethal H5N1 viruses is limited to the respiratory tract [48–50]. These studies demonstrated that some influenza A viruses can replicate systemically in a mammalian species and can be neurotropic without prior adaptation. Dissemination of these viruses to extrapulmonary organs may depend on viremia that occurs early during infection, with more pathogenic viruses replicating more efficiently in other organs [48]. Infection is characterized by a sustained inflammatory response and an impaired adaptive immune response that results in an inability to contain the infection at the primary site of replication. Mutations in the polymerase genes of highly pathogenic viruses, such as the E627K mutation of the PB2 protein and the N66S mutation of the PB1-F2 protein, have been associated with an impaired adaptive immune response and increased host cell apoptosis, which may contribute to enhanced viral replication and extrapulmonary spread (Table 5) [51].

Infection with HPAI or 1918 influenza viruses is characterized by severe lung pathology, including pulmonary edema and extensive inflammatory infiltrates [47,52]. Infection with these viruses results in significantly larger numbers of macrophages and neutrophils in the lungs compared to infection with low pathogenicity viruses, suggesting these cell types play a role in acute lung inflammation. Though infection with H5N1 and 1918 viruses show many similarities in lung cellularity, composition of immune cell sub-populations, and their temporal dynamics, H5N1 viruses induce significantly higher levels of pro-inflammatory cytokines than the 1918 virus [52].

Some HPAI H5N1 viruses are lethal for mice while others are not. Mice infected with lethal H5N1 virus strains exhibit severe lymphopenia compared to non-lethal H5N1 viruses, which display only a transient lymphopenia (Table 6) [49,53]. Infection with lethal H5N1 strains is associated with a reduction in the number of CD4+ and CD8+ T cells and reduced synthesis of the cytokines interleukin-1β and gamma interferon and the chemokine macrophage inflammatory protein in lung and lymphoid tissues. In contrast, chemokine and cytokine levels in the brains of infected mice increased, and evidence of apoptosis in the spleen and lungs of infected mice suggests a mechanism for lymphocyte destruction. The destructive effects on the immune system may contribute to H5N1 virulence in mammalian hosts [50,53].

Mutations in viral gene segments, such as PB2, PB1-F2, and HA, may contribute to the enhanced virulence of H5N1 viruses (Table 5). Mutations have been identified at positions 701 and 627 of the PB2 protein. The acquisition of an Asp-to-Asn mutation at position 701 of the PB2 protein was associated with the ability of H5N1 AIV to replicate efficiently and cause lethal infection in mice [54]. HPAI viruses also possess the E627K mutation in the PB2 protein, which was mentioned earlier to be a characteristic of highly pathogenic influenza viruses. These mutations suggest the PB2 protein is important for both virulence in mice (residue 627) and host range (residue 701) of HPAI viruses [46،54]. However, some H5N1 viruses that do not contain this mutation are still highly virulent in mice, suggesting that other mechanisms contribute to virulence [49]. The precise mechanism by which the E627K substitution contributes to virulence is not known, but it may influence the replication efficiency of H5N1 viruses in murine cells, resulting in a more widespread infection with prolonged neutrophil infiltration in the mouse lung [49].

Studies suggest that cold sensitivity in AIV is mainly determined by residue 627 of the PB2 protein, resulting in a reduced ability of the AIV polymerase complex of to replicate the viral genome at 33°C, the temperature of the upper respiratory tract, and this could contribute to the inability of AIV to grow efficiently in humans [55،56]. Viruses possessing lysine at position 627 of the PB2 protein replicate efficiently in both the lungs and nasal turbinates, as well as in cells at 33°C, while viruses possessing glutamic acid at this position replicate less well in nasal turbinates than in lungs, and less well in cells at 33°C. This suggests that lysine at PB2-627 permits efficient growth in the upper and lower respiratory tract of mammals [55]. An inhibitory activity or factor in human cells may restrict influenza polymerase function containing an avian-like PB2, preventing assembly of polymerase into the RNP complex, blocking viral replication and inhibiting cross-species transmission. The E627K mutation of the PB2 protein may overcome this inhibitory activity and promote viral replication [57]. Position 627 in the PB2 protein may be a key regulator of species-specific polymerase activity.

While the PB1-F2 protein is truncated in seasonal human H1N1 viruses, it is functional in the 1918 influenza virus and may contribute to virulence by functioning as a proapoptotic protein [51]. A point mutation at amino acid 66 of the PB1-F2 protein is associated with the virulence of the 1918 influenza virus and with the accompanying cytokine dysregulation (Table 5) [51]. Interestingly, the 2009 pH1N1 virus does not encode a PB1-F2 protein [58]. Mutant 2009 pH1N1 viruses engineered to express a PB1-F2 protein with either an asparagine or serine at position 66 showed minimal difference in virulence, though they were associated with altered levels of proinflammatory cytokines [58].

The HA gene has also been linked to the virulence of H5N1 and 1918 viruses [28،59]. Viruses bearing wild-type and mutant 1918 HA genes with differing receptor specificities (SAα2,3, SAα2,6, or both) all caused lethality in mice and exhibited similar pathology and cellular tropism [59]. The HA contains virulence determinants independent of receptor binding specificity. One such determinant is a motif of basic amino acids that results in a highly cleavable HA, which causes lethal infection in poultry and mice [46]. The HA precursor protein (HA0) is cleaved into 2 subunits (HA1 and HA2) by host cell proteases; HA cleavage is required for fusion of the viral and endosomal membranes and for viral infectivity [reviewed in 13]. LPAI viruses possess a single Arg residue at the cleavage site, recognized by extracellular, trypsin-like proteases, whose expression is limited to cells of the respiratory and intestinal tract of humans and birds, respectively, limiting infection to these regions [reviewed in 13]. By contrast, HPAI viruses possess multiple basic amino acids at the cleavage site recognized by ubiquitous intracellular subtilisin-like proteases that can cleave the HA in extrapulmonary sites, thereby altering the tissue specificity, triggering systemic infection [reviewed in 13]. While HA cleavability is a major determinant of tissue

tropism of AIV, the multibasic amino acid motif in the HA connecting peptide does not affect the cell tropism of the virus but rather its replicative ability in mice.

The NA is a tetramer with a box like head bearing an enzymatic site, on a stalk, which varies in sequence and length [reviewed in 13]. Typically, viruses with a shortened NA stalk are less efficiently released presumably because the enzyme active site in the head cannot efficiently access its substrate [reviewed in 1 and 13]. However, shorter NA stalk length, characterized by a deletion in the NA stalk, is widespread in terrestrial poultry, with subtype H5 and H7 influenza viruses selecting for these shorter stalk lengths [reviewed in 1 and 13,20]. AIV with shortened stalks were more virulent in poultry and mice compared to those with long NA stalks (Table 6) [20]. A correlation has been observed between NA stalk length and HA glycosylation. Among viruses with a short NA stalk, viruses with fewer HA glycosylation sites were more virulent in mice than viruses with a highly glycosylated HA [20]. NA stalk length does not appear to affect infectivity or efficiency of viral replication. If the findings in mice reflect virulence in humans, the short-stalk NA of H5N1 viruses may contribute to virulence in humans.

4.1.2.2 Mouse adapted human influenza viruses: While seasonal human influenza viruses are generally unable to replicate efficiently in mice, serial passage in mice results in increased virulence and severe disease that resembles HPAI and 1918 influenza virus infection. Historically, H1N1 influenza viruses such as Puerto Rico/8/34, WSN/33, and FM/1/47 have been used in mice since all are well adapted to mice [32]. Several of these viruses were passaged extensively in mice and gained mutations that contribute to their enhanced virulence, including systemic spread and neurotropism. H3N2 viruses adapted to BALB/c mice cause enhanced pulmonary lesions and systemic virus infection [60]. Thus, extrapulmonary spread of the virus resulted from adaptation with enhanced replication. Mutations in the HA (G218E) and NS1 (D125G) proteins were associated with enhanced virulence of H3N2 viruses (Table 5) [60].

4.1.2.3 Other highly virulent influenza viruses: Infection with specific H7, H6, and H2 subtype viruses can cause morbidity and mortality in mice (Table 6) [25–27]. Like H5N1 and 1918 influenza viruses, highly pathogenic H7 viruses can spread to extrapulmonary sites, such as the brain and spleen, and cause lethality in mice [25]. Some highly virulent H7 viruses lack the multibasic cleavage site usually associated with high pathogenicity, suggesting there are other determinants of this phenotype [25]. H6 and H2 subtype viruses cause varying levels of morbidity and mortality with some causing severe disease in mice [26,27]. However, extrapulmonary spread to the brain was not associated with lethal infection with H6 and H2 subtype viruses [26,27].

4.2 Ferrets: influenza virulence

Many influenza viruses, including some seasonal human influenza viruses and certain H3, H6, and H7 subtype AIV replicate in the upper respiratory tract of ferrets without signs of disease [reviewed in 39]. Influenza viruses that can replicate and cause disease in ferrets display varying levels of virulence (Table 6) [39,61,62].

4.2.1 Mild/moderate virulence in ferrets—Virulence varies between subtypes and between viruses of the same subtype. For example, ferrets infected with different H5N1 isolates displayed either mild or severe disease associated with systemic spread and death [61].

Non-adapted H1N1, H2N2, and H3N2 seasonal human influenza viruses generally cause mild to moderate disease in ferrets. As in humans, replication of these viruses is primarily

restricted to the upper respiratory tract [reviewed in 39]. Infected ferrets display signs of clinical illness that are quite similar to those observed in humans, including lethargy, nasal discharge, sneezing, fever, and weight loss [39]. The severity of these clinical signs depends on the specific strain of influenza virus [61–63].

Influenza strains that cause mild disease in ferrets are associated with mild inflammatory changes in the lungs. These viruses induce rapid expression of type I and II interferons and TNFα to high levels, and infection is associated with a strong and rapid upregulation of IL-8 in ferrets [64]. In humans infected with influenza, IL-8 secretion has been mainly detected at later stages of disease and in temporal association with infiltration of the respiratory mucosa. This suggests that the IL-8 induction observed in ferrets may indicate rapid, granulocyte-mediated virus clearance and control of infection in the upper respiratory tract [64].

4.2.2 2009 pH1N1 influenza virus—Studies with 2009 pH1N1 influenza virus in ferrets have yielded mixed results. While some investigators observed mild, non-lethal disease, others reported more severe disease with up to 50% lethality [62,63,65]. Although the strains are genetically closely related, the passage history of the virus, dose and volume of inoculum, and age of ferrets differed. The 2009 pH1N1 influenza virus is more pathogenic than seasonal human influenza viruses in ferrets; with greater morbidity, more extensive viral replication in both the upper and lower respiratory tract, and replication in the intestinal tract (Table 6). While replication of seasonal influenza viruses is confined to the upper respiratory tract, the 2009 pH1N1 influenza virus also replicates in the trachea, bronchi, and bronchioles. With the exception of the intestine, the 2009 pH1N1 virus does not spread to extrapulmonary tissues and disease is not as severe as that seen with 1918 and HPAI viruses [62,63,66]. Pneumonia caused by the 2009 pH1N1 influenza virus is intermediate in severity between that caused by seasonal H1N1 viruses and that caused by HPAI H5N1 viruses. The greater severity compared to seasonal human influenza viruses is likely due to the ability of 2009 pH1N1 virus to replicate well in the lower respiratory tract of ferrets [67].

Ferrets infected with the 2009 pH1N1 influenza virus display mild to moderate clinical signs, and the virus is transmitted efficiently between ferrets via aerosol or respiratory droplets [63]. The host immune response to 2009 pH1N1 viral infection occurs in two phases [68]. In the innate phase of the immune response, there is a robust expression of chemokines (CCL2, CCL8, CXCL7, CXCL10), cytokines (TNFα and IL-1β), and interferon-stimulated genes (ISGs), which correlate with lung pathology [68]. In the second phase, these genes are shut off, and the adaptive immune response is initiated [68]. The timing of this switch, which appears to occur at day 7 post-infection, is important for viral clearance, and a delay or failure to switch to the adaptive immune phase may contribute to higher morbidity and more severe disease in 2009 pH1N1 infection [68]. CXCL10 and CCL2 levels correlate with the severity of respiratory illness in severe cases of H5N1 infection in humans [68].

4.2.3 Severe virulence in ferrets

4.2.3.1 HPAI and 1918 H1N1 influenza viruses: Severe influenza disease in ferrets is characterized by severe systemic pathology, rapid progression of the disease, and lethality [reviewed in 39,69]. As in mice, the 1918 influenza virus and many HPAI viruses cause severe disease in ferrets (Table 6) [reviewed in 1 and 39,69]. Ferrets infected with highly pathogenic viruses suffer from more severe symptoms and pneumonitis than those seen with seasonal influenza that can be lethal [reviewed in 39]. Influenza viruses that cause severe disease in ferrets replicate in both the upper and lower respiratory tract, leading to severe pulmonary damage [reviewed in 1 and 39,69]. This may indicate a correlation between the ability of highly pathogenic viruses to infect the lower respiratory tract and their virulence.

Severe disease is associated with extrapulmonary spread [reviewed in 1 and 39،69]. HPAI H5N1 viruses can infect the brain, spleen, and intestine, while 1918 influenza virus has been isolated from the heart and spleen of ferrets, but not the brain [1،39،69،70]. The data suggest HPAI H5N1 viruses are neurotropic in ferrets and mice [49،61].

Pathological changes and different cytokine responses are observed in ferrets infected with influenza viruses that cause severe disease [64]. The induction of type I and type II IFNs is delayed and not as strong with viruses that cause mild disease [64]. This delay in the immune response might be sufficient to enable the virus to spread to the lower respiratory tract, resulting in more severe disease. In addition, IL-6, which may be an indicator of severe disease in humans, was strongly induced only by the more virulent strains [64]. These findings suggest that more virulent strains may interfere more efficiently with the host response at early stages of disease.

H5N1 viruses isolated from 2004 onwards have displayed varying levels of virulence ranging from mild, non-lethal disease to severe disease associated with extreme lethargy, diarrhea, severe neurological impairment, and death [61]. When H5N1 viruses that caused mild and severe disease in ferrets were compared, amino acid differences were identified in the coding region of multiple genes, including the HA. Among six amino acid differences observed between the HA proteins, one (K250R) that was located at the globular head of the HA may affect viral attachment (Table 6) [61]. A lysine at position 627 of the PB2 protein was not essential for lethality and virulence in ferrets, as some viral isolates possessing glutamic acid at this position were still highly virulent [49،61]. This suggests that other amino acid differences may contribute to virulence and compensate for the lack of lysine at PB2 627.

Multiple residues and motifs in the NS1 protein of H5N1 viruses may play a critical role in influenza virus pathogenesis in ferrets [61]. Pathogenesis depends partly on the ability of a virus to evade or suppress the host immune response. The NS1 protein plays a central role in this process by counteracting the cellular IFN response by binding to double-stranded RNA, suppressing the activation of double-stranded RNA-activated protein kinase, a stimulator of type I IFN, and by preventing the activation of transcription factors such as ATF-2/c-Jun, NFκB, and IRF-3/5/7, all of which stimulate IFN production [reviewed in 13]. These functions of the NS1 protein may be altered in highly virulent H5N1 viruses, with suppression of the immune response resulting in more severe disease.

4.3 Non-human primates: influenza virulence

4.3.1 Seasonal human influenza viruses—Non-human primates are generally less susceptible to human influenza viruses than humans [reviewed in 39], virus replication may be detected without evidence of disease. The ability to cause disease varies based on the species, the route of inoculation, and the influenza virus strain. In general, high doses of virus are administered by multiple routes to ensure infection. For example, Rhesus macaques and cynomolgus macaques infected with H1N1 viruses only showed clinical signs of infection when infected intratracheally and intranasally [reviewed in 39]. Cynomolgus macaques infected intratracheally with an H3N2 virus did not show clinical signs of infection, despite the recovery of virus from nasal swabs and lung lavage [71]. In contrast, seasonal H1N1 virus infection of pigtailed macaques resulted in clinical signs of illness, including weight loss, nasal discharge, fever, associated with pathological changes in the lung similar to those seen in humans [72]. Non-human primates can also develop specific disease symptoms that are not observed in other animal models such as conjunctivitis and rhinorrhea following infection with mildly pathogenic viruses [73].

4.3.2 1918 H1N1 influenza virus—Other influenza virus strains, such as HPAI, 1918 and 2009 pH1N1 viruses replicate and cause mild to severe disease in non-human primates. The 1918 virus is virulent in cynomolgus macaques, and causes acute respiratory distress and lethality (Table 6) [reviewed in 39,74]. The 1918 virus displays replicative capacity and tissue tropism that differs from contemporary human isolates. In non-human primates, the 1918 virus is detected up to 8 days after infection in tissues of the upper and lower respiratory tract, while seasonal strains are restricted to the upper respiratory tract and are generally cleared by day 6 [71]. Non-human primates suffer from similar clinical symptoms as humans, but the mortality is very high, with 50% to 75% lethality within 2 weeks compared to the 1–3% case fatality rate reported in the 1918 pandemic [74]. This high mortality in monkeys may be due to the use of a high dose of 1918 virus administered by the intranasal and intratracheal route.

The ability of influenza viruses to evade the host immune response is important for both host range and pathogenicity. Macaques infected with the 1918 influenza virus mount an atypical immune response characterized by dysregulation of the antiviral response. They showed relative constancy in gene expression profiles and activation of immune-response-related genes compared to the more dynamic response in those infected with seasonal human influenza viruses [74]. Macaques infected with the 1918 influenza virus specifically showed a significant increase in IL-6, a delay in the activation of several key cytokine genes, including IL-8 and CXCL11, reduced induction of IFN-alpha genes, and differential activation of type-I-IFN-stimulated gene expression [74]. The influenza virus NS1 protein, which modulates the IFN-mediated antiviral response, may contribute to the altered immune response in 1918 influenza virus infected primates by interfering with its ability to protect the host from infection and could be a critical determinant of the severity of 1918 virus-induced disease in non-human primates [74].

4.3.3 HPAI H5N1 influenza virus—Cynomolgus and Rhesus macaques have also been used as models for HPAI H5N1 pathogenesis [75–77]. These monkeys exhibit systemic symptoms similar to those experienced by humans, including fever, lethargy, and loss of appetite, but respiratory signs and symptoms are limited (Table 3) [75,76]. Pneumocytes, particularly type I pneumocytes in the alveoli rather than type II pneumocytes and macrophages of the lower airway, but not the ciliary epithelium of the trachea and bronchi, were the primary target cells in the lungs of infected animals [75–77]. Infection in the lungs of macaques commonly results in a necrotizing bronchial pneumonia, suggesting this model may be helpful for understanding the mechanisms responsible for severe influenza pneumonia in humans [75,76]. Infected cells were not seen in the spleen, heart, or brain, suggesting that replication is largely limited to the respiratory tract [75,76]. Failure of H5N1 viruses to attach to the ciliary epithelium of the trachea and bronchi may limit the transmissibility of the H5N1 virus in these animals [77]. Systemic replication is a feature of HPAI H5N1 infection in mice and ferrets, but not macaques, where multiple-organ dysfunction was attributed to diffuse alveolar damage rather than systemic virus replication [49]. Certain LPAI viruses, including subtype H2 avian influenza viruses, can also replicate in non-human primates, however, unlike HPAI viruses, they do not cause disease (Table 6) [78].

Studies in squirrel monkeys initially identified the 627 residue in the PB2 protein as a determinant of host range [45]. Transfer of internal protein genes of an AIV into a human influenza background attenuated the human influenza virus [79]. Single gene reassortant viruses were used to determine which genes contributed to attenuation and host range restriction. Single gene reassortants with avian NP, PB2, or M genes were attenuated [79]. Only two amino acid differences in the M1 protein and one difference in the M2 protein contributed to the attenuation phenotype [79]. Single gene reassortants containing an avian

NS gene segment of allele B were significantly restricted in growth in the respiratory tract of squirrel monkeys while reassortants with an allele A NS gene segment were not [80].

4.3.4 2009 pH1N1 influenza virus—The p2009 H1N1 virus replicates efficiently in the lungs and other respiratory organs of infected non-human primates [65] but the pattern differs from seasonal human influenza viruses, which are typically limited in their ability to infect the lungs of primates [65]. The 2009 pH1N1 virus caused more severe histopathologic lesions in the lungs than seasonal influenza (Table 6) [74,75]. The enhanced severity of 2009 pH1N1 infection could be due to more severe lung lesions. The most upregulated genes in lung samples with histologic lesions belonged to the innate immune response and proinflammatory pathways. 2009 pH1N1 viral antigen was found in both type I and type II pneumocytes, while seasonal influenza virus is restricted to type I pneumocytes [65]. This may contribute to the severe lung lesions seen in this model. However, mortality was not reported in non-human primates infected with 2009 pH1N1 influenza virus, suggesting that disease is still not as severe as that seen with 1918 or HPAI viruses [65]

5. Other animal models

While mice, ferrets, and nonhuman primates have been the focus of this review, they are not the only important animal models for studying influenza infection. Guinea pigs have recently been re-established as a model for influenza transmission [81]. Due to their low cost and reduced size compared to ferrets, guinea pigs represent a suitable alternative host for transmission studies [reviewed in 39,81]. As animals such as domestic poultry (chickens, quail) and pigs are natural or intermediate hosts between wild-birds and humans, in which reassortment and host adaptation may occur [reviewed in 13], their use as animal models may allow better understanding of the molecular mechanisms behind host adaptation and influenza virus transmission to humans. In chickens, AIV exhibit a reduced specificity for SAα2,3 receptors, which may explain how AIV are transmitted to humans who predominantly express SAα2,6 receptors [82]. More recently, cats and dogs have been used as models for influenza infection. Naturally occurring H5N1 infection in cats have been reported following exposure to infected raw poultry or wild birds, with H5N1 transmission occurring between cats [reviewed in 83]. Clinical influenza with sustained transmission in dogs was seen after cross-species transmission of equine H3N8 influenza viruses in Florida in 2004, as well as, natural infection with H3N2 viruses in South Korea [8, reviewed in 83]. Though dogs are susceptible to experimental influenza infection, there is no evidence that dogs transmit the virus to other animals. However, the continued exposure of humans to cats and dogs makes transmissibility studies in these animals of interest.

6. Conclusion

In this review, we have discussed the contributions animal models have made in the study of influenza host range and pathogenesis. There is no single ideal animal model for studying influenza virus infection; each model has advantages and limitations. The phenotypes of host range and pathogenicity are multigenic and host species, influenza virus subtype, and influenza virus strain all have a significant impact on influenza virus replication and disease. These limitations must be carefully considered when selecting an animal model for study. Despite these caveats, animal models have been instrumental in identifying determinants of influenza host range and pathogenicity, and the continued use of animal models will be crucial for the study of influenza viruses in the future.

Acknowledgments

The authors' research was supported by the Intramural Research Program of the NIAID/NIH.

References

1. Belser JA, Szretter KJ, Katz JM, Tumpey TM. Use of animal models to understand the pandemic potential of highly pathogenic avian influenza viruses. Adv Virus Res. 2009; 73:55–97. [PubMed: 19695381]
2. Barnard DL. Animal models for the study of influenza pathogenesis and therapy. Antiviral Res. 2009; 82:A110–22. [PubMed: 19176218]
3. Chen W, Calvo PA, Malide D, Gibbs J, Schubert U, Bacik I, Basta S, O'Neill R, Schickli J, Palese P, Henklein P, Bennink JR, Yewdell JW. A novel influenza A virus mitochondrial protein that induces cell death. Nat Med. 2001; 7:1306–1312. [PubMed: 11726970]
4. Centers for Disease Control and Prevention. Prevention and control of influenza with vaccines-recommendations of the Advisory Committee on Immunization Practices (ACIP), (2010). MMWR. 2010; 59:RR08:1–62.
5. Beare AS, Webster RG. Replication of avian influenza viruses in humans. Arch Virol. 1991; 119:37–42. [PubMed: 1863223]
6. Kida H, Yanagawa R, Matsuoka Y. Duck influenza lacking evidence of disease signs and immune response. Infect Immun. 1980; 30:547–553. [PubMed: 7439994]
7. Subbarao K, Klimov A, Katz J, Regnery H, Lim W, Hall H, Perdue M, Swayne D, Bender C, Huang J, et al. Characterization of an avian infuenza A (H5N1) virus isolated from a child with a fatal respiratory illness. Science. 1998; 279:393–396. [PubMed: 9430591]
8. Crawford PC, Dubovi EJ, Castleman WL, Stephenson I, Gibbs EP, Chen L, Smith C, Hill RC, Ferro P, Pompey J, Bright RA, Medina MJ, Johnson CM, Olsen CW, Cox NJ, Klimov AI, Katz JM, Donis RO. Transmission of equine influenza virus to dogs. Science. 2005; 310:482–485. [PubMed: 16186182]
9. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. J Virol. 1989; 63:4603–4608. [PubMed: 2795713]
10. Garten RJ, Davis CT, Russell CA, Shu B, Lindstrom S, Balish A, Sessions WM, Xu X, Skepner E, Deyde V, Okomo-Adhiambo M, Gubareva L, Barnes J, Smith CB, Emery SL, Hillman MJ, Rivailler P, Smagala J, de Graaf M, Burke DF, Fouchier RA, Pappas C, Alpuche-Aranda CM, López-Gatell H, Olivera H, Lopez I, Myers CA, Faix D, Blair PJ, Yu C, Keene KM, Dotson PD Jr, Boxrud D, Sambol AR, Abid SH, St George K, Bannerman T, Moore AL, Stringer DJ, Blevins P, Demmler-Harrison GJ, Ginsberg M, Kriner P, Waterman S, Smole S, Guevara HF, Belongia EA, Clark PA, Beatrice ST, Donis R, Katz J, Finelli L, Bridges CB, Shaw M, Jernigan DB, Uyeki TM, Smith DJ, Klimov AI, Cox NJ. Antigenic and genetic characteristics of swine-origin 2009 A(H1N1) influenza viruses circulating in humans. Science. 2009; 325:197–201. [PubMed: 19465683]
11. Matrosovich MN, Matrosovich TY, Gray T, Roberts NA, Klenk HD. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. Proc Natl Acad Sci U S A. 2004; 101:4620–4624. [PubMed: 15070767]
12. Rogers GN, Paulson JC, Daniels RS, Skehel JJ, Wilson IA, Wiley DC. Single amino acid substitutions in influenza haemagglutinin change receptor binding specificity. Nature. 1983; 304:76–78. [PubMed: 6191220]
13. Neumann G, Kawaoka Y. Host range restriction and pathogenicity in the context of influenza pandemics. Emerg Infect Dis. 2006; 12:881–886. [PubMed: 16707041]
14. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T. H5N1 virus attachment to lower respiratory tract. Science. 2006; 312:399. [PubMed: 16556800]
15. Shinya K, Ebina M, Yamada S, Ono M, Kasai N, Kawaoka Y. Influenza virus receptors in the human airway. Nature. 2006; 440:435–436. [PubMed: 16554799]
16. Karlas A, Machuy N, Shin Y, Pleissner KP, Artarini A, Heuer D, Becker D, Khalil H, Ogilvie LA, Hess S, Mäurer AP, Müller E, Wolff T, Rudel T, Meyer TF. Genome-wide RNAi screen identifies human host factors crucial for influenza virus replication. Nature. 2010; 463:818–822. [PubMed: 20081832]
17. König R, Stertz S, Zhou Y, Inoue A, Hoffmann HH, Bhattacharyya S, Alamares JG, Tscherne DM, Ortigoza MB, Liang Y, Gao Q, Andrews SE, Bandyopadhyay S, De Jesus P, Tu BP, Pache L, Shih

C, Orth A, Bonamy G, Miraglia L, Ideker T, García-Sastre A, Young JA, Palese P, Shaw ML, Chanda SK. Human host factors required for influenza virus replication. Nature. 2010; 463:813–817. [PubMed: 20027183]

18. Brass AL, Huang IC, Benita Y, John SP, Krishnan MN, Feeley EM, Ryan BJ, Weyer JL, van der Weyden L, Fikrig E, Adams DJ, Xavier RJ, Farzan M, Elledge SJ. The IFITM proteins mediate cellular resistance to influenza A H1N1 virus, West Nile virus, and dengue virus. Cell. 2009; 139:1243–1254. [PubMed: 20064371]
19. Shapira SD, Gat-Viks I, Shum BO, Dricot A, de Grace MM, Wu L, Gupta PB, Hao T, Silver SJ, Root DE, Hill DE, Regev A, Hacohen N. A physical and regulatory map of host-influenza interactions reveals pathways in H1N1 infection. Cell. 2009; 139:1255–1267. [PubMed: 20064372]
20. Matsuoka Y, Swayne DE, Thomas C, Rameix-Welti MA, Naffakh N, Warnes C, Altholtz M, Donis R, Subbarao K. Neuraminidase stalk length and additional glycosylation of the hemagglutinin influence the virulence of influenza H5N1 viruses for mice. J Virol. 2009; 83:4704–4708. [PubMed: 19225004]
21. Graef KM, Vreede FT, Lau YF, McCall AW, Carr SM, Subbarao K, Fodor E. The PB2 subunit of the influenza virus RNA polymerase affects virulence by interacting with MAVS and inhibiting IFN-{beta} expression. J Virol. 2010; 84:8433–8445. [PubMed: 20538852]
22. Ning ZY, Luo MY, Qi WB, Yu B, Jiao PR, Liao M. Detection of expression of influenza virus receptors in tissues of BALB/c mice by histochemistry. Vet Res Commun. 2009
23. Ibricevic A, Pekosz A, Walter MJ, Newby C, Battaile JT, Brown EG, Holtzman MJ, Brody SL. Influenza virus receptor specificity and cell tropism in mouse and human airway epithelial cells. J Virol. 2006; 80:7469–7480. [PubMed: 16840327]
24. Boon AC, deBeauchamp J, Hollmann A, Luke J, Kotb M, Rowe S, Finkelstein D, Neale G, Lu L, Williams RW, Webby RJ. Host genetic variation affects resistance to infection with a highly pathogenic H5N1 influenza A virus in mice. J Virol. 2009; 83:10417–10426. [PubMed: 19706712]
25. Joseph T, McAuliffe J, Lu B, Jin H, Kemble G, Subbarao K. Evaluation of replication and pathogenicity of avian influenza a H7 subtype viruses in a mouse model. J Virol. 2007; 81:10558–10566. [PubMed: 17634234]
26. Gillim-Ross L, Santos C, Chen Z, Aspelund A, Yang CF, Ye D, Jin H, Kemble G, Subbarao K. Avian influenza h6 viruses productively infect and cause illness in mice and ferrets. J Virol. 2008; 82:10854–10863. [PubMed: 18715930]
27. Chen GL, Lamirande EW, Yang CF, Jin H, Kemble G, Subbarao K. Evaluation of replication and cross-reactive antibody responses of H2 subtype influenza viruses in mice and ferrets. J Virol. 2010; 84:7695–7702. [PubMed: 20504935]
28. Tumpey TM, Basler CF, Aguilar PV, Zeng H, Solórzano A, Swayne DE, Cox NJ, Katz JM, Taubenberger JK, Palese P, García-Sastre A. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science. 2005; 310:77–80. [PubMed: 16210530]
29. Belser JA, Wadford DA, Pappas C, Gustin KM, Maines TR, Pearce MB, Zeng H, Swayne DE, Pantin-Jackwood M, Katz JM, Tumpey TM. Pathogenesis of pandemic influenza A (H1N1) and triple-reassortant swine influenza A (H1) viruses in mice. J Virol. 2010; 84:4194–4203. [PubMed: 20181710]
30. Horisberger MA. Interferons, Mx genes, and resistance to influenza virus. Am J Respir Crit Care Med. 1995; 152:S67–71. [PubMed: 7551417]
31. Staeheli P, Grob R, Meier E, Sutcliffe JG, Haller O. Influenza virus-susceptible mice carry Mx genes with a large deletion or a nonsense mutation. Mol Cell Biol. 1988; 8:4518–4523. [PubMed: 2903437]
32. Brown EG, Bailly JE. Genetic analysis of mouse-adapted influenza A virus identifies roles for the NA, PB1, and PB2 genes in virulence. Virus Res. 1999; 61:63–76. [PubMed: 10426210]
33. Keleta L, Ibricevic A, Bovin NV, Brody SL, Brown EG. Experimental evolution of human influenza virus H3 hemagglutinin in the mouse lung identifies adaptive regions in HA1 and HA2. J Virol. 2008; 82:11599–11608. [PubMed: 18829764]

34. Gabriel G, Dauber B, Wolff T, Planz O, Klenk HD, Stech J. The viral polymerase mediates adaptation of an avian influenza virus to a mammalian host. Proc Natl Acad Sci U S A. 2005; 102:18590–18595. [PubMed: 16339318]
35. Smith W, Andrewes CH, Laidlaw PP. A virus obtained from influenza patients. Lancet II. :66–68.
36. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T. Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. Am J Pathol. 2007; 171:1215–1223. [PubMed: 17717141]
37. Xu Q, Wang W, Cheng X, Zengel J, Jin H. Influenza H1N1 A/Solomon Island/3/06 virus receptor binding specificity correlates with virus pathogenicity, antigenicity, and immunogenicity in ferrets. J Virol. 2010; 84:4936–4945. [PubMed: 20200248]
38. Wu R, Zhang H, Yang K, Liang W, Xiong Z, Liu Z, Yang X, Shao H, Zheng X, Chen M, Xu D. Multiple amino acid substitutions are involved in the adaptation of H9N2 avian influenza virus to mice. Vet Microbiol. 2009; 138:85–91. [PubMed: 19342184]
39. Luke CJ, Subbarao K, Rappuoli R, Del Giudice G. The role of animal models in influenza vaccine research, Influenza Vaccines for the Future. 2008:161–202.
40. Tumpey TM, Maines TR, Van Hoeven N, Glaser L, Solorzano A, Pappas C, Cox NJ, Swayne DE, Palese P, Katz JM, García-Sastre A. A two-amino acid change in the hemagglutinin of the 1918 influenza virus abolishes transmission. Science. 2007; 315:655–659. [PubMed: 17272724]
41. Sorrell EM, Wan H, Araya Y, Song H, Perez DR. Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proc Natl Acad Sci U S A. 2009; 106:7565–7570. [PubMed: 19380727]
42. Herlocher ML, Elias S, Truscon R, Harrison S, Mindell D, Simon C, Monto AS. Ferrets as a transmission model for influenza: sequence changes in HA1 of type A (H3N2) virus. J Infect Dis. 2001; 184:542–546. [PubMed: 11494159]
43. Sidwell RW, Huffman JH, Gilbert J, Moscon B, Pedersen G, Burger R, Warren RP. Utilization of pulse oximetry for the study of the inhibitory effects of antiviral agents on influenza virus in mice. Antimicrob Agents Chemother. 1992; 36:473–476. [PubMed: 1605614]
44. Bussey KA, Bousse TL, Desmet EA, Kim B, Takimoto T. PB2 residue 271 plays a key role in enhanced polymerase activity of influenza A viruses in mammalian host cells. J Virol. 2010; 84:4395–4406. [PubMed: 20181719]
45. Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. J Virol. 1993; 67:1761–1764. [PubMed: 8445709]
46. Hatta M, Gao P, Halfmann P, Kawaoka Y. Molecular basis for high virulence of H5N1 Hong Kong influenza A viruses. Science. 2001; 293:1840–1842. [PubMed: 11546875]
47. Dybing JK, Schultz-Cherry S, Swayne DE, Suarez DL, Perdue ML. Distinct pathogenesis of hong kong-origin H5N1 viruses in mice compared to that of other highly pathogenic H5 avian influenza viruses. J Virol. 2000; 74:1443–1450. [PubMed: 10627555]
48. Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM. A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. J Virol. 1999; 73:5903–5911. [PubMed: 10364342]
49. Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P, Chittaganpitch M, Waicharoen S, Nguyen DT, Nguyen T, Nguyen HH, Kim JH, Hoang LT, Kang C, Phuong LS, Lim W, Zaki S, Donis RO, Cox NJ, Katz JM, Tumpey TM. Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. J Virol. 2005; 79:11788–11800. [PubMed: 16140756]
50. Fornek JL, Gillim-Ross L, Santos C, Carter V, Ward JM, Cheng LI, Proll S, Katze MG, Subbarao K. A single-amino-acid substitution in a polymerase protein of an H5N1 influenza virus is associated with systemic infection and impaired T-cell activation in mice. J Virol. 2009; 83:11102–11115. [PubMed: 19692471]
51. Conenello GM, Zamarin D, Perrone LA, Tumpey T, Palese P. A single mutation in the PB1-F2 of H5N1 (HK/97) and 1918 influenza A viruses contributes to increased virulence. PLoS Pathog. 2007; 3:1414–1421. [PubMed: 17922571]

52. Perrone LA, Plowden JK, García-Sastre A, Katz JM, Tumpey TM. H5N1 and 1918 pandemic influenza virus infection results in early and excessive infiltration of macrophages and neutrophils in the lungs of mice. PLoS Pathog. 2008; 4:e1000115. [PubMed: 18670648]
53. Tumpey TM, Lu X, Morken T, Zaki SR, Katz JM. Depletion of lymphocytes and diminished cytokine production in mice infected with a highly virulent influenza A (H5N1) virus isolated from humans. J Virol. 2000; 74:6105–6116. [PubMed: 10846094]
54. Li Z, Chen H, Jiao P, Deng G, Tian G, Li Y, Hoffmann E, Webster RG, Matsuoka Y, Yu K. Molecular basis of replication of duck H5N1 influenza viruses in a mammalian mouse model. J Virol. 2005; 79:12058–12064. [PubMed: 16140781]
55. Hatta M, Hatta Y, Kim JH, Watanabe S, Shinya K, Nguyen T, Lien PS, Le QM, Kawaoka Y. Growth of H5N1 influenza A viruses in the upper respiratory tracts of mice. PLoS Pathog. 2007; 3:1374–1379. [PubMed: 17922570]
56. Massin P, van der Werf S, Naffakh N. Residue 627 of PB2 is a determinant of cold sensitivity in RNA replication of avian influenza viruses. J Virol. 2001; 75:5398–5404. [PubMed: 11333924]
57. Mehle A, Doudna JA. An inhibitory activity in human cells restricts the function of an avian-like influenza virus polymerase. Cell Host Microbe. 2008; 4:111–122. [PubMed: 18692771]
58. Hai R, Schmolke M, Varga ZT, Manicassamy B, Wang TT, Belser JA, Pearce MB, García-Sastre A, Tumpey TM, Palese P. PB1-F2 expression by the 2009 pandemic H1N1 influenza virus has minimal impact on virulence in animal models. J Virol. 2010; 84:4442–4450. [PubMed: 20181699]
59. Qi L, Kash JC, Dugan VG, Wang R, Jin G, Cunningham RE, Taubenberger JK. Role of sialic acid binding specificity of the 1918 influenza virus hemagglutinin protein in virulence and pathogenesis for mice. J Virol. 2009 Apr; 83(8):3754–61. [PubMed: 19211766]
60. Narasaraju T, Sim MK, Ng HH, Phoon MC, Shanker N, Lal SK, Chow VT. Adaptation of human influenza H3N2 virus in a mouse pneumonitis model: insights into viral virulence, tissue tropism and host pathogenesis. Microbes Infect. 2009; 11:2–11. [PubMed: 18983930]
61. Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E. Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004. J Virol. 2005; 79:2191–2198. [PubMed: 15681421]
62. Maines TR, Jayaraman A, Belser JA, Wadford DA, Pappas C, Zeng H, Gustin KM, Pearce MB, Viswanathan K, Shriver ZH, Raman R, Cox NJ, Sasisekharan R, Katz JM, Tumpey TM. Transmission and pathogenesis of swine-origin 2009 A(H1N1) influenza viruses in ferrets and mice. Science. 2009; 325:484–487. [PubMed: 19574347]
63. Munster VJ, de Wit E, van den Brand JM, Herfst S, Schrauwen EJ, Bestebroer TM, van de Vijver D, Boucher CA, Koopmans M, Rimmelzwaan GF, Kuiken T, Osterhaus AD, Fouchier RA. Pathogenesis and transmission of swine-origin 2009 A(H1N1) influenza virus in ferrets. Science. 2009; 325:481–483. [PubMed: 19574348]
64. Svitek N, Rudd PA, Obojes K, Pillet S, von Messling V. Severe seasonal influenza in ferrets correlates with reduced interferon and increased IL-6 induction. Virology. 2008; 376:53–59. [PubMed: 18420248]
65. Itoh Y, Shinya K, Kiso M, Watanabe T, Sakoda Y, Hatta M, Muramoto Y, Tamura D, Sakai-Tagawa Y, Noda T, Sakabe S, Imai M, Hatta Y, Watanabe S, Li C, Yamada S, Fujii K, Murakami S, Imai H, Kakugawa S, Ito M, Takano R, Iwatsuki-Horimoto K, Shimojima M, Horimoto T, Goto H, Takahashi K, Makino A, Ishigaki H, Nakayama M, Okamatsu M, Takahashi K, Warshauer D, Shult PA, Saito R, Suzuki H, Furuta Y, Yamashita M, Mitamura K, Nakano K, Nakamura M, Brockman-Schneider R, Mitamura H, Yamazaki M, Sugaya N, Suresh M, Ozawa M, Neumann G, Gern J, Kida H, Ogasawara K, Kawaoka Y. In vitro and in vivo characterization of new swine-origin H1N1 influenza viruses. Nature. 2009; 460:1021–1025. [PubMed: 19672242]
66. Tumpey TM, García-Sastre A, Taubenberger JK, Palese P, Swayne DE, Basler CF. Pathogenicity and immunogenicity of influenza viruses with genes from the 1918 pandemic virus. Proc Natl Acad Sci USA. 2004; 101:3166–3171. [PubMed: 14963236]
67. van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, de Wit E, Munster V, Bestebroer T, Fouchier RA, Kuiken T, Osterhaus AD. Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and

highly pathogenic avian influenza H5N1 virus. J Infect Dis. 2010; 201:993–999. [PubMed: 20187747]

68. Rowe T, Leon AJ, Crevar CJ, Carter DM, Xu L, Ran L, Fang Y, Cameron CM, Cameron MJ, Banner D, Ng DC, Ran R, Weirback HK, Wiley CA, Kelvin DJ, Ross TM. Modeling host responses in ferrets during A/California/07/2009 influenza infection. Virology. 2010; 401:257–265. [PubMed: 20334888]
69. Watanabe T, Watanabe S, Shinya K, Kim JH, Hatta M, Kawaoka Y. Viral RNA polymerase complex promotes optimal growth of 1918 virus in the lower respiratory tract of ferrets. Proc Natl Acad Sci U S A. 2009; 106:588–592. [PubMed: 19114663]
70. Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM. Pathogenesis of avian influenza A (H5N1) viruses in ferrets. J Virol. 2002; 76:4420–4429. [PubMed: 11932409]
71. Rimmelzwaan GF, Baars M, van Beek R, van Amerongen G, Lövgren-Bengtsson K, Claas EC, Osterhaus AD. Induction of protective immunity against influenza virus in a macaque model: comparison of conventional and iscom vaccines. J Gen Virol. 1997; 78:757–765. [PubMed: 9129647]
72. Baskin CR, García-Sastre A, Tumpey TM, Bielefeldt-Ohmann H, Carter VS, Nistal-Villán E, Katze MG. Integration of clinical data, pathology, and cDNA microarrays in influenza virus-infected pigtailed macaques (Macaca nemestrina). J Virol. 2004; 78:10420–10432. [PubMed: 15367608]
73. Baas T, Baskin CR, Diamond DL, García-Sastre A, Bielefeldt-Ohmann H, Tumpey TM, Thomas MJ, Carter VS, Teal TH, Van Hoeven N, Proll S, Jacobs JM, Caldwell ZR, Gritsenko MA, Hukkanen RR, Camp DG 2nd, Smith RD, Katze MG. Integrated molecular signature of disease: analysis of influenza virus-infected macaques through functional genomics and proteomics. J Virol. 2006; 80:10813–10828. [PubMed: 16928763]
74. Kobasa D, Jones SM, Shinya K, Kash JC, Copps J, Ebihara H, Hatta Y, Kim JH, Halfmann P, Hatta M, Feldmann F, Alimonti JB, Fernando L, Li Y, Katze MG, Feldmann H, Kawaoka Y. Aberrant innate immune response in lethal infection of macaques with the 1918 influenza virus. Nature. 2007; 445:319–323. [PubMed: 17230189]
75. Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD. A primate model to study the pathogenesis of influenza A (H5N1) virus infection. Avian Dis. 2003; 47:931–933. [PubMed: 14575089]
76. Kuiken T, Rimmelzwaan GF, Van Amerongen G, Osterhaus AD. Pathology of human influenza A (H5N1) virus infection in cynomolgus macaques (Macaca fascicularis). Vet Pathol. 2003; 40:304–310. [PubMed: 12724572]
77. Chen Y, Deng W, Jia C, Dai X, Zhu H, Kong Q, Huang L, Liu Y, Ma C, Li J, Xiao C, Liu Y, Wei Q, Qin C. Pathological lesions and viral localization of influenza A (H5N1) virus in experimentally infected Chinese rhesus macaques: implications for pathogenesis and viral transmission. Arch Virol. 2009; 154:227–233. [PubMed: 19130169]
78. Murphy BR, Hinshaw VS, Lewis Sly D, London WT, Hosier NT, Wood FT, Webster RG, Chanock RM. Virulence of avian influenza A viruses for squirrel monkeys. Infect Immun. 1982; 37:1119–1126. [PubMed: 7129631]
79. Murphy BR, Buckler-White AJ, London WT, Snyder MH. Characterization of the M protein and nucleoprotein genes of an avian influenza A virus which are involved in host range restriction in monkeys. Vaccine. 1989; 7:557–561. [PubMed: 2609730]
80. Treanor JJ, Snyder MH, London WT, Murphy BR. The B allele of the NS gene of avian influenza viruses, but not the A allele, attenuates a human influenza A virus for squirrel monkeys. Virology. 1989; 171:1–9. [PubMed: 2525836]
81. Lowen AC, Mubareka S, Tumpey TM, García-Sastre A, Palese P. The guinea pig as a transmission model for human influenza viruses. Proc Natl Acad Sci U S A. 2006; 103:9988–9992. [PubMed: 16785447]
82. Matrosovich MN, Krauss S, Webster RG. H9N2 influenza A viruses from poultry in Asia have human virus-like receptor specificity. Virology. 2001; 281:156–162. [PubMed: 11277689]
83. Harder TC, Vahlenkamp TW. Influenza virus infections in dogs and cats. Vet Immunol Immunopathol. 2010; 134:54–60. [PubMed: 19896216]

84. Zheng B, Chan KH, Zhang AJ, Zhou J, Chan CC, Poon VK, Zhang K, Leung VH, Jin DY, Woo PC, Chan JF, To KK, Chen H, Yuen KY. D225G mutation in hemagglutinin of pandemic influenza H1N1 (2009) virus enhances virulence in mice. Exp Biol Med (Maywood). 2010; 235:981–988. [PubMed: 20660098]

85. Mänz B, Matrosovich M, Bovin N, Schwemmle M. A polymorphism in the hemagglutinin of the human isolate of a highly pathogenic H5N1 influenza virus determines organ tropism in mice. J Virol. 2010; 84:8316–8321. [PubMed: 20519408]

86. Jagger BW, Memoli MJ, Sheng ZM, Qi L, Hrabal RJ, Allen GL, Dugan VG, Wang R, Digard P, Kash JC, Taubenberger JK. The PB2-E627K mutation attenuates viruses containing the 2009 H1N1 influenza pandemic polymerase. MBio. 2010; 18:e00067–10. pii. [PubMed: 20689744]

Table 1

Summary of influenza proteins and their functions

Genome segment	Proteins encoded	Functional Unit	Protein function
1	PB2	Polymerase subunit	Binds caps of host cell mRNAs
2	PB1	Polymerase subunit	Polymerase catalytic subunit
	PB1-F2	Non-structural protein	Proapoptotic factor, possible immune evasion function
3	PA	Polymerase subunit	Polymerase subunit
4	Hemagglutinin (HA)	Surface glycoprotein	Viral attachment and fusion
5	Nucleoprotein (NP)	Internal protein	Viral RNA synthesis
6	Neuraminidase (NA)	Surface glycoprotein	Virion release
7	Matrix protein 1 (M1)	Internal protein	Regulates RNP[a] nuclear import
	Matrix protein 2 (M2)	Surface protein	Ion channel that facilitates viral RNP uncoating
8	NS1	Non-structural protein	Evasion of host immune response
	Nuclear export protein (NEP or NS2)	Nuclear export protein	Viral RNA nuclear export

[a]RNP = ribonucleoprotein

Table 2

Definitions of influenza host range, pathogenicity, and virulence

Host Range	The ability of an influenza virus to infect specific hosts
Pathogenicity	The ability of an influenza virus to cause disease in the host
Virulence	A measure of the severity of disease, from mild to severe

Table 3

Commonly used animal models for influenza

Animal model	Parameters	Advantages	Disadvantages
Mice	• **Weight loss** • Oxygen saturation (SaO2) in blood • **Level of viral replication** • **Lethality (mouse lethal dose 50 (MLD_{50})** • **Seroconversion** • Symptoms (lethargy, ruffled fur, hunching)	• Inexpensive • Well-characterized genetics • Abundance of reagents • Inbred strains	• Not natural host for influenza • Generally lack clinical symptoms of human influenza • Most strains show hypothermia instead of fever • Develop subtype-specific and heterosubtypic immunity [1] • Do not transmit virus [39] • Ability to infect can be dependent on mouse strain
Ferrets	• Weight loss • Fever • Activity level (e.g. lethargy) • Pulse/respiratory rates • Respiratory signs such as sneezing, coughing, and rhinorrhea • **Lethality** • **Viral titer in the respiratory tract**	• Similar sialic acid receptor distribution as humans • Similar clinical symptoms as humans • Similar pathological changes in the respiratory tract as humans	• Not standardized with respect to ferret age, inoculum titer, and volume • Lack of specific immunological reagents • Relatively expensive • Require special housing, • Few suppliers • Outbred
Non-human primates	• **Loss of appetite and weight** • Fever • **Viral titer in the respiratory tract** • **Seroconversion** • **Lethality (rare)**	• Closest to humans in physiology and genome sequence • Display clinical symptoms seen in humans • Limited extent of pathology and viral replication resembles human disease [76] • Size allows collection of samples for multiple analyses and for evaluating in different areas of the lungs • Similar cytokine/chemokine response as humans [76]	• Expensive • Difficult to work with. • Less susceptible to human influenza viruses than humans • Disease outcome and clinical signs of illness is dependent on species, influenza strain, and route of inoculation • Sialic acid receptor distribution and pattern of viral attachment may not be similar to humans

* Parameters highlighted in bold are those that are common among all three animal models. Italicized parameters are those that are specific to particular animal models.

Table 4

Influenza-specific host factors in animal models

Host factor	BALB/c Mice	B6 Mice	Ferrets	NHPs[a]
SAα2,3 receptor distribution	Trachea, lungs, spleen, liver, kidney, cerebellum	Ciliated cells in the airways, type II alveolar epithelial cells	Low levels of expression in trachea, bronchus, and lung alveoli	Virus has been detected in various tissues of the URT[b] and LRT[c], however, the specific distribution of SA receptors has not yet been determined
SAα2,6 receptor distribution	Trachea, lungs, spleen, liver, kidney, cerebellum	None in the lungs	Predominant in the respiratory tract (trachea, bronchus, lung alveolus, ciliated cells)	
Mx proteins	Present (Mx1)	Present (Mx1)	Unknown	Unknown

[a] NHPs = Non-human primates,

[b] URT = upper respiratory tract,

[c] LRT = lower respiratory tract

Table 5

Summary of influenza virus protein mutations that affect host range, pathogenicity, or virulence in mice

Virus	Requires adaptation?	HA	NA	Pols	NP/M/NS
1918	No	• D225G changes receptor binding specificity [84]		• PB1-F2 (N66S) increases virulence Encodes PB2 627K	
Seasonal	Yes				
Mouse adapted strains	Yes	• HA1 (residues 162, 210, 218) and HA2 (residue 154) mutations affect median pH of fusion and receptor specificity [33] • HA2 mutation (residue 47) increases viral yield [32]	• N368I increases viral yield [32]	• PB2 (K482R) increases viral yield [32] • PB1 (D538G) increases viral yield and affects interference [32] • Mutations in PB1 and PB2 are host restrictive [32] • PB1 (13P and 678N) enhance polymerase activity [34] • PB2 (701N and 714R) enhance polymerase activity [34] • PB2 333I decrease polymerase activity [34] • PA 615N, enhances polymerase activity [34]	• M1 (T139A) increases viral yield and macrophage recruitment [32] • NS1 (D125G) increases virulence • NP (319K) enhances polymerase activity [34]
p2009 H1N1	No	• D225G increases virulence [84]		• PB2 (T271A) enhances viral yield • Encodes PB2 627E. PB2 (E627K and D701N) attenuate infection [86]	
HPAI H5N1	No	• K222E affects viral yield and organ tropism [85]		• PB2 (E627K) increases virulence in some strains (Non-lethal strains encode PB2 627E) • PB2 (D701N) enhances polymerase activity • PB1-F2 (N66S) increases virulence	

Table 6

Characterization of influenza virus virulence in animal models.

Virus subtype		Mice	Ferrets	Non-human primates
H1N1	Seasonal	• No replication • Truncated PB1-F2	• Replication limited to respiratory tract • Mild to moderate virulence • Mild inflammatory changes	• Many viruses replicate but are not virulent • Replication restricted to URT[a] • Clinical symptoms depend on route of infection
	Mouse-adapted	• Viruses replicate • Systemic spread • Neurotropic • Lethal	N/A	N/A
	1918	• Systemic spread • Enhanced inflammatory response • Impaired adaptive immune response • Lethal	• Systemic spread • Severe pathology • Lethal	• Replication in URT and LRT[b] • Acute respiratory distress • Atypical immune response • Lethal
	p2009	• Replication limited to respiratory tract • Non-lethal • Does not express PB1-F2 protein	• High titer replication in URT and LRT • Mild to severe disease • Replication in intestinal tract • Varied reports on lethality	• Replicates in lungs and other respiratory tissues • More severe lung lesions than seasonal influenza • Non-lethal
H3N2	Seasonal	• Do not replicate without adaptation	• Some viruses replicate in URT but are not virulent • Replication limited to respiratory tract • Mild inflammatory changes • Mild to moderate virulence	• Many viruses replicate but are not virulent
	Mouse-adapted	• Systemic spread • Enhanced pulmonary lesions • Neurotropic	N/A	N/A

Virus subtype		Mice	Ferrets	Non-human primates
		• Lethal		
H5N1	Non-lethal	• Replication limited to respiratory tract • Transient lymphopenia	• Mild disease • Encodes HA 250K [61]	
	Lethal	• Systemic spread • Severe lymphopenia • Neurotropic • Enhanced inflammatory response • Impaired adaptive immune response • Encodes PB1-F2 • HA contains multi-basic cleavage site • NA possesses shortened stalk length	• Severe pathology • Systemic spread • Encodes PB2 627K • Lethal • Neurotropic • Encodes HA 250R [61]	• Replication limited to respiratory tract
Other Subtypes	Non-lethal	• Some H2 and H6 subtypes do not replicate [26–27] • H9N2 replication limited to respiratory tract [38]	Certain H3, H6, and H7 subtype AIVs can replicate in URT but are not virulent [39]	Certain LPAI viruses, including subtype H2 AIV, replicate but are not virulent [78]
	Lethal	• Specific H7, H6, and H2 subtype viruses cause morbidity and mortality [25–27]. • HPAI H7 viruses cause systemic spread and lethality [25]		

[a] URT = upper respiratory tract,

[b] LRT = lower respiratory tract