The Role of Animal Models In Influenza Vaccine Research

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Abstract A major challenge for research on influenza vaccines is the selection of an appropriate animal model that accurately reflects the disease and the protective immune response to influenza infection in humans. Vaccines for seasonal influenza have been available for decades and there is a wealth of data available on the immune response to these vaccines in humans, with well-established correlates of protection for inactivated influenza virus vaccines. Many of the seminal studies on vaccines for epidemic influenza have been conducted in human subjects. Studies in humans are performed less frequently now than they were in the past. Therefore, as the quest for improved influenza vaccines continues, it is important to consider the use of animal models for the evaluation of influenza vaccines, and a major challenge is the selection of an appropriate animal model that accurately reflects the disease and the protective immune response to influenza infection in humans.

The emergence of highly pathogenic H5N1 avian influenza (AI) viruses and the threat of a pandemic caused by AI viruses of this or another subtype has resulted in a resurgence of interest in influenza vaccine research. The development of vaccines for pandemic influenza presents a unique set of obstacles, not the least of which is that the demonstration of efficacy in humans is not possible. As the correlates of protection from pandemic influenza are not known, we rely on extrapolation of the lessons from seasonal influenza vaccines and on data from the evaluation of pandemic influenza vaccines in animal models to guide our decisions on vaccines for use in humans. The features and contributions of commonly used animal models for influenza vaccine research are discussed. The recent emergence of the pandemic 2009 H1N1 influenza virus underscores the unpredictable nature of influenza viruses and the importance of pandemic preparedness.

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1 Influenza Viruses

Influenza is a negative-sense, single-stranded RNA virus belonging to the family *Orthomyxoviridae*. *Orthomyxoviridae* consist of four genera: influenza A, influenza B, influenza C and Thogoto viruses. The proteins of influenza A viruses are encoded by genes on eight RNA segments. Influenza A viruses are widely distributed in nature and can infect a wide variety of birds and mammals, including humans. Influenza A virus subtypes are classified on the basis of the antigenicity of their surface glycoproteins, hemagglutinin (HA) and neuraminidase (NA) [1, 2] into 16 HA subtypes and 9 NA subtypes, and all of these subtypes have been found to infect birds [2, 3]. Waterfowl and shorebirds are the natural reservoirs of AI viruses.

In their natural hosts, most AI infections are not associated with clinical disease, and the viruses are generally thought to be in evolutionary stasis [4]. In humans, relatively few subtypes of influenza A viruses have caused sustained outbreaks of disease; viruses bearing H1, H2, and H3 HA genes and N1 and N2 NA genes have circulated in the human population during the twentieth century. H1N1 viruses appeared in 1918 and circulated until 1957, when they were replaced by H2N2 viruses. These, in turn, were replaced in 1968 by H3N2 viruses, which continue to circulate. In 1977, H1N1 viruses reappeared and have continued to co-circulate with the H3N2 viruses. Influenza A and B viruses continue to cause epidemics in humans each winter.

In addition to the seasonal influenza epidemics, the potential also exists for an influenza pandemic at any time. A pandemic occurs when an influenza strain with a novel HA subtype (with or without a novel NA subtype) appears and spreads in a susceptible human population. In the twentieth century, influenza pandemics occurred in 1918, 1957, 1968, and most recently, in 2009, with the emergence of the swine-origin pandemic H1N1 influenza virus. The influenza pandemic of 1918 was associated with severe morbidity and significant mortality but the pandemics of 1957 and 1968 were milder [5]. To date, disease caused by the pandemic 2009 H1N1 influenza virus does not appear to be more severe than disease seen with epidemic influenza, but there is a significant difference in the age groups most affected, with the majority of cases of pandemic 2009 H1N1 infection and hospitalization occurring in children and young adults and the highest mortality occurring in adults aged 24–49 years.

AI viruses in their natural reservoir in waterfowl and shorebirds are one source from which novel HA and NA subtypes are introduced into the human population. An influenza virus with a novel HA and/or NA can be introduced into the human population by direct spread from either wild birds or domestic poultry, as was seen when an H5N1 AI virus infected humans in 1997 [6]. Alternatively, avian and human influenza viruses can reassort, generating a virus that can efficiently spread in humans, as happened in the 1957 H2N2 and 1968 H3N2 pandemics [7]. The pandemic 2009 H1N1 influenza virus is derived from influenza viruses that were circulating in pigs rather than birds. This virus is a reassortant, bearing gene segments that were originally derived from avian, human and classical swine influenza viruses [8].

Influenza A viruses also infect and cause disease in a wide variety of mammalian species, including swine, horses, ferrets, mink, dogs, seals, and whales. The currently circulating highly pathogenic AI H5N1 viruses that emerged in Asia in 2003 can also infect and cause lethal infection in felids, including tigers, leopards and domestic cats [9, 10].

Although several animal species can be infected with influenza A viruses naturally and experimentally, an ideal animal model for studying infection and immunity to human influenza has not been identified. Several animal species are permissive to infection with influenza A and B viruses to varying degrees and some exhibit clinical signs of illness and pathological changes in the respiratory tract that are similar to those seen in human influenza. In this chapter, we discuss the main features of the animal models used for the evaluation of influenza vaccines, their advantages and disadvantages, and their contribution to research on vaccines against influenza in humans. We also discuss the role of animal models in the development of vaccines against pandemic influenza. Veterinary vaccines for swine, equine, avian and canine influenza can be evaluated in their natural hosts and are not discussed.

2 Influenza Vaccines

Vaccines have been available for epidemic or "seasonal" influenza since the 1940s. Inactivated influenza virus vaccines are still largely the same as they were when first developed. They are still generally produced in embryonated hen's eggs. There has been much investment recently in the development of cell-based influenza vaccines, of which at least two are licensed in Europe and several others are in development in Europe and the United States. Live attenuated influenza vaccines were first licensed in the United States in 2003, and are currently approved for annual use in healthy individuals between 2 and 49 years of age.

A serum hemagglutination inhibiting (HAI) antibody titer of 1:32 or 1:40 or greater is associated with protection from seasonal influenza [11–13], and this is used as a measure to predict the protective efficacy of the seasonal inactivated influenza virus vaccines. The correlates of protection for live attenuated vaccines are less clear-cut. These vaccines elicit systemic and mucosal immune responses and mucosal antibodies in the respiratory tract are believed to play a major role in the protection afforded by these vaccines [14–16].

Antigenic drift describes the gradual change in the antigenicity of an influenza virus which allows the virus to escape neutralization by antibodies that have already been induced by prior infection or immunization with previously circulating strains. Antigenic drift results from point mutations in and around antibody-combining sites in the HA and NA proteins. Influenza virus vaccines are unusual in that one or more of the components of the trivalent vaccine formulation may have to be changed annually to keep pace with antigenic drift of the virus, but as long as a licensed manufacturing process is used, the change in composition of the vaccine is considered a strain change and is not treated as a new vaccine. Approval of seasonal

influenza vaccines for use in humans requires limited testing in animals, and an evaluation of immunogenicity in humans is required in Europe but not in the United States.

In recent years, a resurgence of interest in the improvement of seasonal influenza vaccines, and the looming threat of a possible influenza pandemic have spurred efforts to develop vaccines that could thwart the spread of an emerging pandemic virus. Extensive pre-clinical characterization of these new vaccines in animals will be necessary. Many researchers are engaged in efforts aimed at developing "universal" influenza vaccines that can protect against both epidemic and pandemic strains by targeting the more conserved antigens of the virus, such as nucleoprotein (NP) or the matrix protein (M), thus eliminating the need for having to constantly update the composition of the annual seasonal influenza vaccine. The immune responses to candidate universal vaccines are entirely different from those elicited by the currently licensed seasonal inactivated influenza virus vaccines, where protective immunity is based mainly on neutralizing antibodies produced against the HA protein. Animal models in which different types of immune responses can be evaluated are needed.

One of the major challenges faced during the development of pandemic influenza vaccines is that the correlates of protection from AI viruses of pandemic potential are not known. Efficacy of these novel influenza vaccines cannot be established in humans, so estimates of efficacy are based on the information gleaned from challenge studies in animals.

3 Animal Models for Influenza

Despite the diversity of mammalian species infected by influenza viruses in nature only a few species are amenable to study in the laboratory. Tables 1–3 and the following sections summarize the features of the most commonly used small animal models in the study of influenza, and their respective utilities in the evaluation of influenza vaccines are summarized in Table 4. Commonly used laboratory animal species may not be fully permissive for infection with wild-type, non-adapted isolates of influenza viruses, and can vary in susceptibility to infection with specific virus strains and subtypes. Other variables that can influence the outcome of infection are the use of anesthesia, route of virus administration and the volume of inoculum used.

3.1 Rodent Models

Rodent models of infectious diseases are attractive for a number of scientific and practical reasons. They are small and relatively inexpensive to purchase and house. Many inbred strains are available and a battery of immunological reagents are available for some species.

Table 1 The use of the mouse model for the evaluation of vaccines against influenza

Influenza virus subtypes tested	Findings	References
Human influenza H1N1, H3N2, H2N2	Human influenza virus isolates require adaptation to cause illness (lethality) in mice Infection under anesthesia results in viral pneumonia Clinical signs include ruffled fur, hunching, labored breathing, unsteady gait, hypothermia, and weight loss Inflammation is observed in the respiratory tract	[18, 31, 32, 181]
Reconstructed 1918 H1N1 pandemic virus	Causes illness in mice and replicates efficiently in the respiratory tract without prior adaptation Up to 13% loss of body weight is observed Lethal to mice with an MDT[a] of 4.5 days No extrapulmonary spread observed Necrotizing bronchitis and bronchiolitis, and moderate to severe peribronchial and alveolar edema present	[138]
HPAI[b] H5N1	Most isolates cause severe illness and death without prior adaptation Replicate efficiently in the respiratory tract without prior adaptation Cause significant weight loss Most isolates are lethal in mice with a MDT of 6-8 days Some isolates are detected in extrapulmonary sites including the brain Variable virulence in mice is observed with isolates from Hong Kong from 1997, and 2003–2004, and viruses isolated from Europe and South America	[100–104, 133, 148]
H7	HP[c] and LP[d] isolates replicate efficiently in respiratory tract of mice without prior adaptation, with some viruses causing weight loss and death Extrapulmonary spread to the brain and spleen observed following intranasal infection with some isolates Histopathologic observations following intranasal infections with human isolates include necrosis and inflammation throughout the respiratory tract, but no lesions in the brain, heart, spleen, liver or kidneys Histopathological lesions are observed following intranasal infection with HP avian isolates	[115–119]
H9N2	Replicate efficiently in lungs of mice without prior adaptation Conflicting reports of lethality in mice Adaptation by passage in mouse lungs results in increased virulence Replication in brain reported following intranasal infection with non-adapted and mouse-adapted viruses	[132–137, 182, 183]
H6	Varying replication efficiency in respiratory tract depending on isolate Replication more efficient in the lower respiratory tract than in the upper respiratory tract	[132, 145]

(*continued*)

Table 1 (continued)

Influenza virus subtypes tested	Findings	References
	A/teal/W312/HK/97 (H6N1) and A/quail/HK/1721-30/99 (H6N1) lethal for mice when administered at high titers	
	Significant weight loss (average 24%) is observed in infected mice	
Pandemic 2009 H1N1	Replicate efficiently in the upper and lower respiratory tract	[171, 172]
	Some isolates cause weight loss and are lethal when administered at high titers	

[a]*MDT* mean time to death
[b]*HPAI* highly pathogenic avian influenza
[c]*HP* highly pathogenic
[d]*LP* low pathogenicity

Table 2 The use of the ferret model for the evaluation of vaccines against influenza

Influenza virus subtypes tested	Findings	References
Human influenza H1N1, H3N2, H2N2 viruses	Efficient replication of non-adapted isolates in respiratory tract	[17, 147, 184, 185]
	Isolated report of the presence of an H3N2 human influenza virus in the brain	
	Signs of illness include fever, sneezing, rhinorrhea, and weight loss	
	Mild inflammatory changes are observed upon histopathological examination of lungs of infected animals	
Reconstructed 1918 H1N1 pandemic virus	Replication to high titers in respiratory tract	[156]
	Severe disease observed including lethargy, anorexia, severe weight loss and high fever	
	Infection is lethal in 2/3 of inoculated animals; death occurs by Day 11	
	Virus is not detected in brain or heart	
	Necrotizing bronchiolitis, and moderate to severe alveolitis with edema observed upon histopathological examination	
HPAI[a] H5N1	Efficient replication in respiratory tract and evidence of extrapulmonary spread to brain, spleen and intestines	[104, 147, 149]
	Most isolates cause severe disease, including fever, rhinitis, sneezing, severe lethargy, hind limb paresis and diarrhea	
	Many isolates cause lethal infection in ferrets	
	Histopathologic observations include inflammatory changes in the lungs (bronchioloitis, bronchitis, interstitial pneumonia) and inflammation in the brain	

(*continued*)

Table 2 (continued)

Influenza virus subtypes tested	Findings	References
H6	Replicate to varying levels in the respiratory tract, with lower titers of virus in the upper respiratory tract than in the lungs Transient weight loss observed with Eurasian and North American isolates Transient elevation in body temperature	[145, 155]
H7	Replicate to varying levels in the respiratory tract Some HP H7 viruses replicate in the brain. A/NL/219/2003 (H7N7) HPAI causes severe disease with neurologic symptoms and mortality HP H7 viruses generally replicate to higher titers in the lungs than LP H7 viruses and duration of replication is longer	[115, 116]
Pandemic 2009 H1N1	Viruses replicate efficiently in the upper and lower respiratory tract Viruses replicate to higher titers in the lungs than seasonal H1N1 influenza viruses Some isolates caused signs of illness (weight loss, fever), severe illness and death One isolate was detected in rectal swabs	[171, 172, 176]
AI[b] subtypes H1N1, H2N1, H6N2, H2N2, H2N3, H3N2, H10N7, H3N6, H7N7, seal H7N7 isolate	Efficient replication in the upper respiratory tract No signs of illness with any of these isolates	[155]

[a]*HPAI* highly pathogenic avian influenza
[b]*AI* avian influenza

Table 3 The use of the hamster model for the evaluation of vaccines against influenza

Influenza virus subtypes tested	Findings	References
Human influenza H3N2	Non-adapted isolates replicate in the upper and lower respiratory tract. No clinical signs of infection are observed	[35–37, 161]
HPAI[a] H5N1	Non-adapted A/HK/483/97 (H5N1) resulted in lethal infection with deaths of all inoculated animals by Day 6 post-inoculation Virus is detectable in the lungs and brain	[161]
H9N2	Non-adapted A/HK/1073/99 (H9N2) replicates to high titers in the lungs but is not detected in the brain Infection is not lethal	[161]
H9N5	Non-adapted A/dk/HK/702/79 (H9N5) replicates efficiently in the lungs Infection is not lethal	[161]

[a]*HPAI* highly pathogenic avian influenza

Table 4 Comparison of the utility of commonly used animal models in the evaluation of influenza vaccines

Species	Utility in vaccine evaluation
Mouse	Determination of level of replication of live attenuated vaccine candidates in comparison to wild-type viruses
	Evaluation of antibody responses to vaccination by HAI[a] assay, Nt Ab[b] assay, ELISA[c]
	Evaluation of cellular immune responses to vaccination
	Evaluation of vaccine efficacy and effects of adjuvants
	General safety test for manufactured candidate vaccine
Ferret	Determination of level of replication of live attenuated vaccine candidates in comparison to wild-type viruses
	Evaluation of antibody responses to vaccination by HAI assay, Nt Ab assay, ELISA
	Limited evaluation of cellular immune responses to vaccination
	Evaluation of vaccine efficacy and effects of adjuvants
	Toxicology studies
Hamster	Determination of level of replication of temperature-sensitive live attenuated vaccine candidates
	Evaluation of vaccine immunogenicity by HAI assay, Nt Ab assay and ELISA
	Evaluation of vaccine efficacy

[a]*HAI* hemagglutination inhibition
[b]*NtAb* neutralizing antibody
[c]*ELISA* enzyme linked immunosorbent assay

3.2 Mice

Mice have been used for influenza vaccine research from the earliest days of the study of influenza virus biology. Shortly after the first human influenza virus was isolated from ferrets in 1933 by Wilson Smith and colleagues at the National Institute for Medical Research in London [17], it was discovered that human influenza viruses would cause disease in mice only if they were first adapted to the species by serial passages in the lungs [18]. This was subsequently found to be true for all human influenza virus isolates. One of the most commonly used human influenza viruses in mice is influenza A/Puerto Rico/8/34 (PR8), an H1N1 virus with a complex passage history, including several passages in ferrets, and hundreds of passages in eggs and mice (CB Smith, CDC, Atlanta, GA, personal communication). This virus is well adapted to mice and causes a lethal infection. The need for adaptation through serial passage of human influenza viruses is one of the major drawbacks of using mice in influenza research, because many mutations can arise during adaptation to the murine host; [19–22] these can alter their replication kinetics, and can result in the ability of the virus to escape the host innate immune responses [23].

Influenza viruses that cause disease and are lethal for mice provide a useful endpoint for vaccine efficacy studies. Depending on the strain of virus used, mice may become lethargic, anorexic, develop ruffled fur, and may also exhibit neurological symptoms of infection, in addition to weight loss, which is often the primary objective measure of the severity of infection. Body temperature is not a useful

measurement in mice because hypothermia can occur following infection with mouse-adapted viruses. Irrespective of whether an influenza virus induces morbidity or mortality in mice, the level of replication of influenza viruses in the lungs is the most informative endpoint for efficacy studies in mice since even a modest reduction in titer of infectious virus in the lungs can be associated with survival from lethal infection [24, 25]. Mice immunized with influenza viruses or vaccines develop serum HAI and neutralizing antibodies, the titers of which correlate with protection from subsequent challenge. Studies by Virelizier [26] demonstrated that antibody alone could protect against influenza infection in mice. Passive transfer of immune serum to naive mice resulted in a reduction in the replication of virus in the lungs and protected the recipient mice from lethal influenza pneumonitis, but did not prevent tracheitis or replication of virus in the upper respiratory tract [27]. The observation that passively transferred serum antibodies can reduce pulmonary viral replication but not viral replication in the upper respiratory tract is not unique to influenza A. Similar observations have been reported with influenza C virus [28], respiratory syncytial virus (RSV) [29] and severe acute respiratory syndrome-associated coronavirus (SARS-CoV) infections [30]. Measuring the amount of virus in various tissues in cases where high levels of serum antibody are present, for example, when vaccines are administered with adjuvant, should be done by quantitative molecular methods to rule out the possibility of ex vivo neutralization by serum antibody during tissue preparation. Such ex vivo neutralization has been shown to account for a reduction of up to 300-fold in detectable virus in the lungs of mice that had undergone passive transfer of immune serum against SARS-CoV [30]. The use of nasal and bronchiolar wash samples, instead of tissue homogenates, for viral quantitation was also employed as a solution to this issue [28].

The level of anesthesia can influence the outcome of influenza infection in mice. Mice infected under anesthesia develop pneumonia, while infection is limited to the upper respiratory tract when awake mice are infected [31, 32]. The volume of inoculum administered intranasally also influences the extent to which virus is distributed in the respiratory tract [32]. Immunologically, the lack of a functional Mx gene in standard laboratory strains of mice is a disadvantage of this model for studies in which the innate immune response to infection is important [33, 34]. However, the ready availability of mice, their relatively low cost, the available variety of genetic backgrounds and targeted genetic defects, and the immunological reagents available still make the mouse an attractive and heavily utilized animal model for studies on influenza.

3.3 Hamsters

Influenza virus infection of hamsters with non-adapted human influenza viruses does not result in clinical disease, but the virus replicates to high titers in the nasal turbinates and lungs following an intranasal infection [35–37]. As with mice, the hamster represents a readily available small animal model that can be used for

pre-clinical evaluation of candidate vaccines, but it has not been as extensively used as mice for studies of inactivated influenza virus vaccines. The body temperature of Golden Syrian hamsters is about 39°C, while that of mice is 37°C. Thus, hamsters have been used for the evaluation of live attenuated temperature-sensitive vaccines with shut-off temperatures $\geq$38.8°C [38].

3.4 Guinea Pigs

Guinea pigs can be infected with non-adapted human influenza viruses, although the amount of virus needed to infect guinea pigs is about ten times more than the amount needed to infect hamsters or ferrets [39]. Infection of guinea pigs with A/England/42/72 (H3N2) did not result in febrile illness or other clinical signs of influenza infection. The virus was isolated from the nasal washes of animals infected with influenza A/England/42/72 (H3N2), A/Hong Kong/1/1968 (H3N2) or A/FM/1/47 (H1N1) viruses, but titers of virus shed in the nasal secretions were not as high as those observed following experimental infection of ferrets. Infection of guinea pigs with influenza A/HK/1/68 (H3N2) virus resulted in pneumonia, which developed slowly and was reversible. This model was used to study the effects of environmental pollutants or drugs on the respiratory tract [40]. Lowen and colleagues [41] reported that guinea pigs of the Hartley strain are highly susceptible to non-adapted influenza A/Panama/2007/99 (H3N2) virus. Intranasal infection resulted in virus replication in the nose and lungs, with higher titers of virus being recovered from the lungs. The virus could be recovered from the upper respiratory tract for up to 9 days post inoculation, whereas shedding declined to undetectable levels in the lungs by day 5. Virus replication was not associated with any effects on body temperature or weight of the animals, and no other clinical signs of illness were observed.

3.5 Rats

Common laboratory strains of rat are described as “semi-permissive” for influenza infection, and infant rats are of some utility in the evaluation of live attenuated influenza vaccines, but they have not been used extensively to study influenza infection [42–44].

The cotton rat (*Sigmodon hispidus*) has been used in the laboratory as a model for several infectious diseases (reviewed in [45]). In particular, the cotton rat model was extensively used in the development of therapeutic antibody treatments for RSV and has provided much useful information for vaccine development against this pathogen. Sadowski and co-workers reported that intranasal administration of human influenza virus to lightly anesthetized, outbred young adult cotton rats resulted in virus replication in the respiratory tract, the production of pulmonary

lesions and a strong immune response [46]. In recent years, there has been some renewed interest in the cotton rat as a laboratory animal model for human influenza virus infection. Species-specific reagents that permit more detailed analysis of viral pathogenesis and immune responses in this species have been developed [45] and inbred cotton rats are now available. The advantages of this model include the fact that cotton rats can be infected by non-adapted human influenza viruses, inbred animals are available, the virus replicates in the upper and lower respiratory tract, some clinical parameters can be measured, and viral infection results in histopathological changes in the lungs that are similar to those seen during natural infection of humans [47]. To date only a limited number of human influenza viruses have been evaluated in cotton rats.

3.6 Ferrets

Ferrets are exquisitely susceptible to infection with human influenza viruses. The initial isolation of a human influenza virus by Smith and colleagues was from ferrets [17]. The ferret model of influenza has remained the same since this fortuitous discovery, and, in the opinion of many researchers, the ferret remains the ideal small animal model for influenza research. Ferrets can be infected with non-adapted human influenza virus isolates. Influenza virus infection in ferrets is primarily an upper respiratory tract infection, and infected ferrets exhibit clinical signs of infection similar to those seen during human influenza including fever, rhinitis and sneezing. The disadvantages of the ferret as a model for studying influenza vaccines include expense, special housing requirements, a limited number of suppliers, difficulties in obtaining animals that are seronegative for influenza virus, their exquisite sensitivity to other respiratory pathogens and ease of acquiring infection from their handlers, and the lack of species-specific reagents, although this last point does not present an obstacle for the evaluation of HAI and neutralizing antibody responses. In addition, the high body temperature of ferrets (average temperature of 38.8°C) may limit their utility in the evaluation temperature-sensitive live attenuated influenza vaccines.

3.7 Non-Human Primates

Non-human primates have not been used extensively for influenza vaccine research. From a practical standpoint, these animals are expensive and they have not proven to be the best model for the study of vaccines for influenza. Old World and New World species of monkeys have been evaluated as models of human influenza infection. It was determined early in the days of the study of influenza virus biology that non-human primate species were not as susceptible to human influenza viruses as their human relatives. Burnet reported in 1941 [48] that clinical signs of infection

were only apparent in cynomolgus macaques when they were infected via the intratracheal route as opposed to the intranasal route. Interestingly, mortality was observed in animals inoculated with the "W.S. Egg" strain, which was a mouse-adapted human influenza virus that had been passaged in eggs. Burnet reported that pathological changes consistent with those seen in human influenza infection were observed in the lungs of infected monkeys. The observation that intratracheal infection in monkeys might be required to achieve clinical signs of infection was supported by studies conducted by Saslaw and colleagues [49] in Rhesus macaques. Intratracheal infection of Rhesus macaques with a lung filtrate from mice infected with mouse-adapted A/PR/8/34 (H1N1) virus resulted in clinical signs of illness on day 2 post infection (p.i.), which resolved by day 4 p.i., whereas no signs of illness were apparent in monkeys inoculated with the same virus preparation intranasally, although both groups of animals showed hematological and serological evidence of infection.

Cynomolgus macaques were explored as a model for the evaluation of the immunogenicity and efficacy of an immunostimulating complex (ISCOM) influenza vaccine by Rimmelzwaan and colleagues [50]. Cynomolgus macaques inoculated intratracheally with the human influenza A/Netherlands/18/94 (H3N2) virus did not develop clinical signs of illness but virus could be recovered from lung lavage, nasal swabs and pharyngeal swab samples. Histopathological examinations were not performed.

Pigtailed macaques (*Macaca nemestrina*) were infected with a recombinant human influenza A/Texas/91 (H1N1) virus following virus administration via the trachea, tonsils and conjunctiva [51]. The animals exhibited clinical signs of infection, including loss of appetite, weight loss, nasal discharge and moderate fever, and histopathological observations that were consistent with progressive pneumonia. Virus was recovered from lung tissue at day 4 p.i. but not at day 7 p.i.

New World monkeys – including squirrel and cebus monkeys – have been evaluated as models for influenza vaccine studies. Murphy et al. [52] demonstrated that adult squirrel monkeys could be infected with intratracheally administered human influenza viruses. Mild illness that manifested as afebrile coryza was seen and, although radiographic evidence of pneumonia was not observed, the animals shed virus from the respiratory tract. Further studies evaluated the ability of AI viruses to replicate and cause illness in this species [53]. Different viruses caused varying degrees of clinical illness; some influenza viruses were completely attenuated in squirrel monkeys, while others replicated efficiently and caused clinical signs which were of a severity similar to that seen in human H3N2 influenza infection. Squirrel monkeys were employed to evaluate the level of attenuation of avian/human influenza virus reassortants, in a study comparing the replication of reassortants in chimpanzees and human volunteers [54]; the findings in squirrel monkeys were not predictive of the level of attenuation of the reassortant viruses in humans.

Cebus apella and *Cebus albifrons* monkeys were evaluated as models for influenza infection by Grizzard et al. [55]. The monkeys were inoculated either intranasally or intratracheally with two human influenza A viruses: A/Victoria/75

(H3N2) and A/New Jersey/76 (H1N1). All animals that received the A/Victoria/75 (H3N2) strain developed clinical signs of illness, and showed evidence of infection by either virus shedding or serology. Radiographic evidence of pulmonary disease was only seen in animals inoculated intratracheally with A/Victoria/75 (H3N2). Eight of ten animals inoculated intratracheally with the A/New Jersey/76 (H1N1) virus had mild upper respiratory tract illness, but only one of ten animals shed virus. However, all of these animals seroconverted. Histopathological evidence of inflammation in the lungs and trachea was seen in animals inoculated intratracheally with either strain, although the lesions in the animals that received A/Victoria/75 (H3N2) were more severe.

Chimpanzees are considered to be a valuable animal model to study infections of humans because of their close evolutionary relationship with the human species. However, the use of chimpanzees as animal models in research is logistically difficult. They are extremely expensive animals that require long-term care and stringent isolation since they are susceptible to several human pathogens. Chimpanzees have been used for some studies of influenza [54, 56, 57]. Influenza A and B viruses replicated to high titer in seronegative chimpanzees, but viral replication was not associated with illness. The advantages of studying influenza in this species include the fact that chimpanzees have the same body temperature as humans, their lower respiratory tract can be repeatedly sampled safely, they display permissiveness for vectored vaccines, similar to humans (for example, vaccinia-based vaccines), they are evolutionarily close to humans, and this may mean that similar host-range restrictions for replication of viruses may be present which could facilitate the selection of live attenuated candidate vaccines for testing in humans.

There is renewed interest in the use of non-human primates for evaluation of vaccines for pandemic influenza (see *Vaccines for pandemic influenza* below).

4 Animal Models in Influenza Vaccine Research

The three general areas of vaccine research and development in which animal models are utilized are the evaluation of vaccine safety, immunogenicity and efficacy. The following sections describe the use of animal models in each of these aspects of the pre-clinical evaluation of influenza vaccines.

4.1 Safety

In the early days of clinical testing of live attenuated vaccines against seasonal influenza, it was recognized that an animal model that could predict the attenuation of these vaccines would allow more rapid progression to immunogenicity and efficacy testing. Ideally, systematic comparisons of the behavior of attenuated virus vaccine candidates in animal models and in humans are needed to achieve

this end. Researchers began to address this question in the late 1970s and early 1980s, and the infant rat was extensively investigated as a model to predict the restriction in the replication of live attenuated influenza vaccines in humans [42–44]. In general, attenuation in the infant rat model correlated with attenuation in humans, although there were exceptions. Other species evaluated for this purpose include mice, hamsters, ferrets, and chimpanzees.

Although vaccine safety can only be fully assessed when a vaccine is administered to human subjects, regulatory authorities usually recommend standard tests for pre-clinical evaluation of the safety of new canidiate vaccines. The primary safety concern for inactivated influenza virus vaccines is reactogenicity, and for live attenuated influenza vaccines, it is their level of attenuation and their genetic stability. Standard toxicology tests on new vaccine candidates are often performed in rabbits, although current WHO guidelines for nonclinical evaluation of vaccines recommend that toxicology studies be performed in an animal species that most closely reflects the immune response to the vaccine in humans, or is "sensitive to the biological effects of the vaccine", and use the same dose and route of administration to be studied in clinical trials [58]. The design and results of such studies should be reviewed with special attention to experimental details such as the route of administration, volume and quantity of virus in the inoculum, and whether or not anesthesia was used, particularly for live attenuated vaccines, because each of these factors can influence the outcome. Toxicity following administration of very high doses of live influenza virus to animals via a variety of routes has been reported in the literature. For example, administration of 10^9 EID_{50} of influenza virus administered intranasally resulted in complete pulmonary consolidation and death in mice, and this pathology occurred despite restricted replication of virus in lung tissue [59]. Henle and Henle [60] reported inflammation in the gut, damage to the liver and spleen, and death in mice given high doses of influenza virus intraperitoneally. Similar findings were observed in rats, rabbits and guinea pigs. Lung inflammation was observed in ferrets administered high titer live attenuated influenza viruses intranasally [61] and systemic signs of illness were reported in human volunteers who received attenuated influenza viruses at doses that exceeded 10^7 $TCID_{50}$ [62–64]. In these studies, signs of clinical illness, including fever and other systemic signs, appeared within 48 h of administration of the virus, which is more rapid, in general, than the appearance of symptoms associated with a productive influenza virus infection. The systemic symptoms did not correlate with the titer of virus shed in respiratory secretions, or with the occurrence of respiratory symptoms. The occurrence of systemic illness in humans following administration of high doses of influenza virus in the absence of high levels of virus replication may be explained by the innate immune response to an abortive infection of epithelial cells.

The current procedures for marketing approval of vaccines for seasonal influenza do not involve extensive safety testing in animals. In the US, a standard general safety test, which is designed to detect extraneous toxic components in the vaccine preparation, is usually performed with the final drug product in mice and guinea pigs [65]. This test is performed for both inactivated and live attenuated

virus vaccines. For inactivated influenza virus vaccines, the vaccine can be administered via either the subcutaneous or intraperitoneal route for the guinea pig test, whereas only intraperitoneal route can be used for other types of vaccine. The vaccine formulation must also be certified to be free of endotoxin.

New vaccine candidates or novel preparations (including vaccines prepared by currently licensed methodologies that are now formulated with adjuvant) require extensive pre-clinical safety testing. In addition to tests such as repeat dose toxicology testing and general safety testing, some tests would be appropriate depending on the specific type of vaccine, e.g., demonstration of attenuation of live attenuated vaccines compared to the wild-type parent virus in more than one animal species [16, 66, 67], and biodistribution studies for plasmid-based vaccines [68–72].

Ferrets have been used to assess the attenuation of cold-adapted live attenuated vaccines against influenza [73]. These studies showed that cold-adapted 6-2 reassortant vaccine viruses generated from human influenza viruses failed to replicate in the lower respiratory tract of ferrets. Since ferrets are a good model for influenza infection in humans, they can also be used in toxicological studies of influenza vaccines.

The attenuation phenotype of several live attenuated influenza vaccine candidates was evaluated using the hamster model [35, 37]. For the small number of temperature-sensitive, cold-adapted reassortant influenza viruses tested in hamsters, and later in humans, there was a general correlation between the level of replication in hamsters and humans. However, in studies with AI/human influenza virus reassortants, the findings in hamsters did not accurately predict the level of attenuation of the viruses in humans [74]. Such data are important because they demonstrate that the genetic determinants of attenuation of influenza viruses are different in different species.

Non-human primate species have not been extensively used in studies the safety of influenza vaccines. Chimpanzees were used in several studies to evaluate the level of attenuation and the safety of candidate live attenuated vaccines [74]. Regulatory authorities in Europe require neurovirulence testing of live attenuated influenza vaccines and inactivated vaccines that are to be administered intranasally [75]. Since influenza viruses are not central nervous system pathogens in humans, the wisdom of such requirements, which were designed to determine the safety of live attenuated vaccines for truly neurotropic viruses such as poliovirus, can be questioned. The neonatal rat was recently proposed as a model for the study of neurovirulence of intranasally administered influenza vaccines [76], and a few influenza strains have been evaluated in this model. Some viruses replicated in the brain following intranasal administration, but pronounced lesions or dramatic behavioral changes were not demonstrated in infected animals.

4.2 Immunogenicity

The vast majority of studies conducted in animals for influenza vaccine research are those that evaluate the immune response to candidate vaccines. Although it is clear

that the immune responses to vaccines in animals are not often identical to and may not be directly predictive of those seen in humans, the first step in the proof-of-principle for a new vaccine is to establish its immunogenicity in animals before proceeding to clinical evaluation. The immune responses measured in the animal model should be relevant to the desired response in humans. Such studies may provide useful information regarding the regimen and routes of vaccination and can guide the design of clinical trials.

4.3 Strain-Specific Immunity Directed Against the HA

It is well established that the primary correlate of protection for inactivated whole-virus or subunit influenza vaccines administered parenterally is serum antibody directed against the HA protein. Most studies that are conducted to evaluate immune responses to influenza vaccines are done in mice and ferrets. The measurement of antibody responses in animal models is very straightforward, since HAI and neutralizing antibody assays do not require species-specific reagents. Limited studies have been conducted to evaluate the guinea pig as a model to study immunity to influenza virus. Phair and colleagues [39] demonstrated that infection of guinea pigs with unadapted human influenza viruses resulted in resistance to challenge with a homologous virus, and that passive transfer of hyperimmune serum to naive guinea pigs also conferred protection against infection. However, the levels of HAI antibody detected in the serum following infection were lower than those observed in ferrets or hamsters, and infected guinea pigs did not produce detectable levels of local antibody in their nasal secretions. In addition, high levels of nonspecific inhibitors of hemagglutination were present in guinea pig sera, making measurement of specific HAI antibodies problematic [39]. Phair et al. did, however, demonstrate that guinea pigs exhibited a delayed-type hypersensitivity response to influenza infection which resembled that seen in humans, although this response did not appear to be involved in resistance to infection.

Humoral immune responses to the HA of human influenza viruses and vaccines have been studied extensively in ferrets. Early studies determined that naive ferrets were not protected against influenza infection by vaccination with killed virus [77]. These observations were confirmed in later studies using formalin-inactivated vaccines [78]. However, killed vaccine, administered with an adjuvant to naive ferrets, provided partial protection against infection [79]. Thus, immune responses, in the ferret, to vaccination with inactivated virus vaccines against human influenza viruses do not appear to be identical to those seen in humans, since humans do not generally require an adjuvant to achieve protective levels of HAI antibodies. In contrast to the findings with inactivated influenza viruses, immunization with live influenza virus resulted in protection against subsequent challenge [78]. An explanation for this difference may be that in ferrets, influenza infection is primarily an upper respiratory tract infection, and adjuvant is required to elicit higher levels of

serum antibody needed to restrict replication of virus in the upper respiratory tract. Several studies have demonstrated that higher levels of serum antibody are required to provide protection against respiratory viruses in the nose of animals than in the lungs [27–29].

4.4 Heterosubtypic Immunity

In recent years, particularly since the emergence of the highly pathogenic H5N1 viruses in Asia in 2003, and the challenges in developing H5N1 vaccines, there has been a resurgence of interest in heterosubtypic immunity – the ability of an immune response elicited by a particular influenza A virus to protect against an influenza A virus of a different subtype. Heterosubtypic immunity against influenza has been demonstrated in a number of studies in mice but the precise mechanism of this immunity is not clear [24, 80–82]. Previously, it was thought that this phenomenon was mediated by cellular immune responses, but recent studies suggest that antibodies are the primary mediators of heterosubtypic immunity [82] and that the diversity of the antibody repertoire is important [83].

Heterosubtypic immunity has also been observed in ferrets [84, 85], although there was some debate as to the length of time for which such immunity persists. McLaren and Potter [84] reported that it did not persist beyond 10 weeks after vaccination, but in another study, protection against infection with a heterosubtypic virus was observed 18 months following immunization [86]. In both cases, heterosubtypic immunity did not prevent infection but it did limit virus replication following challenge.

The utility of the cotton rat model in addressing the issue of heterosubtypic immunity was explored [87]. The endpoints in this study were respiratory rate, virus replication in the lungs and nasal tissues, and pulmonary histopathology. A statistically significant reduction in respiratory rate was seen following challenge with A/Wuhan/359/95 (H3N2) in cotton rats that had been immunized with either the homologous virus or with a virus of a different subtype, A/PR/8/34 (H1N1), 4 weeks earlier, compared to non-immunized animals. This reduction in respiratory rate correlated with a statistically significant reduction in virus titers in the lungs and nasal tissues in immunized animals. Cotton rats that were immunized with the heterosubtypic A/PR/8/34 (H1N1) virus had the same extent of alveolitis, interstitial pneumonia and airway debris as non-immune, infected animals, and, like the cotton rats that were immunized with homologous virus, they had more severe early peribronchiolitis than was observed during primary infection. This peribronchiolitis could be indicative of a memory response in the heterosubtypic immune animals. However, the heterosubtypic-immune cotton rats had less bronchiolar epithelial damage than those animals immunized with homologous virus.

The role of heterosubtypic immunity through prior exposure or vaccination in humans, although inferred from retrospective analysis of data from influenza

pandemics [88], is extremely complex and cannot be readily determined. Studies in young infants and children on the effect of pre-existing immunity on replication and immunogenicity of heterosubtypic attenuated influenza viruses suggested that heterosubtypic immunity in humans is weak [89].

4.5 *Immune Responses to Other Influenza Proteins*

An approach that is being explored in the development of novel vaccines for influenza is that of universal influenza vaccines that target the conserved proteins of the virus – NP, M1, and M2. A number of modalities, such as NP and M DNA vaccines [90–92], baculovirus-expressed recombinant M2 protein [93], M2 peptides [94] and recombinant M2 protein incorporated into hepatitis B core antigen [95–97], have been tested in mice, and prevent death but not illness following challenge with a heterologous virus. In the case of candidate universal vaccines for influenza, new animal models and assays that can measure antibody and cellular responses to viral antigens other than the HA and NA are needed . As the immune responses to these conserved antigens are not well characterized in humans, at present, it is not clear whether these responses are accurately reflected in animal models. Undoubtedly, more information will be obtained in this area in the future as candidate universal vaccines are evaluated in clinical trials.

Recently, there has also been interest in the role of immune responses to the NA component of seasonal vaccines in protection against related subtypes of influenza, including potential pandemic strains [98]. Antibodies to the NA protein can modulate the severity of influenza illness [99] but the NA content of inactivated influenza virus vaccines is not currently standardized.

4.6 *Efficacy*

Animal models are also used to evaluate the efficacy of new candidate influenza vaccines. The most commonly used animal models for such studies are mice and ferrets. In mice and ferrets, it has been established that antibodies against the HA can prevent infection or ameliorate disease following challenge with influenza virus. Reduction in virus titer in the lower respiratory tract following a challenge correlates with protection, so quantitative virology is the most relevant measure of vaccine efficacy for vaccines designed to generate antibody responses to HA. Additional endpoints such as morbidity, mortality and pathological findings may provide supporting evidence of protection from infection and disease. Although demonstration of vaccine efficacy in an animal model is not an absolute requirement in the pre-clinical evaluation of a candidate vaccine from a regulatory standpoint, it does provide evidence that immune responses to the vaccine are biologically relevant.

5 Vaccines for Pandemic Influenza

The direct transmission of HPAI H5N1, H7N7 and low pathogenicity AI (LPAI) H9N2 viruses from birds to humans, associated in many cases with severe morbidity and mortality, has raised concerns about the emergence of one of these viruses as a pandemic virus and has, therefore, prompted efforts to develop vaccines against AI viruses of pandemic potential. Evaluation and characterization of a suitable animal model for these other influenza virus subtypes is a critical step in the development of such vaccines.

6 Animal Models

In the following section we describe the features of the animal models that have been developed to study AI viruses, and their contributions to the evaluation of pandemic vaccines. In addition, the predictive value of the various animal models in the evaluation of safety and immunogenicity of several live attenuated pandemic influenza candidate vaccines, that have been evaluated in clinical trials, will be discussed.

6.1 Mice

Mice have been used in pre-clinical studies of inactivated and live attenuated pandemic influenza virus vaccines. Reports in the scientific literature that describe characterization of the replication, pathogenicity and the immune response of AI viruses in mice focus on viruses of the H5, H6, H7, and H9 subtypes.

6.1.1 H5N1 Viruses and Vaccines

Several studies demonstrated that H5N1 viruses that were isolated from human cases in Hong Kong in 1997 cause disease and death in mice without prior adaptation [100–102]. These viruses varied for their ability to cause disease and death in BALB/c mice and generally fell into two distinct groups – those that were highly virulent, and those with low virulence for mice – and one virus (A/HK/156/97) was of intermediate virulence in two of the studies [101, 102]; however, Gao et al. [100] found this isolate to be one of the most highly virulent in this model. The 50% lethal dose of H5N1 viruses that were highly virulent for mice was 10–1,000 times lower than that of low virulence strains, they replicated to titers that were up to 1,000 times higher in the lungs of mice early in the course of infection, and they replicated in extrapulmonary sites, including the brain. Viral antigen was observed by immunohistochemistry in the lungs of mice infected with A/HK/483/97 (H5N1),

a highly virulent strain, and A/HK/486/97 (H5N1), a less virulent strain, and was associated with necrotic bronchi. Viral antigen was also observed, in both glial cells and neurons, in the brain of mice infected with the highly virulent influenza A/HK/483/97 (H5N1) virus, a finding also reported by Gao et al. [100]. In addition, Gao et al. reported the presence of viral antigen in cardiac myofibers of mice infected with the highly virulent influenza A/HK/483/97 (H5N1) virus. The ability of the H5N1 viruses to replicate and cause disease and death in mice did not correlate with their ability to kill chickens [102], and the relevance of replication of these viruses in extrapulmonary sites in mice to the disease in humans is not clear, although a general correlation between the level of virulence in mice and the severity and outcome of disease in humans was observed with 11 of 15 viruses evaluated [101]. Dybing and colleagues [103] reported that infection of mice with highly pathogenic H5 AI viruses that were isolated from Scotland [influenza A/ck/Scotland/59 (H5N1)], Italy [influenza A/ck/Italy/1485-330/97 (H5N2)], Queretaro [influenza A/ck/Queretaro/7653-20/95 (H5N1)] and England [influenza A/tk/England/91 (H5N1)], caused little or no disease in BALB/c mice. HPAI H5N1 influenza viruses isolated from humans in Asia in 2004 caused weight loss, ruffled fur, listlessness and pronounced leukopenia, and were lethal in mice without prior adaptation, and replicated outside the respiratory tract [104]. In the same study, HPAI H5N1 viruses isolated from birds, and a single human isolate, were less virulent for mice.

Lu et al. [102] used the BALB/c mouse model to evaluate the immunogenicity and efficacy of a vaccine against H5N1 influenza, based on an antigenically related non-pathogenic AI virus, A/duck/Singapore-Q/F119-3/97 (H5N3). They found that two doses of inactivated vaccine were required to elicit HAI antibody responses of a magnitude that would be protective in human influenza in the majority of vaccinated animals, and that the addition of an alum adjuvant resulted in higher levels of HAI antibody and a greater seroconversion rate. These findings generally agreed with the observations made in humans when a similar vaccine was tested in clinical studies: two doses of vaccine were necessary to achieve acceptable levels of antibody, and the addition of adjuvant, in this case MF59 (instead of alum used in the studies in mice), increased the magnitude of the antibody response as well as the seroconversion rate [105–107]. The efficacy of this vaccine in mice was determined by measuring the level of virus replication in the lungs and protection against lethal challenge with an H5N1 isolate that was highly virulent for mice.

The efficacy of several different H5N1 virus vaccines has been evaluated in mice and in all cases, the vaccines were found to be immunogenic and protective in mice (reviewed in [108]). When tested in Phase I studies in humans, inactivated H5N1 virus vaccines were found to be suboptimally immunogenic, requiring high doses [109, 110] to elicit neutralizing and HAI antibody responses. The administration of whole virion vaccines and inactivated virus vaccines with adjuvant increased the immunogenicity in mice and in humans [109, 111]. It is unclear whether data obtained in mice with pandemic influenza vaccines are predictive of vaccine immunogenicity in humans since pre-clinical data for the specific vaccine formulations that have been tested in humans to date have not been reported.

Cold-adapted live attenuated vaccine candidates against H5N1 AI viruses have been evaluated in pre-clinical studies in mice [66]. H5N1 vaccine candidates, bearing the modified HA and the NA from various HPAI H5N1 human isolates and the six internal protein genes from the A/Ann Arbor/6/60 cold-adapted (*ca)* donor virus, were restricted in replication in the lungs of mice compared to the corresponding wild-type virus, were found be immunogenic and conferred protection against challenge with homologous and heterologous wild-type viruses, although two doses of the vaccine virus were required to fully protect mice against replication of homologous wild-type viruses in the lungs [66]. Another live attenuated cold-adapted candidate H5N1 vaccine, a 7:1 reassortant virus which derived the HA from the low pathogenicity virus A/duck/Potsdam/86 (H5N2) and the remaining genes from the A/Leningrad/17/57 (H2N2) cold-adapted virus that is the donor virus for the seasonal live attenuated vaccine used in Russia, was evaluated in mice [112–114]. Only a single dose of this live vaccine virus was evaluated, but similar findings were reported: it was restricted in replication in the respiratory tract of mice and was immunogenic. The ability of the live attenuated H5N2 virus to elicit local IgA antibody responses in nasal washes has been demonstrated in mice. Modest levels of neutralizing antibodies were detected 6 weeks after a single dose of the H5N2 live attenuated vaccine, and the vaccine conferred protection against lethal challenge with a wild-type HPAI H5N1 virus. The predictive value of the mouse model for the evaluation of the safety and immunogenicity of these vaccines is discussed below in the section entitled "Clinical Evaluation of Live Attenuated Candidate Vaccines for Pandemic Influenza".

6.1.2 H7 Viruses and Vaccines

Representative low pathogenicity and highly pathogenic H7 AI viruses from both the Eurasian and North American lineages replicated in mice without prior adaptation [115, 116]. Highly pathogenic H7 viruses demonstrated extrapulmonary spread to the spleen and brain, as has been observed with HPAI H5N1 isolates, although H7 viruses were detected in the brain earlier during infection (day 1 p.i. for H7 and day 4 for H5) [116]. de Wit et al. [117] reported that intranasal infection of mice with the non-adapted HPAI A/Netherlands/219/2003 H7N7 virus, that was isolated from a fatal human case, resulted in severe illness, as indicated by weight loss, lethargy, ruffled fur, and lethality. The rate of loss in body weight was similar over a range of doses of virus between 3×10^3 and 3×10^6 EID_{50}. The virus was detected in the spleen, liver, kidneys and brain, as well as in the lungs of mice. This model was used for the evaluation of the immunogenicity and efficacy of candidate H7 influenza vaccines [117]. A single dose of an ISCOM vaccine and two doses of a subunit vaccine failed to protect mice against lethal infection with the A/NL/219/2003 (H7N7) virus, with one exception. Mice vaccinated with two doses of 1 μg or 5 μg ISCOM vaccine exhibited a small temporary loss in body weight but otherwise appeared healthy after challenge. Vaccination with two doses of the ISCOM vaccine resulted in at least a 1,000-fold reduction in virus replication in the lungs, and near-complete

reduction of extrapulmonary replication of the challenge virus. However, in all vaccinated mice, virus was still present in the lungs at high titers.

Munster et al. [118] reported that the human HPAI H7N7 viruses A/NL/219/2003 and A/NL/33/2003 both caused lethal infection in mice when administered intranasally at a high dose (dose not specified). At a dose of 5×10^2 $TCID_{50}$, influenza A/NL/219/2003 virus, which was isolated from a fatal human case, resulted in loss of body weight, ruffled fur, lethargy, and respiratory problems from day 2 p.i. and infected mice were euthanized on day 5 p.i., whereas in mice that were infected intranasally with 5×10^2 $TCID_{50}$ of influenza A/NL/33/2003 virus, isolated from a human with conjunctivitis in the same outbreak, no signs of illness or loss in body weight were observed up to day 7 p.i. The influenza A/NL/219/2003 virus replicated to a titer that was more than 1,000-fold higher compared to the titer in the lungs of mice infected with the influenza A/NL/33/2003 virus, and it was isolated from the brain, spleen, liver, and kidney of all infected animals. Influenza A/NL/33/2003 virus was isolated from the brain of only one out of three mice, and was not detected in the other organs examined. Histopathological findings in all mice infected with influenza A/NL/219/2003 virus included necrosis and inflammation throughout the respiratory tract that was pronounced in the trachea and became progressively milder in the bronchi, bronchioles, and alveoli. In contrast, lesions in the respiratory tract were only observed in one out of four mice infected with the influenza A/NL/33/2003 virus, and were characterized as mild to moderate cell necrosis, with neutrophil infiltrates in the trachea, bronchi, and bronchioles. Lesions were not observed upon histopathological examination of the brain, heart, spleen, liver or kidneys of mice infected with either virus. Viral antigen expression was limited to the tissues of the respiratory tract in mice infected with either virus, but was more abundant in mice infected with the influenza A/NL/219/2003 virus. Rigoni and colleagues [119] reported that HPAI H7N1 viruses isolated from chickens and ostriches could infect and replicate in mice without adaptation, and were associated with disease signs of varying severity. Bronchitis, tracheitis, alveolitis, and brain lesions were observed in mice infected with three HPAI H7N1 influenza viruses. However, the influenza A/ostrich/2332/00 virus caused more severe lesions and spread more rapidly in the lungs and brain than the other two viruses (influenza A/ostrich/984/00 and influenza A/ck/5093/99) [119].

Low pathogenicity H7 viruses replicated to high titers in the upper and lower respiratory tract of mice, but were not lethal, even at high doses. Immunogenicity of these viruses was also evaluated in mice [116].

Several reassortant viruses, bearing the HA or NA genes from H7 avian influenza viruses and the internal protein genes from A/PR/8/34 (H1N1) [PR8], have been described. Jadhao et al. [120] and Pappas et al. [121] reported the evaluation of the egg-based PR8 reassortant H7 influenza virus vaccines in mice. An H7N7-PR8 reassortant was generated which derived its HA from the low pathogenicity A/mallard/Netherlands/12/2000 (H7N3) virus and its NA from the low pathogenicity A/mallard/Netherlands/2/2000 (H10N7) virus. An H7N2-PR8 vaccine was generated with the HA and NA from the low pathogenicity A/turkey/Virginia/4529/02

(H7N2) virus. Mice immunized with two doses of the formalin-inactivated H7N7-PR8 or H7N2-PR8 vaccines, with or without alum, mounted a serum HAI antibody response that increased after the second vaccination. Antibody responses were generally higher when the vaccine was administered with an adjuvant. Mice that received two doses of the vaccine were protected from lethal challenge with highly pathogenic H7 influenza viruses. Evaluation of these vaccines in clinical trials is planned.

The immunogenicity and efficacy of a cell-based H7N1 avian influenza split virion vaccine, derived from the HPAI A/chicken/Italy/13474/99 (H7N1) virus, have been studied in mice [122]. Low titers of HAI antibodies were detected in the sera after two doses of 12 or 20 μg of HA. Titers were generally higher if vaccine was administered with an adjuvant. Vaccinated mice shed significantly less virus than unvaccinated animals following intranasal challenge with the HPAI A/ck/Italy/13474/99 (H7N1) virus and were protected from both disease and weight loss. Vaccination also conferred significant protection against lethal challenge. The same vaccine was tested in a Phase I clinical trial in sixty healthy adults [123]. Two doses were administered, with or without adjuvant. Serum HAI and neutralizing antibody titers, after two doses, were low, but were higher in the individuals who received an adjuvant (21 vs. 50% for the 12 μg dose and 23 vs. 62% for the 24 μg dose). Antibody secreting cells were also detected in those individuals with detectable HAI or neutralizing antibody titers, which were associated with IL-2 production.

The mouse model has also been employed for the evaluation of the attenuation, immunogenicity and protective efficacy of a candidate cold-adapted, live attenuated, influenza vaccine of the H7N3 subtype [124]. The HA and NA genes of this vaccine virus were derived from the low pathogenicity AI virus A/chicken/British Columbia/CN-6/2004 (H7N3), and its six internal protein genes were from the A/Ann Arbor/6/60 *ca* virus that is the backbone of FluMist®. In mice, the vaccine virus did not cause weight loss, and was restricted in replication in the lower respiratory tract compared to the low pathogenicity wild-type parent virus and an antigenically related HPAI H7N3 wild-type virus, and it appeared to have delayed replication kinetics in the upper respiratory tract compared to the wild-type parent virus. A single dose of the H7N3 *ca* vaccine virus was immunogenic in mice and provided complete protection against lethality and pulmonary replication following challenge with H7 influenza viruses of the North American lineage. Two doses of vaccine were required to confer protection against H7 influenza viruses of the Eurasian lineage.

6.1.3 H9 Viruses and Vaccines

Human infections with H9N2 AI viruses were first reported in 1999 [125, 126] and, although the illness in the infected individuals was relatively mild, there is still concern over the pandemic potential of H9 viruses because viruses of this subtype

are highly prevalent in birds [127–131]. The pathogenicity of human and avian H9 influenza viruses in mice has been studied by several laboratories, with a view of establishing an animal model that can be used to study strategies for prevention of pandemic influenza, including vaccines and antiviral drugs. Some H9 influenza viruses replicate in the respiratory tract of mice without prior adaptation [128, 132–134], but serial passage of the A/quail/Hong Kong/G1/97 (H9N2) virus in mice resulted in an increase in the virulence and in the extrapulmonary spread and lethality of this virus in intranasally infected mice [132, 133]. Data from different laboratories that have used the same H9N2 virus to infect mice are not consistent. Some of the factors that can influence the outcome of infection are anesthesia, dose, volume and route of virus administration, and passage history. It is difficult to compare studies when complete information is not provided. For example, in studies reported by Lu et al. [134], the human influenza A/Hong Kong/1073/99 (H9N2) virus replicated efficiently in the lungs of mice but failed to cause death or signs of disease, significant weight loss or to spread to extrapulmonary sites. However, Leneva et al. [132] reported that infection of mice with this virus resulted in 40% mortality and significant weight loss in the surviving mice. In these discordant studies, mice were anesthetized with CO_2 [134] or with metofane [132], were infected by the same route using virus that had been propagated in embryonated eggs, at approximately the same dose (10^6 EID_{50}), but inoculum volumes used were not stated in either study, so it is not clear why this virus was lethal in one study and not in the other. Similarly, a lethal challenge of mice with the human influenza A/Hong Kong/1073/99 (H9N2) virus was reported as part of a study to determine the efficacy of an M2 liposome vaccine [135], although this virus did not cause disease or lethality in the hands of other investigators [134, 136, 137] . All laboratories delivered virus intranasally to anesthetized mice. However, in the study reported by Ernst et al. [135], mice were anesthetized intraperitoneally with ketamine/xylazine, whereas in the other two studies, inhalational anesthesia was used, which may have resulted in a lighter state of anesthesia.

The mouse model has been used to evaluate the level of attenuation and the protective efficacy of a candidate cold-adapted, live attenuated, H9N2 vaccine bearing the HA and NA from the influenza A/ckHK/G9/97 (H9N2) virus and the internal protein genes from the influenza A/Ann Arbor/6/60 cold-adapted virus [136]. The H9N2 live attenuated vaccine was restricted in replication and protected mice from challenge with homologous and heterologous wild-type H9N2 influenza viruses.

6.1.4 1918 H1N1 Pandemic Virus

Like the highly pathogenic H5N1 AI viruses, the fully reconstructed recombinant 1918 H1N1 pandemic influenza virus was highly lethal in mice without prior adaptation [138]. The mean time to death in mice infected intranasally was 4.5 days. However, in contrast to the highly pathogenic H5N1 influenza viruses, this virus was not detected in extrapulmonary tissues. Histopathological findings

included necrotizing bronchitis and bronchiolitis, moderate to severe alveolitis and severe peribronchial and alveolar edema.

The mouse model appears to be potentially useful for the evaluation of pandemic influenza vaccines. Most AI viruses studied in mice, to date, can replicate without adaptation, although the outcome of infection with some AI viruses is clearly different, depending not only on the particular virus being studied but also on the laboratory in which the studies were conducted. It is important that AI viruses continue to be evaluated in mice, using standardized inoculation procedures and doses and with the measurement of the same endpoints so that the utility of this model can be maximized for the evaluation of pandemic influenza vaccines.

6.1.5 H6 Viruses and Vaccines

Although most of the pandemic influenza vaccine development efforts have focused on the subtypes of AI that have caused infections in humans, namely H5N1, H7, and H9 viruses, in theory, AI viruses of all subtypes have the potential to cause pandemics and therefore it is prudent to develop animal models to study the pathogenicity of these viruses and to evaluate experimental vaccines that may be needed in the future. There is concern regarding the pandemic potential of H6 AI viruses, since these viruses are highly prevalent in many avian species around the world [139–143]; they have a high propensity to reassort, and an H6N1 virus, A/teal/W312/Hong Kong/97, has been implicated as the donor of the internal protein genes of the H5N1 AI viruses that emerged in 1997 [140, 144]. In addition, there is serological evidence of human infections with H6 AI viruses in China [143].

The replication, pathogenicity and immunogenicity of several H6 AI viruses have been studied in mice [145]. Fourteen temporally and antigenically diverse H6 AI viruses of various NA subtypes, from both the Eurasian and North American lineages, were evaluated in BALB/c mice. Following intranasal inoculation of 10^5 $TCID_{50}$ of virus, replication of varying efficiency was observed in the respiratory tract of mice. Eleven of the 14 viruses replicated in the lower respiratory tract, ten in the upper respiratory tract; only one of the viruses failed to replicate to detectable levels in mice. Higher titers of the viruses were observed in the lungs of mice compared to the nasal turbinates. Two viruses from Hong Kong, A/teal/W312/HK/97 (H6N1) and A/quail/HK/1721-30/99 (H6N1) caused significant weight loss, illness, and death in mice, but their replication appeared to be limited to the respiratory tract. H6 AI viruses that replicated well in the lungs elicited high neutralizing antibody titers in infected mice, but the immunogenicity of H6 viruses did not correlate with their efficiency of replication in the respiratory tract. The cross-reactivity of the neutralizing antibodies was not an accurate predictor of protection. Live attenuated, cold-adapted candidate vaccines were generated from three of the H6 AI viruses studied [146]. Immunogenicity and efficacy of the candidate vaccines were evaluated in mice. A single intranasal dose of each vaccine virus elicited serum neutralizing and HAI antibody, and fully protected mice against replication of the wild-type parent H6 AI virus in the lower respiratory

tract. Cross-reactive antibody titers against heterologous H6 viruses were significantly lower than against the homologous parent virus. A second dose of vaccine in mice boosted the antibody titers, and improved cross-protection against the heterologous H6 AI viruses. As had been seen in the initial studies in mice, the level of neutralizing antibody elicited by the H6 candidate vaccines was a poor predictor of their ability to cross-protect against antigenically distinct H6 AI viruses. A candidate A/teal/HK/97 (H6N1) cold-adapted vaccine elicited the broadest cross-protective response, and this vaccine virus is currently undergoing evaluation in human clinical trials.

6.2 Ferrets

6.2.1 H5N1 Viruses and Vaccines

The ability of a limited number of AI subtypes to replicate and cause disease in ferrets has been investigated, and not surprisingly, the behavior of H5 subtype viruses has been the most studied. Zitzow and colleagues [147] demonstrated that two H5N1 influenza viruses isolated from human cases of infection in Hong Kong in 1997 were capable of replication not only in the respiratory tract, but also in the brain, spleen and intestines of ferrets. Virus replication was associated with clinical signs of disease such as severe lethargy, sneezing, rhinitis, hind limb paresis and, in some cases, diarrhea, and some H5N1 viruses were lethal to ferrets. However, the hierarchy in the severity of disease seen with the different H5N1 1997 isolates upon infection of mice, was not observed in ferrets: influenza A/HK/483/97 and A/HK/486/97 were equally virulent after intranasal infection of ferrets, whereas the A/HK/483/97 virus was more virulent in mice than the A/HK/486/97 virus was in several studies [100–102, 148]. As with mice, the significance, with respect to humans, of disease signs and the extrapulmonary replication of H5N1 viruses in ferrets is not clear, particularly since, in the same study, Zitzow et al. reported the isolation of a human H3N2 influenza virus from the brain of ferrets following intranasal infection. Similar studies have been conducted using human and avian H5N1 viruses isolated in 2004–2005 [104, 149]. Govorkova et al. [149] evaluated four human H5N1 influenza isolates and nine avian H5N1 isolates from Asia from 2004. A wide spectrum of infectivity, severity of disease and lethality was observed in ferrets inoculated with these viruses. The H5N1 viruses isolated from humans and two of the avian isolates caused severe disease in ferrets with some lethality. However, it is difficult to draw general conclusions regarding the behavior of these viruses in this model because of the small numbers of animals used (only two animals per group for all but one of the viruses tested), and the variability in infectivity of the viruses examined. For example, although the influenza A/Vietnam/3046/2004 virus caused severe disease in two out of two the inoculated ferrets, it was lethal in only one animal, and virus was only recovered from the nasal washes. In contrast, the influenza A/Vietnam/3062/2004 virus, which was also lethal in one out of two

ferrets inoculated, was recovered from the lungs, brain, spleen, and intestine of these animals. Similarly, Maines et al. [104] evaluated H5N1 isolates from Asia from 2004 using the ferret model. Although the viruses used in this study were different from those used by Govorkova et al. (with the exception of A/Vietnam/1203/2004), similar findings were reported: the human isolates caused severe disease, with some lethality, in ferrets. Again, small numbers of animals were used (three per group for most of the isolates tested) and some variability in infectivity and severity of disease was observed. In the study conducted by Zitzow et al., gross pathological changes observed in ferrets infected with highly virulent HPAI H5N1 viruses included focal areas of redness in the lungs, consolidation of the lungs and rare discoloration of the liver, petechiae on the liver and lesions on the intestines and kidneys [147]. Maines et al. [104] reported the presence of hemorrhage in the adipose tissue surrounding the liver, kidney and bladder in two-thirds of infected ferrets. Histopathological findings in the lungs of infected ferrets included acute bronchiolitis, bronchopneumonia, interstitial pneumonia with suppurative exudates in the bronchi, bronchioles and adjacent alveolar spaces, prominent epithelial necrosis and marked intraalveolar edema by day 3 p.i., and bronchitis, bronchiolitis and pneumonia observed on days 6–7 p.i. [104, 147, 149]. Inflammatory changes were also evident in the brain of ferrets infected with highly virulent HPAI H5N1 viruses at days 5–6 p.i., including in the glial nodules with perivascular infiltration of lymphocytes and polymorphonuclear leukocytes in the brain parenchyma, neuronophagia and lymphocytic infiltrates in the choroid plexus [147, 149]. Viral antigen was observed by immunohistochemistry in neurons in the same areas of the brain as the inflammation [104]. Govorkova et al. [149] reported histopathological changes in the liver, including diffuse vacuolization of the hepatocellular cytoplasm, mononuclear infiltrates, periportal hemorrhage, and hepatocellular necrosis. Generally, the viruses isolated from avian species caused less severe disease than those isolated from humans.

The number of ferrets inoculated with each virus was small and ferrets are an outbred species, so the significance of the variability in data such as virus replication and clinical illness are difficult to interpret. Until the scientific community has more experience with the behavior of AI viruses in animal models, it would be prudent to compare new isolates with well-characterized strains and to study these pathogens in more than one model.

The ferret model has also been used to evaluate the efficacy of several experimental inactivated [150, 151] and live attenuated [66, 112, 114] vaccines against H5N1 influenza. Inactivated H5N1 vaccines were immunogenic and protective in the ferret model [150, 151]. However, inactivated H5N1 vaccines that were tested in clinical trials were suboptimally immunogenic [109, 110]. The attenuation of cold-adapted live attenuated H5N1 vaccines has been demonstrated in ferrets. These vaccine candidates were also immunogenic and protective against challenge with homologous and heterologous H5N1 wild-type viruses in ferrets [66]. Protection from lethal H5N1 infection and the level of replication of the challenge virus in the lungs and other tissues are the endpoints used for evaluation of efficacy in this model. Van Riel et al. [152] demonstrated that the pattern of attachment of H5N1 influenza human isolates in

the respiratory tract of ferrets was similar to that seen in the human respiratory tract; the virus attached predominantly to type II pneumocytes, alveolar macrophages and nonciliated cuboidal epithelial cells of the terminal bronchioles in the lower respiratory tract and became progressively rarer more proximally, i.e., towards the trachea. This pattern of H5N1 virus attachment, predominantly in the lower respiratory tract, is thought to be related to the distribution of α-2,3 sialic acid receptors [153]. However, other investigators found that H5N1 influenza viruses were able to infect ex vivo cultures of the human upper respiratory tract, i.e., nasopharyngeal, adenoid and tonsillar tissues, despite the lack of α-2,3 sialic acid receptors in these tissues [154]. The tropism of H5N1 influenza viruses in the respiratory tract of humans and other species remains equivocal and further studies, in which a number of different isolates are evaluated in larger numbers of animals, are needed.

6.2.2 H7 Viruses and Vaccines

The behavior of AI viruses of the H7 subtype has been studied in ferrets. Human isolates of highly pathogenic H7N7 influenza viruses replicated to higher titers in the upper and lower respiratory tract of ferrets than low pathogenicity H7N2 influenza viruses isolated from humans. The H7N7 viruses also replicated in non-respiratory tissues [115]. The H7N7 isolate A/NL/219/2003 caused severe illness, including significant weight loss, caused neurological symptoms and was lethal in 2 out of 3 ferrets inoculated. Another highly pathogenic H7N7 AI virus, A/NL/230/2003, and the low pathogenicity H7N2 viruses evaluated in this study, did not cause severe disease and were not lethal in this model. Joseph et al. [116] demonstrated that the pattern of antigenic relatedness of H7 subtype AI viruses, determined using post-infection ferret sera, was similar to that observed in mice. The ferret model was used to evaluate attenuation, immunogenicity and efficacy of the H7N3 *ca* live attenuated vaccine virus [124]. The vaccine virus was restricted in replication in the upper respiratory tract of ferrets and did not replicate to detectable levels in the lungs or in the brain. Neutralizing antibodies were detected in the sera of ferrets immunized with a single dose of the H7N3 *ca* vaccine 4 weeks after immunization, and a second dose of vaccine provided a boost in the antibody response. Two doses of vaccine significantly reduced the replication of homologous and heterologous highly pathogenic H7 influenza viruses in the lungs of ferrets and prevented their spread to the brain and the olfactory bulb.

Ferrets immunized with an inactivated vaccine derived from an H7N1-PR8 reassortant based on HPAI A/chicken/Italy/13474/99 (H7N1), with alum adjuvant, mounted a serum HAI (GMT 76) and neutralizing antibody (range 42–200) response after two 24 μg doses of vaccine [122]. Cross-reactive HAI titers against heterologous Eurasian and North American H7 viruses were detectable but low (titer 8–160). Vaccination of ferrets resulted in reduced signs of illness, shedding of virus from the upper and lower respiratory tract and systemic spread following challenge with HPAI A/chicken/Italy/13474/99 (H7N1).

6.2.3 Other AI Subtypes

There are few reports in the scientific literature that describe the replication and clinical signs resulting from infection of ferrets with other AI subtypes. Hinshaw et al. [155] demonstrated that AI viruses of the H2, H3, H6, H7, and H10 subtypes, as well as an H7N7 virus isolated from a seal, replicated in the upper respiratory tract of ferrets, but elicited low or undetectable levels of antibody. None of these AI isolates tested caused any signs of disease in infected ferrets. Replication, pathogenesis, and immunogenicity of AI viruses of the H6 subtype were evaluated in the ferret model. Following evaluation in the mouse model of infection, four AI viruses of the H6 subtype that replicated to varying degrees in mice were studied in ferrets [145]. As in mice, the viruses replicated to lower titers in the upper respiratory tract than in the lungs, although the difference in titers was much less than in mice (~10-fold lower titers in ferrets vs. 10–1,000-fold difference seen in mice). All four viruses replicated to a peak titer of about 10^7 $TCID_{50}$/g in ferret lungs, although the peak titer occurred at different timepoints post-infection. Transient weight loss and fever were observed in ferrets infected with the A/teal/HK/97 and A/quail/HK/99 viruses that were lethal in mice, but also in ferrets that received the influenza A/mallard/Alberta/85 (H6N2) virus, which caused no signs of illness in mice. Ferrets infected with influenza A/duck/HK/77 (H6N9) did not exhibit weight loss or fever, but, unlike mock-infected ferrets, they failed to gain weight during the period of observation. Antibody responses elicited by an infection in ferrets generally correlated with those seen in mice, but, as in the mouse model, the antibody responses did not correlate with virus replication. In the ferret model, live attenuated, cold-adapted H6 AI candidate vaccine viruses were attenuated compared to the corresponding wild-type H6 virus. None of the vaccine viruses caused signs of illness in ferrets, nor did they replicate in the lungs. A single intranasal dose of the vaccine viruses elicited serum neutralizing and HAI antibodies in ferrets, and, as in mice, conferred complete protection in the lower respiratory tract following wild-type virus challenge. The levels of neutralizing antibody induced in ferrets by these vaccine viruses did not accurately predict the outcome of challenge with heterologous H6 viruses. The H6 AI viruses generally behaved in a similar fashion in ferrets and in mice, but species-specific differences in the cross-reactive antibody responses were observed.

6.2.4 1918 H1N1 Pandemic Virus

The reconstructed 1918 H1N1 influenza virus replicated to high titers in the upper respiratory tract of ferrets following intranasal inoculation [156]. All inoculated ferrets exhibited severe signs of disease that included lethargy, anorexia, sneezing, rhinorrhea, severe weight loss and high fever from day 2 p.i., and two out of three animals succumbed to infection by day 11. Unlike the highly pathogenic H5N1 viruses in ferrets, viral replication was not detected in tissues outside the respiratory tract. Necrotizing bronchiolitis, moderate to severe alveolitis and edema were

observed in the lungs of infected ferrets on day 3 p.i. The presence of viral antigen in the upper and lower portions of the bronchi, bronchial and bronchiolar epithelium and in the hyperplasic epithelium within the alveoli was observed.

6.3 Cats

There are few reports in the literature on influenza infection in cats. In studies conducted by Paniker and Nair in the 1970s [157, 158], intranasal infection of anesthetized cats with influenza A/Hong/Kong/1968 (H3N2) virus freshly isolated from human cases or laboratory- and egg-adapted isolates did not result in clinical signs of influenza but virus was recovered from pharyngeal secretions, and infection induced HAI antibodies and was transmitted to contact animals. Infected cats did not display clinical signs of influenza. Hinshaw and colleagues [155] later demonstrated that intranasally administered H7N7 and H7N3 AI viruses replicated in the upper respiratory tract of cats without clinical signs of disease, and the cats developed HAI antibodies after infection.

6.3.1 H5N1 AI Viruses

There was little interest in influenza infection and immunity in cats until the recent re-emergence of highly pathogenic avian H5N1 viruses in Asia, when it was reported that a number of big cats, namely tigers and leopards in the zoos in Thailand, became infected with HPAI H5N1 viruses, apparently after they were fed infected chicken carcasses [9]. Infection in many of these felids was fatal, and later, anecdotal reports of H5N1 infection in domestic cats in areas where there were outbreaks of H5N1 infection in avian populations contributed to a surge in interest in H5N1 influenza in cats. The pattern of attachment of a human H5N1 influenza virus to respiratory tract tissues of a cat was similar to that seen with human tissue [152].

Experimental infection of European short haired cats with an H5N1 virus isolated from a human in Vietnam in 2004 resulted in clinical disease, virus replication in respiratory and extra-pulmonary tissues, and pathological changes consistent with H5N1 infections in humans [10, 159]. Clinical signs, including significant elevation in body temperature, decreased activity, conjunctivitis and labored breathing were seen in cats experimentally infected intratracheally or by feeding on infected chicks [10]. Similar disease symptoms were observed in sentinel cats that became infected from being housed with cats that had been infected intratracheally. Illness in contact cats became apparent about 3 days later than in the cats infected via the intratracheal route. Peak viral titers in the throat swabs of the intratracheally infected cats were ~$10^{4.5}$ $TCID_{50}$/ml, whereas the peak titers observed in nasal swabs ranged from $10^{2.5}$ to $10^{5.0}$ $TCID_{50}$/ml [159]. The virus was also recovered from rectal swabs of cats infected by feeding on infected chicks, but the titers of virus in these samples varied widely. In addition, cats infected through feeding had lesions in the intestines. In animals infected

intratracheally or by feeding, the virus was also recovered from extra-pulmonary tissues, most often from the brain, liver, kidney and heart. Infected sentinel cats did not have detectable virus in tissues outside the respiratory tract; however, pathological changes were observed in the adrenal glands in one of the two sentinel cats infected in this manner. These studies demonstrated that HPAI H5N1 viruses are capable of extrapulmonary spread in cats, and can cause severe disease and even death in animals infected intratracheally or by feeding on infected bird carcasses. These observations also raise the possibility that the gastrointestinal tract may serve as a source for HPAI infection in cats.

Karaca et al. [160] studied the immunogenicity of a fowlpox-based H5 vaccine in cats. HAI antibodies were detected in serum of cats following a single subcutaneous dose of the vaccine, and a significant boost in antibody titers was observed following a second vaccination.

It remains to be seen if cats will be used extensively in the evaluation of vaccines against pandemic influenza.

6.4 Hamsters

6.4.1 H9 Viruses and Vaccines

Saito and colleagues conducted a study to evaluate the replication and pathogenicity of influenza viruses of various subtypes in Syrian hamsters [161]. The influenza A/HK/1073/99 (H9N2) virus replicated to high titers in the lungs, but was not lethal to hamsters and was not detected in the brain. The HPAI H5N1 influenza A/HK/483/97 virus, that was highly virulent in mice, was also lethal in hamsters, with all animals succumbing to infection by day 6 p.i., and, as in mice, virus was recovered from the brain of the infected hamsters. Avian H9N2 and H9N5 isolates could replicate in the lungs of hamsters, but did so to lower titers compared to human isolates. The human H9N2 virus elicited low levels of neutralizing antibody in infected hamsters, whereas the avian H9N2 isolate did not elicit detectable neutralizing antibody. The behavior of this limited number of AI isolates in the Syrian hamster model suggests that the effects of this viruses may be similar to that observed in mice, and further evaluation of this model for evaluating the efficacy of pandemic influenza vaccines is warranted.

6.4.2 Non-Human Primates

There is renewed interest in the use of non-human primates for immunogenicity studies of pandemic vaccines; this is based on the presumption that immune responses in these animals, which have a closer evolutionary relationship to humans, may be more predictive of the responses in humans than in smaller animals like mice and ferrets. To date, few data are available on the serological responses of non-human primates to AI virus vaccines.

6.4.3 H5N1 AI Viruses

The use of cynomolgus macaques as a model for influenza virus infection in humans was revisited following the emergence of the highly pathogenic H5N1 AI viruses in 1997 [162]. The initial human H5N1 influenza isolate, A/Hong Kong/156/1997, isolated from a fatal case of influenza in a child [6], was inoculated at multiple sites, including the trachea, tonsils and conjunctiva. Three of four animals developed fever within 2 days, and one showed signs of anorexia and acute respiratory distress. High titers of virus were recovered from lungs on day 4 p.i., and the virus was also isolated from the trachea, tracheobronchial lymph nodes and the heart. The virus was not recovered from these tissues on day 7 p.i. The virus was also recovered from bronchioalveolar lavage from 2 out of 2 animals on days 3 and 5 p.i.; from pharyngeal swabs of two animals on day 5 p.i., and from nasal swabs of one animal on days 3 and 7. Viral RNA was detected by RT-PCR in the brains of two animals on day 4 p.i., and in the spleen of all four animals tested on day 7 p.i. Pathological changes in the lungs of infected animals included pulmonary consolidation, necrotizing broncho-interstitial pneumonia and flooding of alveoli with edema fluid, fibrin, erythrocytes, cell debris, macrophages, and neutrophils, and inflammatory changes were seen in multiple organs [163].

Infection of Rhesus macaques with avian H5N1 isolates, reported by Chen et al. [164], indicated that results of intranasal inoculation varied depending on the influenza virus isolate used. Clinical signs of infection, including elevation in body temperature, anorexia and increased respiratory rate were observed in macaques inoculated with the following H5N1 viruses: A/bar-headed goose/Qinghai/1/2005, A/great cormorant/Qinghai/3/2005 and A/duck/Guangxi/35/2001. Pathological changes were seen in the lungs of all of the infected animals, but were more pronounced in the monkeys inoculated with the duck isolate. However, the only virus to be re-isolated from infected animals was A/duck/Guangxi/35/2001, and this virus was isolated from respiratory tract secretions and tissues and also from the spleen, liver and the heart.

The Rhesus macaque model has been used to evaluate the immunogenicity and efficacy of a candidate live attenuated cold-adapted H5N1 vaccine [113]. The vaccine virus derives its HA and NA from the clade 2.3 A/Anhui/2/2005 (H5N1) virus and the six internal protein genes from the cold-adapted A/AA/6/60 *ca* virus. The multibasic cleavage site in the HA gene was removed. Animals were inoculated intranasally with 10^7 EID_{50} of the vaccine virus on days 0 and 28. Serum antibodies in the vaccinated macaques were detected by ELISA 2 weeks following the first dose, with an apparent boost after the second dose. Four weeks after the first dose of vaccine all animals had detectable levels of neutralizing antibodies in the serum. After the second dose of vaccine, HAI and neutralizing antibodies were detected in the sera of all the vaccinated animals. HAI and neutralizing titers against a heterologous H5N1 virus were two to fourfold lower than against the homologous virus. T cell responses, measured by IFN-γ ELISPOT, were detected following the second dose of the vaccine. Three weeks after the second vaccination, the macaques were challenged intratracheally with 10^6 EID_{50} of either the parent A/Anhui/2/2005

(H5N1) virus, or the A/bar headed goose/Qinghai/1/2005 (H5N1) virus. Control animals had symptoms of illness including anorexia, fever and loss of appetite from day 1 post-challenge. Four control animals were euthanized on day 3 post-challenge and the remaining 4 animals gradually recovered. None of the vaccinated animals exhibited any clinical signs of illness. Pathological changes in the lungs of the unvaccinated control animals were more severe than in the vaccinated animals, and viral antigen was only detected in cells of the control animals. Virus was not isolated from any organs of vaccinated animals, whereas high titers of virus were detected in the respiratory tissues of the control animals.

Rudenko et al. described the evaluation of the safety, immunogenicity and protective efficacy of a live attenuated cold-adapted vaccine virus, which is based on the low pathogenicity A/duck/Potsdam/86 (H5N2) virus, as a candidate vaccine against H5N1 in Java macaques [165]. This vaccine candidate is a 7:1 reassortant, and derives its HA from A/duck/Potsdam/86 (H5N2) and its NA and internal protein genes from the donor virus for the live attenuated influenza vaccines used in Russia, A/Leningrad/17/57 (H2N2). Monkeys vaccinated with two doses of the H5N2 cold-adapted vaccine virus, 21 days apart, did not exhibit signs of illness, and virus was recovered from 2 out of 4 animals, at titers between $10^{1.2}$ and $10^{4.2}$ EID_{50}/ml between days 3 and 5 after the first dose. The dose of vaccine virus used was not reported. The H5N2 cold-adapted vaccine virus elicited only modest HAI responses in the vaccinated macaques. The animals were challenged with the HPAI A/chicken/Kurgan/02/2005 (H5N1) isolate. Vaccinated animals developed a fever, but it was of a lower grade and of a shorter duration than that observed in the control animals, and shedding of challenge virus occurred in vaccinated animals, but it was for a shorter duration than in the control animals. These data suggest that there may be a small protective effect of the H5N2 cold-adapted vaccine virus against heterologous H5 virus challenge, however, the numbers of animals used was small and the immune responses that were observed were not consistent between animals. The H5N2 cold-adapted vaccine has been evaluated in Phase 1 and Phase 2 clinical trials in small numbers of volunteers [166]. The vaccine was evaluated at two different dose levels, and two doses were found to be safe and immunogenic in 47–55% of subjects. HAI antibodies were detected in the serum of the vaccine recipients and IgA antibodies were detected by ELISA in nasal wash samples. Qualitatively, these responses were similar to those observed in the mouse model. The level of replication of the vaccine virus in humans was not reported.

6.4.4 1918 H1N1 Pandemic Virus

Cynomolgus macaques were evaluated as a model for studying the reconstructed 1918 H1N1 pandemic influenza virus [167]. Monkeys were infected by multiple routes – intratracheally, orally, on the tonsils and conjunctiva – based on the earlier studies with HPAI H5N1 influenza viruses in this species [162]. Animals infected with the reconstructed 1918 virus had severe clinical illness, high levels of virus replication in the respiratory tract and severe pathological changes in the lungs

compared to control animals infected with a recombinant human H1N1 influenza virus, A/Kawasaki/173/01 [167].

There may be a place for non-human primates as models for the evaluation of pandemic influenza vaccines, but the currently available data are not sufficient to support the use of these animals for immunogenicity or efficacy studies. Further studies are needed to characterize AI infection and the immune responses to AI viruses and vaccines in these species.

Clinical Evaluation of Live Attenuated Candidate Vaccines for Pandemic Influenza

The development of live attenuated vaccines against influenza viruses with pandemic potential has rapidly progressed from pre-clinical evaluation to early stage clinical testing in recent years. Data from both mouse and ferret models suggested that vaccine viruses of H5, H6, H7, and H9 subtypes, though restricted in replication in the respiratory tract compared to wild-type viruses, elicited serum antibody responses and were protective against both lethal challenge and pulmonary and extra-pulmonary replication following wild-type virus challenge. In addition, cross-protection against heterologous wild-type viruses was observed to varying degrees. Studies in non-human primates also showed that live attenuated H5 influenza virus vaccines could replicate in the respiratory tract and elicit serum HAI responses [113, 165].

In clinical trials involving small numbers of healthy adults, live attenuated cold-adapted H5N1 vaccine candidates, based on the clade 1 viruses A/Vietnam/1203/2004 and A/Hong Kong/213/2003, were found to be highly restricted in replication and poorly immunogenic [168]. A live vaccine virus, based on the A/Vietnam/1203/04 (H5N1) virus, when administered in two doses at $10^{7.5}$ $TCID_{50}$ per dose, failed to elicit neutralizing antibody in the serum of vaccinees and elicited serum HAI antibody in only 10% of the study subjects. Serum IgA and nasal wash IgA responses were detected in 52% and 19% of subjects, respectively; serum or local IgA responses had not been measured in ferret studies. Although the underlying reasons have not yet been elucidated, the poor predictive value of the mouse and ferret models with respect to replication and immunogenicity of these particular vaccine candidates was unexpected.

Rudenko et al. reported that a live attenuated cold-adapted vaccine, with the HA from a low pathogenicity avian H5N2 virus, elicited serum HAI and neutralizing antibodies in about 50% of volunteers after two doses, and resulted in the production of local IgA in the respiratory tract in 65% of vaccinees [166]. The level of shedding of the vaccine virus in volunteers was not determined. The reasons for the superior immunogenicity of the H5N2 *ca* vaccine virus, compared to the H5N1 vaccines based on the A/AA/6/60 *ca* in these small clinical studies, are not fully understood. It is possible that the donor cold-adapted H2N2 virus used to generate the H5N2 *ca* vaccine virus is less attenuated than the A/AA/6/60 *ca* donor virus, resulting in a vaccine virus that replicates more efficiently, however, this cannot be confirmed since replication of the H5N2 *ca* vaccine virus in humans was not reported. Given the poor predictive value of the mouse and ferret models with

respect to the replication and immunogenicity of clade 1 live attenuated H5N1 vaccines in humans [66, 168], it will be interesting to see how the A/Anhui/2/2005 cold-adapted vaccine described by Fan et al. [113] behaves in human clinical trials.

The H7N3 *ca* vaccine virus was highly restricted in replication in Phase I clinical studies but elicited an immune response in over 90% of subjects [169]. However, serum IgA, and not HAI or neutralizing antibody, was the most frequently observed indication of immunogenicity of this vaccine in humans, with a serum IgA response being detected in 71% of subjects, and 62% and 48% of the subjects developing a fourfold or greater rise in HAI or neutralizing antibody, respectively. Studies in mice and ferrets did not accurately predict such restricted replication of the vaccine virus and it is difficult to determine the predictive value of the mouse and ferret studies in terms of immunogenicity, since the number of human subjects in whom the vaccine was evaluated was small, and serum IgA responses were not studied in animals.

An H9N2 *ca* vaccine was evaluated in a Phase I clinical trial in humans [170]. Despite being highly restricted in replication, the vaccine virus was immunogenic in all subjects in at least one assay (HAI or neutralization assay). Again, the degree of restriction of replication of the vaccine virus in humans was not predicted by studies in mice.

Pandemic 2009 H1N1 Influenza Vaccines

The emergence of the pandemic 2009 H1N1 influenza virus prompted a rapid response from the research community and vaccine manufacturers to develop a vaccine against the emerging virus. Inactivated and live attenuated vaccines, based on the A/California/07/2009 (H1N1) virus, were produced using the same manufacturing process and regulatory infrastructure as for seasonal influenza vaccines in order to make vaccine available expeditiously. The pandemic 2009 H1N1 vaccines were evaluated in limited clinical trials to support licensure but extensive pre-clinical testing in animals was not performed. Inactivated and live attenuated vaccines for the novel H1N1 influenza virus were licensed in September 2009 in the US. Animal models, however, will be needed for the continued study of the pandemic 2009 H1N1 influenza virus and for the evaluation of alternative approaches to develop vaccines against this pathogen. Several laboratories have reported studies of pandemic 2009 H1N1 influenza virus isolates in laboratory animals. These studies are summarized in the following section.

6.5 Mice

Pandemic 2009 H1N1 influenza viruses isolated from humans replicated efficiently in the respiratory tract of BALB/c mice without prior adaptation [171, 172]. However, differences in the severity of disease caused by the A/California/04/2009 (H1N1) isolate were reported by the two laboratories. Maines et al. reported that the A/California/04/2009 (H1N1) isolate was highly infectious in the mouse model, with a 50% mouse infectious dose (MID_{50}) of between $10^{0.5}$ and $10^{1.5}$

plaque forming units (PFU), but this virus and two other isolates (A/Texas/15/2009 and A/Mexico/4108/2009) were not lethal in mice; whereas Itoh and co-workers reported that the A/California/04/2009 (H1N1) virus was lethal in mice at an LD_{50} of $10^{5.8}$ PFU, and that mortality was also observed in mice infected with another isolate, WSLH34939 (LD_{50} of $10^{4.5}$ PFU). In both studies, weight loss was observed in mice following infection with A/California/04/2009, but the disease was far more severe in the study conducted by Itoh et al. In both studies, the peak virus titer in the lungs at day 3 post-infection was similar (between $10^{5.8}$ and $10^{7.8}$ PFU), and virus replication was restricted to the respiratory tract. Higher levels of virus replication and more severe pathological changes were observed in the lungs of mice infected with the pandemic 2009 H1N1 influenza virus compared to those inoculated with a recent seasonal human H1N1 influenza virus. Prominent bronchitis and alveolitis, with positive staining for viral antigen were observed on day 3 p.i. in mice infected with A/California/04/2009, with signs of regeneration present by day 6 p.i. [171]. Mice that were inoculated with the seasonal H1N1 virus had progressed to bronchitis and peribronchitis by day 6 p.i., but there was much less extensive staining for virus antigen in the tissues from these animals.

An inactivated split-virion vaccine for pandemic 2009 H1N1 influenza, administered with or without adjuvant (MF59), was evaluated in mice [173]. These studies suggested that a single dose of vaccine required an adjuvant to elicit a serum HAI response that was predictive of protection. Interim data from human clinical studies demonstrated that a single dose of 7.5 μg of HA, administered with MF59, did indeed elicit serum antibody responses that were predictive of protection, according to the criteria for the licensure of seasonal influenza vaccines [174]. However, data from recipients of the same vaccine administered without adjuvant were not reported [174]. Interim data from clinical trials of an inactivated pandemic 2009 H1N1 influenza vaccine suggest that, surprisingly, a single dose of unadjuvanted vaccine is sufficiently immunogenic to meet the criteria established for the licensure of seasonal influenza vaccines [175]. The preliminary data from clinical trials of the pandemic 2009 H1N1 vaccine show evidence of immunologic priming to the novel H1N1 virus. The complex previous immunologic experience of humans with influenza viruses, either by prior infection or vaccination, cannot be emulated easily in experimental animals.

6.6 Ferrets

The replication and virulence of pandemic 2009 H1N1 viruses, compared to recent seasonal H1N1 human influenza viruses, were evaluated in ferrets [171, 172, 176]. Beyond a mild level of inactivity, overt clinical signs of influenza were not observed in ferrets inoculated with A/California/04/2009 [171, 172]. More pronounced clinical features, some resulting in euthanasia, were observed in ferrets inoculated with virus isolates from Texas and Mexico [172], but not in animals that received pandemic 2009 H1N1 virus isolates from Wisconsin, the Netherlands and

Japan [171]. The clinical symptoms seen in ferrets did not reflect the severity of infection in the patients from whom the viruses were isolated.

In general, the pandemic 2009 H1N1 viruses and recent seasonal H1N1 influenza viruses replicated efficiently and to similar levels in the upper respiratory tract of ferrets, but the pandemic 2009 H1N1 viruses achieved higher titers in the lungs [171, 172, 176]. The virus was also detected in rectal swabs and tissue samples taken from the intestinal tract of infected ferrets [172]. There have been sporadic reports of gastrointestinal symptoms in human cases of pandemic 2009 H1N1 influenza infection [177], but this does not appear to be common, and the significance of this observation, with respect to pathogenesis of infection with these viruses, is not clear.

Pathologic changes were observed in the respiratory tract of ferrets inoculated with either seasonal or pandemic H1N1 viruses, but the changes were more extensive and more severe in ferrets infected with the pandemic 2009 H1N1 viruses. Itoh et al. reported similar levels of viral antigen in the nasal mucosa of animals that received either seasonal or pandemic virus. The lungs of ferrets inoculated with the seasonal H1N1 virus A appeared mostly normal, whereas A/California/04/2009-infected ferrets had more severe bronchopneumonia with prominent expression of viral antigen in the peribronchial glands and in a few alveolar cells. Similarly, ferrets inoculated with a pandemic H1N1 isolate from the Netherlands had mild to moderate, multi-focal, necrotizing rhinitis, tracheitis, bronchitis and bronchiolitis on day 3 p.i. with viral antigen observed in many cells in the nasal cavity, trachea, bronchus, and bronchioles while the pathologic changes and the presence of viral antigen were limited to the upper respiratory tract and were less extensive, respectively, in ferrets inoculated with seasonal H1N1 influenza [176]. By day 7 p.i., most of the virus-infected cells had been cleared from the respiratory tract of ferrets inoculated with either the seasonal or pandemic H1N1 virus. The pandemic 2009 H1N1 influenza viruses were also found to efficiently transmit via direct contact and respiratory droplets. In summary, the pandemic 2009 H1N1 viruses replicated more efficiently than seasonal H1N1 influenza viruses in the lower respiratory tract of ferrets. This increased level of replication was associated with more severe pathologic changes in the lower respiratory tract, but did not generally result in more severe clinical illness.

6.7 Non-Human Primates

As described above, there have been several reports of the use of non-human primate species as models for studies of influenza infection and for the evaluation of experimental influenza vaccines. To date, there is only one report of infection of non-human primates with pandemic 2009 H1N1 influenza viruses. Itoh and colleagues studied the infection of cynomolgus macaques with A/California/04/2009 (H1N1) virus [171]. As with the previous studies of avian H5N1 influenza viruses in this model, multiple routes of inoculation were used to establish infection:

animals were inoculated with a total dose of $10^{7.4}$PFU via the intratracheal, intranasal, ocular and oral routes. Macaques inoculated with the A/California/04/2009 (H1N1) virus experienced a greater increase in body temperature than animals that received a recent seasonal H1N1 virus, but they exhibited no other clinical signs of infection. The pandemic 2009 H1N1 virus replicated more efficiently in both the upper and lower respiratory tracts of macaques, achieving titers of between $10^{4.3}$ and $10^{6.9}$PFU in the lungs on day 3 p.i. High titers of virus ($>10^5$PFU) were still detected in the oro/nasopharynx, tonsil and bronchi of one animal on day 7 p.i., but it had been cleared from the other respiratory tissues.

Pathologic changes were observed in animals inoculated with either pandemic or seasonal H1N1 influenza viruses, but these lesions were more severe in the animals that received the pandemic 2009 H1N1 virus. On day 3 p.i., an edematous exudate and inflammatory infiltrates in the alveolar spaces with severe thickening of the alveolar walls were observed. Cells which appeared to be type I pneumocytes, that were positive for viral antigen, were distributed in the inflammatory lesions, and many type II pneumocytes were also positive for virus antigen. A thickening of the alveolar wall was also observed in large sections of lungs from monkeys infected with the seasonal H1N1 influenza virus, with prominent inflammatory cells in the alveolar wall. However, cells staining positive for viral antigen were sparse, and were only type I, not type II, pneumocytes. By day 7 p.i., the lung pathology remained more severe in the animals that received the pandemic virus than in those infected with the seasonal influenza virus, and many antigen-positive cells were still visible; however, regenerative changes were also evident.

7 Correlates of Protection from AI Viruses and Regulatory Concerns

Despite the fact that the correlates of protection from AI virus infections in humans are not known, the criteria for licensing pandemic influenza vaccines are based on the previous experience with vaccines against seasonal influenza. In Europe and the United States, regulatory authorities have published guidances for vaccine manufacturers that attempt to balance the need for expedited approval of pandemic influenza vaccines with the requirements for the demonstration of safety and immunogenicity of candidate vaccines.

In the United States, for example, a guidance for vaccine manufacturers, published in 2007 [178], states that licensure of both inactivated and live attenuated vaccines for pandemic influenza should be based on the percent of subjects achieving an HAI antibody titer of 1:40 or greater, and on the rate of seroconversion, which is defined as a fourfold or greater rise in post-vaccination HAI antibody titer. This could be particularly problematic for live attenuated AI vaccines, since experience with seasonal live influenza vaccines indicates that serum antibody levels do not correlate with the efficacy of such vaccines. Results from clinical

trials conducted so far with live attenuated AI vaccines suggest that the measurement of immune responses other than serum HAI and neutralizing antibody, for example, serum IgA levels in pre-clinical studies, may be of value. Efficacy studies in animal models, although not an absolute requirement, may at least provide evidence that biologically relevant immune responses are elicited by candidate vaccines.

This guidance is intended to allow for rapid marketing approval of pandemic influenza vaccines that are produced using manufacturing processes that are already validated for seasonal influenza vaccines so that the licensure of the pandemic vaccine is essentially a strain change. Such approval requires much more limited testing of the candidate vaccines in animal models. In the European Union, manufacturers are required to submit information on the production and pre-clinical testing of a "mock-up" pandemic vaccine. In the event of a pandemic, a vaccine made in the same way as the mock-up vaccine, but based on the nascent pandemic virus, will be produced and will be subject to limited pre-clinical characterization, including immunogenicity studies in animals on at least one batch of the product [179]. Efficacy studies of the actual pandemic vaccine formulation in animals are not required. However, extensive pre-clinical testing of the vaccine candidate is required for new vaccine modalities and formulations, including formulations of approved vaccines with adjuvants.

In the US, a regulatory mechanism was introduced under what is commonly referred to as the "animal rule" [180] for obtaining marketing approval of vaccines for which efficacy studies in healthy human volunteers are either unethical or not feasible. This regulation stipulates that, in cases where efficacy of vaccines in humans cannot be definitively determined, marketing approval for a vaccine may be granted based on "adequate and well-controlled animal studies", provided the basis for vaccine efficacy is reasonably well understood, and that the animal responds to the vaccine in a manner that is predictive of the response in humans. Studies in more than one animal species would typically be required, unless a single animal model is available that can faithfully predict the efficacy of a vaccine in humans. It is unclear, at this time, whether this rule will eventually be applied to vaccines for pandemic influenza. In any event, it is critical that the predictive value of the available animal models for immunogenicity and efficacy of pandemic influenza vaccines be determined systematically using the same vaccine formulations that are progressing into clinical studies.

8 Conclusion

Although several animal species support the replication of human and AI viruses, a survey of the literature leads to the conclusion that there is no single ideal animal model for the evaluation of influenza vaccines. Some animal models are more suitable than others in predicting the attenuation of live virus vaccines, or more closely reflect the human immune response to vaccines. Animal models certainly

play a crucial role in the evaluation of influenza vaccines, but the limitations of the models must be taken into account when decisions regarding which vaccine candidates should move forward into clinical trials are made.

The evaluation of vaccines for pandemic influenza presents additional challenges, in that, the correlates of protection from AI viruses are not known, and so there may be a greater need for reliance on data from animal studies for these vaccines. It is critical that the behavior of AI viruses with pandemic potential be characterized in a range of animal models. Even from limited observations, it is clear that replication of AI viruses and their ability to cause disease in animals depends on the host species, and is subtype and even strain specific. To date, the level of replication and the immunogenicity of live attenuated AI candidate vaccine viruses seen in animal models have not accurately predicted the behavior of these vaccine viruses in humans. Therefore, pre-clinical safety, immunogenicity and efficacy data from animal studies must be carefully considered in the evaluation of pandemic influenza vaccines.

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