

The ferret as a model organism to study influenza A virus infection

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Influenza is a human pathogen that continues to pose a public health threat. The use of small mammalian models has become indispensable for understanding the virulence of influenza viruses. Among numerous species used in the laboratory setting, only the ferret model is equally well suited for studying both the pathogenicity and transmissibility of human and avian influenza viruses. Here, we compare the advantages and limitations of the mouse, ferret and guinea pig models for research with influenza A viruses, emphasizing the multifarious uses of the ferret in the assessment of influenza viruses with pandemic potential. Research performed in the ferret model has provided information, support and guidance for the public health response to influenza viruses in humans. We highlight the recent and emerging uses of this species in influenza virus research that are advancing our understanding of virus-host interactions.

Introduction

Influenza viruses are responsible for seasonal epidemics and sporadic pandemics of varying severity in humans. In a typical year, seasonal influenza viruses cause 250,000 to 500,000 deaths globally (approximately 36,000 in the United States), with >90% of these deaths occurring in the elderly (Thompson et al., 2004). Rates of infection and mortality can increase greatly during a pandemic. The influenza pandemic of 1918-1919 was the worst pandemic of modern times, which eventually infected about one-third of the world's population, killing 20-50 million people (Johnson and Mueller, 2002). Although the recent 2009 H1N1 pandemic virus did not achieve similar levels of mortality (probably owing to both immunological reasons and advances in modern medicine over the past century), both pandemic viruses targeted young, healthy adults – a demographic not usually associated with seasonal influenza-related mortality (Dawood et al., 2009). Highly pathogenic avian influenza (HPAI) H5N1

viruses have caused over 560 human infections, with a 60% mortality rate; among many of these fatalities were young, previously healthy, adults (WHO, 2011). The heterogeneity of influenza viruses in their natural reservoir of wild waterfowl, and the growing diversity of influenza virus lineages affecting mammalian species (including swine, equine, canine and others), suggests that future human infection with viruses of pandemic potential will continue to occur. For these reasons, the availability of small mammalian models to study the virulence of emerging influenza viruses, in addition to understanding the molecular determinants that confer heightened pathogenicity and transmissibility among selected strains, is essential.

Ferret models (*Mustela putorius furo*) have been established for numerous viruses that cause respiratory infections, including human and avian influenza viruses, coronavirus, nipah virus, morbillivirus and others (Bossart et al., 2009; Martina et al., 2003; Smith and Sweet, 1988; Svitek and von

Messling, 2007; Zitzow et al., 2002). Ferrets are an attractive mammalian model for these studies owing to their relatively small size and the fact that they emulate numerous clinical features associated with human disease; this is especially the case with regard to influenza. Ferrets and humans share similar lung physiology, and human and avian influenza viruses exhibit similar patterns of binding to sialic acids (the receptor for influenza viruses), which are distributed throughout the respiratory tract in both species (Maher and DeStefano, 2004; van Riel et al., 2007). The high susceptibility of ferrets to influenza virus, and the ability of sick ferrets to transmit virus to healthy ferrets, was first demonstrated in 1933 (Smith et al., 1933). Since this time, the use of the ferret model has been indispensable in experimental studies to elucidate virus-host interactions following influenza virus infection (Belser et al., 2009; Smith and Sweet, 1988). In this Primer article, we discuss the advantages of using the ferret for research with influenza A viruses, emphasizing the effectiveness of working with a model that can present both the pathogenic and transmissible features of influenza virus infection. We further highlight recent studies that are expanding the utility of the ferret to address novel research questions in this model.

Advantages of the ferret as a small animal model for influenza virus pathogenesis and transmission

Several mammalian models are available for influenza virus research, with their use depending on the specific research questions being addressed. Mice have traditionally been used as a model for influenza virus pathogenesis and offer many advantages over other laboratory species, including low cost, broad availability of reagents and the availability of transgenic mice with targeted gene disruptions (Belser et al., 2009). However, most human influenza strains do not replicate efficiently in mice without prior virus adaptation, limiting the utility of mice as models in which to study this group of

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viruses. Furthermore, many clinical signs of influenza virus infection in humans are not present in mice, and they are a poor model for virus transmission (Lowen et al., 2006; Schulman and Kilbourne, 1963). Guinea pigs have emerged as an alternative small mammalian model for this virus. Unlike mice, they support replication of human influenza viruses without prior adaptation and can be used to model virus transmission to susceptible contact animals (Lowen et al., 2006). Despite this, guinea pigs are a poor model for viral pathogenesis and also lack many clinical signs of infection that are observed in humans (Van Hoeven et al., 2009a). Although not widely used for the study of influenza A viruses, the cotton rat model supports the replication of human influenza viruses without prior adaptation and is suitable for the study of both innate and adaptive immune responses (Eichelberger, 2007; Sadowski et al., 1987). However, similar to the mouse model, cotton rats lack a sneeze reflex and are not well suited to study virus transmissibility (Eichelberger, 2007). In contrast to these small mammalian models, non-human primates offer the ability to study virus infection in an animal that is similarly complex to humans, but size, cost and ethical considerations limit the use of this species in most settings (Kobasa et al., 2007; Rimmelzwaan et al., 2003). Each of the above models offers unique advantages, but none possesses the ability to study both the pathogenesis and transmission of influenza A viruses in most laboratory settings.

The ferret is currently the only small mammalian model available that is equally well suited to studying both of these aspects of influenza pathobiology. Human and avian influenza viruses replicate efficiently in the respiratory tract of ferrets without prior adaptation, and extra-pulmonary spread of virus results following infection with some HPAI viruses. Unlike other small mammalian models, numerous clinical signs found in humans following seasonal or avian influenza virus infection are present in the ferret following experimental inoculation with these strains by the intranasal route (Table 1). Elevated body temperatures are detected as early as 1 day post-inoculation, and more virulent viruses generally elicit high fevers that can persist for several days (Sweet et al., 1979; Zitzow et al., 2002). Nasal discharge and sneezing are also frequently observed in infected ferrets up to 1 week post-

inoculation, with sneezing detected more frequently following seasonal influenza virus infection (Maines et al., 2006). Transmission models have been established using the ferret to measure the capacity of influenza viruses to spread to naïve contact ferrets in the presence of direct contact (i.e. pair-housing an infected and a naïve ferret) or by the spread of respiratory droplets in the absence of direct contact (i.e. separating infected and naïve ferrets with a perforated wall to allow air exchange only) (Herlocher et al., 2001; Maines et al., 2006; Yen et al., 2007). Successful virus transmission is detected by testing contact ferrets for the presence of virus in nasal secretions and seroconversion at the end of the observation period. Daily clinical assessment of inoculated and contact ferrets provides an additional means to evaluate the effects of virus infection and transmission effectiveness.

The ability to use one mammalian model to evaluate both pathogenicity and transmissibility has allowed for a fuller understanding of the capacity of influenza viruses to cause disease, and the ability to place experimental results obtained from this model in the context of human infection. Facilitated by the use of reassortant viruses, research in the ferret model has identified roles for virus gene segments and individual amino acids that contribute towards both virus pathogenicity and transmissibility (Salomon et al., 2006; Tumpey et al., 2007; Van Hoeven et al., 2009b). Furthermore, the efficacy of novel vaccine preparations has been demonstrated in the ferret model: recent studies have demonstrated the ability of vaccination to limit virus spread to susceptible ferret contacts (Ellebedy et al., 2010; Laurie et al., 2010; Pearce et al., 2011). Indeed, ferrets have proved instrumental in the evaluation of vaccine effectiveness and evaluation of vaccine candidates for human use (Bodewes et al., 2010; Robertson et al., 2011; Subbarao and Luke, 2007). Ferret studies have also been instrumental in demonstrating the impact of mutations that confer resistance to antiviral drugs on overall virus fitness and their ability to transmit to uninfected animals (Herlocher et al., 2002; Herlocher et al., 2004; Yen et al., 2005). The polygenic nature of influenza virus pathogenesis and transmission underscores the importance of using a mammalian model capable of assessing both of these parameters.

Recent advances and future uses of the ferret model in influenza research

A greater understanding of influenza viruses and ferret biology as well as the continued development of sophisticated laboratory equipment has led to the ability of scientists to examine numerous properties not previously studied in the ferret model. Below we describe selected areas of investigation that are likely to expand our understanding of influenza virus infection in the coming years.

Aerosol delivery systems

Intranasal administration of virus in a liquid suspension has long been the traditional method for influenza virus inoculation in the ferret model. However, this practice is not truly reflective of 'natural' virus infection in humans, which commonly occurs following contact (including direct and indirect contact with contaminated surfaces) with or inhalation of virus-containing respiratory secretions from an infected individual. Exposure of naïve ferrets to aerosolized influenza virus has been performed using a nose-only inhalation exposure system (Lednický et al., 2010; Tuttle et al., 2010). However, only recently has a rigorous method of ensuring standard doses of virus inoculum during aerosol delivery been reported, and a comprehensive comparison of intranasal and aerosol methods of inoculation been conducted (Gustin et al., 2011). The development of a method to measure the number of viable influenza viruses in droplets and droplet nuclei exhaled from infected animals has furthered the utility of this model (Gustin et al., 2011). The development of influenza virus aerosol delivery systems that rigorously control for virus challenge inoculums, as well as the development of analytical systems for the ferret model, allow a more accurate recreation of natural influenza infection in the laboratory to study influenza virus transmission and perform risk assessments of emerging influenza viruses that pose a threat to public health.

Induction of immune responses

Reports of hypercytokinemia following H5N1 virus infection in humans, resulting in a deleterious 'cytokine storm,' have underscored the need to study the induction of innate immune responses in severely infected hosts (de Jong et al., 2006).

Table 1. Clinical, laboratory and virological features shared by humans and small mammalian models following influenza virus infection

Human clinical feature	Occurrence of clinical features in animal models			References
	Ferrets	Mice	Guinea pigs	
Fever	Present following infection with human and avian influenza viruses	Not detected	Not detected	Sweet et al., 1979; Zitzow et al., 2002
Nasal secretions	Present following infection with human and avian influenza viruses	Not detected	Not detected	Van Hoeven et al., 2009a; Zitzow et al., 2002
Coughing	Sneezing detected more frequently among highly transmissible viruses	Not observed	Generally not observed	Maines et al., 2006; Sun et al., 2010
Gastrointestinal complications (rare)	Present during infection with the 2009 H1N1 virus strain	Not detected	Not detected	Belser et al., 2011; Maines et al., 2009; Sun et al., 2010
Serum abnormalities	Present; more pronounced following infection with virulent viruses	Not examined	Not examined	Belser et al., 2011
Neurological complications (rare)	Present following infection with highly pathogenic avian influenza viruses	Similar to ferret model	Not detected	Lu et al., 1999; Maines et al., 2005
Weight loss and/or anorexia	Present; more pronounced (>10%) following infection with virulent viruses	Similar to ferret model	Minimal (<10%) even following infection with virulent viruses	Lu et al., 1999; Van Hoeven et al., 2009a
Lethargy	Present; more pronounced following infection with virulent viruses	Similar to ferret model	Not detected	Maines et al., 2005; Van Hoeven et al., 2009a
Lymphopenia	Present; more pronounced following infection with virulent viruses	Similar to ferret model	Not examined	Tumpey et al., 2000; Zitzow et al., 2002
Hypercytokinemia	Divergent innate immune responses detected following infection with virulent viruses	Present following infection with virulent viruses	Not examined	Svitek et al., 2008; Tumpey et al., 2000
Transmission to susceptible contacts	Present following infection with most human, but not most avian, influenza viruses	Not reliably detected	Present, generally similar to ferret model	Herlocher et al., 2001; Lowen et al., 2006; Maines et al., 2006; Schulman and Kilbourne, 1963
Distribution of sialic acid (SA) receptors in respiratory tract	Predominance of α 2-6 SA in ferret upper respiratory tract	Predominance of α 2-3 SA differs from human respiratory tissue	High presence of α 2-6 SA in upper respiratory tract	Ibricevic et al., 2006; Leigh et al., 1995; Sun et al., 2010

Shaded regions represent advantageous characteristics of a model.

Moreover, knowledge about specific immune responses to vaccines, which correlate with protection against infection, will help to improve the design of novel vaccine candidates. In vivo assessments of immune function (i.e. innate and adaptive responses) following influenza virus infection have been primarily limited to the mouse model owing to a lack of ferret-specific reagents and incomplete genome sequencing of this species. However, the regulation of ferret proinflammatory mediators has been measured by real-time PCR and microarray analysis (Cameron et al., 2008; Svitek et al., 2008). Canine reagents have also been used successfully to assess ferret host responses, owing to the high levels of homology between canine and ferret nucleotide sequences, and numerous mouse

monoclonal antibodies have demonstrated cross-reactivity with proteins expressed by ferret lymphocytes (Fang et al., 2010; Rutigliano et al., 2008). Continued immunology-based research in the ferret will result in a valuable outbred mammalian model for the study of innate and adaptive immune responses, leading to an improved understanding of human infection.

Laboratory abnormalities

Severe human infection with influenza viruses can result in substantial abnormalities of the lymphohematopoietic system, including lymphopenia, leukopenia, anemia and quantitative alterations of numerous serum analytes. Although lymphopenia has been previously demonstrated in ferrets, comprehensive analysis of white and red

blood cell populations in whole blood from severely infected ferrets has only recently been performed (Belser et al., 2011; Zitzow et al., 2002). Furthermore, comprehensive analyses of serum chemistry profiles have allowed for detailed measurement of numerous parameters in the ferret that are frequently observed in severe human influenza virus infection (Belser et al., 2011). The ability to measure these parameters throughout the duration of an experiment by collecting samples during scheduled anesthesia of ferrets makes this an important contribution towards the standard pathotyping of viruses in this model.

Opportunistic infections

The 1918 H1N1 virus was extraordinarily virulent, but much of the mortality associated

with this pandemic was due to bacterial pneumonia that developed post-infection (Morens et al., 2010). Studies in mouse and ferret models corroborate the finding that previous infection with influenza virus can render a host more susceptible to secondary bacterial infection (Peltola et al., 2006). Although research in the ferret model has been limited to date, recent work has further demonstrated that prior infection with influenza virus can influence the severity and transmissibility of *Streptococcus pneumoniae* in ferrets, a finding that corroborates historical data in humans documenting that the incidence of pneumonia increases during pandemic influenza (McCullers, 2006; McCullers et al., 2010). Although histopathology provides evidence of bacterial involvement following wild-type virus infection, further work is needed to more precisely measure the onset of naturally occurring opportunistic infections following influenza virus infection in this model.

Immunocompromised hosts

Humans with immunocompromised immune systems represent an especially susceptible population to influenza virus infection, and pose numerous challenges with respect to effectively preventing and treating infection. Although ferrets have not been used extensively to research influenza virus infection in the context of immunosuppression, this remains an understudied area that could benefit from such a model. Immunocompromised ferrets (achieved either by treatment with chemotherapy drugs or by depleting CD8⁺ and/or CD4⁺ T cells) were found to safely tolerate live, attenuated influenza vaccines (Huber and McCullers, 2006; Min et al., 2010). Although the research in this model is largely limited to drug- or antibody-induced immunosuppression, additional future studies evaluating influenza virus pathogenicity and transmissibility in the context of immunosuppression should allow for a greater understanding of influenza virulence in immunodeficient populations.

Conclusion

Influenza viruses continue to emerge and cause human infection, so there remains a need to characterize the pathogenicity and transmissibility of these viruses in animal models. This information serves a vital role in public health with respect to evaluating the efficacy of novel vaccines and therapeutics against currently circulating strains. As

Advantages of using ferrets compared with other small mammalian models for research of influenza A viruses

- Ferrets are highly susceptible to both human and avian influenza viruses.
- Pathogenesis and transmission studies can both be performed in the ferret model.
- Numerous clinical signs of human infection with influenza viruses are present in the ferret.
- Ferrets are well suited to vaccine efficacy studies.

described in this Primer article, the ferret is an excellent small mammalian model that can recapitulate many of the hallmarks of human influenza virus infection. Given the wealth of information that can be generated while studying respiratory virus infection in this model, ferrets offer numerous advantages over other small mammalian models and are an ideal laboratory species to measure the most crucial aspects of influenza virus infection in the context of improving public health.

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