Short Communication

Pathogenesis of Influenza A/H5N1 Virus Infection in Ferrets Differs between Intranasal and Intratracheal Routes of Inoculation

Rogier Bodewes,* Joost H.C.M. Kreijtz,*
Geert van Amerongen,* Ron A.M. Fouchier,*
Albert D.M.E. Osterhaus,*†
Guus F. Rimmelzwaan,*† and Thijs Kuiken*

From the Department of Virology,* Erasmus Medical Center,
Rotterdam; and ViroClinics Biosciences B.V.,† Rotterdam,
The Netherlands

Most patients infected with highly pathogenic avian influenza A/H5N1 virus develop severe pneumonia resulting in acute respiratory distress syndrome, with extrapulmonary disease as an uncommon complication. Intranasal inoculation of ferrets with influenza A/H5N1 virus causes lesions in both the respiratory tract and extrapulmonary organs (primarily brain). However, the route of spread to extrapulmonary organs and the relative contribution of extrapulmonary disease to pathogenicity are largely unknown. In the present study, we characterized lesions in the respiratory tract and central nervous system (CNS) of ferrets ($n = 8$) inoculated intranasally with influenza virus A/Indonesia/5/2005 (H5N1). By 7 days after inoculation, only 3 of 8 ferrets had a mild or moderate bronchointerstitial pneumonia. In contrast, all 8 ferrets had moderate or severe CNS lesions, characterized by meningoencephalitis, choroiditis, and ependymitis, and centered on tissues adjoining the cerebrospinal fluid. These findings indicate that influenza A/H5N1 virus spread directly from nasal cavity to brain, and that CNS lesions contributed more than pulmonary lesions to the pathogenicity of influenza A/H5N1 virus infection in ferrets. In comparison, intratracheal inoculation of ferrets with the same virus reproducibly caused severe bronchointerstitial pneumonia. The method of virus inoculation requires careful consideration in the design of ferret experiments as a model for influenza A/H5N1 in humans. (*Am J Pathol* 2011, 179:30–36; DOI: 10.1016/j.ajpath.2011.03.026)

Since the first human cases of infection with highly pathogenic avian influenza A/H5N1 virus were recog-

nized in 1997, infection has been confirmed in more than 500 people, of whom approximately 60% have died (World Health Organization; http://www.who.int/csr/disease/avian_influenza/country/cases_table_2011_03_16/en/index.html, last accessed March 23, 2011). Typically, infected individuals develop a severe pneumonia, which often is fatal.^{1,2} Different experimental animal species also develop severe pneumonia after inoculation with highly pathogenic avian influenza A/H5N1 virus.^{3–7} In addition to the respiratory tract, influenza A/H5N1 virus has been detected in various extrapulmonary organs, particularly the central nervous system (CNS), both in a few human cases and in multiple experimental animal species.^{2,4,6,8–10}

The ferret (*Mustela putorius furo*) is considered a good animal model for influenza virus infection in humans and is frequently used to study the pathogenesis of influenza.^{11–14} In most pathogenesis studies with influenza A/H5N1 viruses in ferrets, intranasal inoculation is performed.^{6,7,15} After intranasal inoculation, it is assumed that the virus spreads from the nose to the lower respiratory tract and, if pathogenic, causes pneumonia. From there, virus might spread systemically and cause extrapulmonary disease, including encephalitis. However, the route of spread to the CNS and the relative contribution of CNS disease to the pathogenicity of influenza A/H5N1 virus infection for ferrets are largely unknown. We therefore designed a study to determine the character and severity of lesions caused by influenza A/H5N1 virus in the respiratory tract and CNS after intranasal inoculation. To this end, ferrets were inoculated intranasally with highly pathogenic avian influenza virus A/Indonesia/5/2005 (H5N1) and multiple tissues were collected for macroscopic, microscopic, and immunohistochemical (IHC) evaluation after eutha-

Supported in part by the VIRGO consortium (T.K.), an Innovative Cluster approved by the Netherlands Genomics Initiative, and in part by the Government of The Netherlands (BSIK 03012).

Accepted for publication March 23, 2011.

Address reprint requests to Thijs Kuiken, D.V.M., Ph.D., Department of Virology, Erasmus Medical Centre, PO Box 2040, 3000 CA Rotterdam, The Netherlands. E-mail: t.kuiken@erasmusmc.nl.

nasal at 7 days post inoculation (dpi). Results were compared with those of a previous study from our research group,¹⁶ in which ferrets had been inoculated intratracheally with the same virus.

Materials and Methods

Virus Preparation

Influenza virus A/Indonesia/5/2005 (H5N1, clade 2.1) was propagated in confluent Madin-Darby canine kidney (MDCK) cells. After cytopathic changes were complete, culture supernatants were harvested, cleared by low-speed centrifugation, and stored at -80°C . The virus titer was determined in MDCK cells as described previously.¹⁷

Experimental Protocol

Healthy outbred female ferrets between 6 and 12 months of age were purchased from a commercial breeder. All ferrets tested negative for the presence of antibodies against recent influenza A/H1N1 and A/H3N2 viruses, influenza virus A/Indonesia/5/2005, and Aleutian disease virus. During the experiment, ferrets were housed together and received food and water *ad libitum*. Eight ferrets were inoculated intranasally with 5×10^6 median tissue culture infectious dose (TCID₅₀) influenza virus A/Indonesia/5/2005 in a total volume of 0.5 mL PBS under anesthesia with ketamine and medetomidine; sedation was reversed with atipamezole. Inoculation was performed by instilling 250 μL inoculum into each nostril while holding the ferret vertically with the head in line with the rest of its body. After instillation of the inoculum, the ferret was laid on its back with the head slightly raised to prevent the inoculum from dripping out of the nose. After inoculation, ferrets were checked daily for the presence of clinical signs. Before inoculation with influenza A/H5N1 virus and at 2, 4, 6, and 7 dpi, ferrets were weighed under anesthesia with ketamine. At 7 dpi, or earlier in case of ferrets that became moribund, animals were weighed and then euthanized by exsanguination under anesthesia with ketamine and medetomidine. Experimental procedures were approved by an independent animal ethics committee and were performed under biosafety level 3 conditions.

Pathological Examination and IHC

Necropsies were performed according to standard procedures. Area of affected lung was estimated visually. Samples of olfactory bulb, cerebrum, cerebellum, brainstem, lungs (all lobes of the right lung and the accessory lobe, after inflation with 10% neutral-buffered formalin), spleen, liver, and duodenum were fixed in 10% neutral-buffered formalin, embedded in paraffin, and sectioned at 4 μm . Tissue sections were stained with H&E for histological evaluation or with an immunoperoxidase method using a monoclonal antibody directed against the nucleoprotein of the influenza A virus for detection of

virus-infected cells. An IgG2a isotype control for each tissue and a positive control tissue were included in each staining procedure.¹⁰

Virology

Nasal swabs were collected from ferrets at 2, 4, 6, and 7 dpi and were stored at -70°C in Hank's balanced salt solution containing 0.5% lactalbumin, 10% glycerol, 200 U/mL penicillin, 200 $\mu\text{g}/\text{mL}$ streptomycin, 100 U/mL polymyxin B sulfate, 250 $\mu\text{g}/\text{mL}$ gentamicin, and 50 U/mL nystatin (ICN Pharmaceuticals, Zoetermeer, The Netherlands). Samples from cerebrum (temporal lobe), lung (left cranial, medial, and caudal lobes), spleen, and duodenum were collected from ferrets at necropsy, snap-frozen on dry ice with ethanol, and stored at -70°C until further processing. Tissue samples were weighed and subsequently homogenized with a FastPrep-24 homogenizer (MP Biomedicals, Eindhoven, The Netherlands) in the same medium as used for nasal swabs and then were centrifuged briefly. Quadruplicate 10-fold serial dilutions of nasal samples and organ samples were used to infect MDCK cells, and hemagglutination activity of the culture supernatants collected 5 dpi was used as indicator of infection, as described previously.¹⁷ The titers were calculated according to the Spearman-Kärber method and were expressed as log TCID₅₀ per gram for tissue or per milliliter for swabs.¹⁸

Comparison of Pulmonary Disease in Intranasally and Intratracheally Inoculated Ferrets

Results of pathological and virological analyses in the lungs of these ferrets were compared with those of six ferrets from a previous study¹⁶ that were inoculated intratracheally with exactly the same batch of highly pathogenic avian influenza virus A/Indonesia/5/2005 (H5N1). In that study, 3 mL of inoculum containing 1×10^5 TCID₅₀ virus was instilled into the trachea by catheter. The higher volume of inoculum was used to facilitate spread into the lungs. The lower dose of virus was used because intratracheal inoculation of this virus is known to be highly lethal to ferrets.¹⁹ Even so, all six ferrets were moribund by 4 dpi and either had died or were euthanized on humane grounds at that time point. Although the volumes used for intranasal (0.5 mL) and intratracheal (3 mL) inoculation differed, they are representative of the currently used methods in experimental influenza virus infections of ferrets.

Presence of gross lung lesions, area of affected lung tissue, relative lung weight, histopathological changes in the lungs, and virus titers in the lungs were measured according to the same methods as used for the intranasally inoculated ferrets. Semiquantitative comparison of pulmonary lesions in both intranasally and intratracheally inoculated ferrets was performed without knowledge of the identity of the ferrets. Four lung sections per ferret (a longitudinal section and a cross-section of the right cranial lung lobe and a longitudinal section and a cross-

Table 1. Comparison of Pulmonary Disease between Ferrets Inoculated Intranasally or Intratracheally with Influenza Virus A/Indonesia/5/2005 (H5N1)

Route of inoculation	Inoculation (TCID ₅₀)	Animals with macroscopic lung lesions (n/N)	Area of lung tissue affected (%; mean ± SD)	Relative lung weight (%; mean ± SD)	Histopathology score (mean ± SD)	Virus titer (TCID ₅₀ /g lung; mean ± SD)
Intranasal	5 × 10 ⁶ in 0.5 mL	3/8	8 ± 14	0.9 ± 0.5	1.1 ± 1.9	1.5 ± 1.1
Intratracheal*	1 × 10 ⁵ in 3 mL	6/6	59 ± 21	1.7 ± 0.2	4.6 ± 1.0	7.4 ± 0.6

Lungs of intranasally inoculated ferrets were examined at 5 to 7 dpi and those of intratracheally inoculated ferrets were examined at 4 dpi.

*Bodewes et al.¹⁶

TCID₅₀, median tissue culture infectious dose.

section of the right caudal lung lobe) were scored for size and severity of inflammatory foci as described previously,²⁰ with the following modification. Five arbitrarily chosen fields in each lung section were examined for the presence of inflammation by light microscopy using a 10× objective. Each field was scored for size of inflamed area (1 = smaller than or equal to the area of a 10× objective, 2 = larger than the area of a 10× objective and smaller than or equal to the area of a 2.5× objective, and 3 = larger than the area of a 2.5× objective) and for severity of inflammation (1 = mild, 2 = moderate, and 3 = marked). The average cumulative value of size and severity of inflammation per field provided the histopathology score per ferret.

Results

Clinical Signs

From 1 dpi onwards, ferrets developed clinical signs including anorexia, diarrhea, and lethargy, as well as neurological signs. Neurological signs consisted of un-

steady gait and uncontrolled movements. All eight ferrets had lost between 13% and 24% of their original body weight by 7 dpi. Two ferrets were found dead, at 5 dpi and at 7 dpi. One ferret was euthanized at 6 dpi because of the presence of severe clinical signs, in accordance with animal welfare regulations.

Macroscopic and Microscopic Observations

At necropsy, three of the eight ferrets had multifocal dark red areas of consolidation in the lungs. The estimated area of affected lung tissue per ferret was 8 ± 14% (mean ± SD) and the relative lung weight was 0.9 ± 0.48% (mean ± SD) (Table 1). No macroscopic lesions were seen in other organs, except for diffuse hepatic pallor in all eight ferrets.

On histological examination, three of the eight ferrets had a mild or moderate multifocal bronchointerstitial pneumonia; the remaining five ferrets had no pulmonary lesions (Figure 1, A and B; Tables 1 and 2). In the affected areas, alveolar lumina were filled with cellular debris, edema fluid, erythrocytes, fibrin, and inflamma-

Figure 1. Comparison of histological lesions and viral antigen expression in lungs of ferrets inoculated with influenza virus A/Indonesia/5/2005 (H5N1) intranasally (A–C; present study) or intratracheally (D–F; Ref. 16). **A** and **B**: Absence of lesions in the lung of an intranasally inoculated ferret. **C**: Absence of cells expressing virus antigen in alveoli of an intranasally inoculated ferret. **Inset**: Rare type II pneumocyte expressing virus antigen. **D**: Bronchointerstitial pneumonia, with inflammatory exudate in bronchiolar and alveolar lumina, in the lung of an intratracheally inoculated ferret. **E**: Neutrophils, macrophages, cellular debris, and erythrocytes in the alveolus of an intratracheally inoculated ferret. **F**: Epithelial cells, mainly type II pneumocytes, expressing virus antigen in alveoli of an intratracheally inoculated ferret. Original magnification: ×200 (A and D); ×1000 (B, C, E, and F).

Table 2. Presence of Histological Lesions and Viral Antigen Expression in Tissues of Ferrets ($n = 8$) Inoculated Intranasally with Influenza Virus A/Indonesia/5/2005 (H5N1)

Tissue	Histological lesion (no. of ferrets positive)	Virus antigen expression (no. of ferrets positive)
Leptomeninges	8	3
Choroid plexus*	5	4
Ependyma*	6	5
Olfactory bulb	8	6
Cerebrum	8	5
Cerebellum	0	0
Brain stem	3	1
Lung	3	3
Spleen	0	0
Liver	0	0
Duodenum	0	0

*Tissue was not collected from one of the eight ferrets.

tory cells (many macrophages and some neutrophils); alveolar walls showed epithelial necrosis, moderate hypertrophy and hyperplasia of type II pneumocytes, and infiltration with moderate numbers of macrophages and neutrophils. Bronchiolar lumina contained cellular debris, macrophages, and neutrophils; bronchiolar walls had loss of epithelium and infiltration with moderate numbers of macrophages and neutrophils. Bronchial lumina contained a few inflammatory cells and fibrin; bronchial walls contained a few inflammatory cells.

All eight ferrets had a moderate or severe diffuse non-suppurative leptomenigitis in cerebrum, cerebellum, brainstem, and olfactory bulb (Figure 2A). The condition was characterized by an infiltrate in the subarachnoid space ranging from 4 to 20 cells in thickness and consisting of many large mononuclear cells (macrophages), a few lymphocytes, and rare neutrophils. Multifocally, there was dissociation of the superficial mesothelial cells of the arachnoid membrane and endothelial hypertrophy of the meningeal blood vessels.

Five of seven ferrets had a moderate or severe, necrotizing choroiditis (tissue was not collected from one of the eight ferrets) (Figure 2D). The connective tissue of affected choroid plexi had necrosis of cells and infiltration, with many macrophages; cellular debris, inflammatory cells, fibrin, and erythrocytes were present on the epithelial surface.

Six of seven ferrets had a moderate or severe necrotizing ependymitis (tissue was not collected from one of the eight ferrets). This was characterized by segmental loss of the ependymal cell lining and infiltration by many macrophages, although at some locations higher proportions of lymphocytes or neutrophils were present (Figure 2G).

All eight ferrets had a multifocal necrotizing encephalitis, which was moderate in cerebrum and olfactory bulb and mild in the brainstem. Affected areas were characterized by infiltration with neutrophils, necrosis of neurons, gliosis, and vacuolization of neuropil (Figure 2, J and M). Affected areas in the cerebrum were superficial and localized adjacent to the inflammatory lesions pres-

ent in leptomeninges, ependyma, and olfactory bulb. Affected areas in the olfactory bulb were present throughout the tissue.

All eight ferrets had mild diffuse hepatic lipidosis, consistent with anorexia. None of the eight ferrets had microscopic lesions in spleen or duodenum.

Expression of Viral Antigen

Virus antigen expression was detected in the CNS of all eight ferrets and in the respiratory tract of three of the eight ferrets (Table 2). In the CNS, moderate or abundant virus antigen expression was detected in mesothelial cells of cerebral leptomeninges overlying the frontal lobe (Figure 2, B and C), in epithelial cells of choroid plexus (Figure 2, E and F) and ependyma (Figure 2, H and I), and in neurons and glial cells of cerebrum (Figure 2, K and L), brainstem, and olfactory bulb (Figure 2, N and O). Of note, virus-positive cells in cerebrum and brainstem were located superficially, adjacent to meninges, ependyma, and olfactory bulb, whereas virus-positive cells in the olfactory bulb were located in the central part of this tissue.

In the respiratory tract, scant virus antigen expression was detected in the lungs, mainly in type II pneumocytes (Figure 1C). None of the eight ferrets showed virus antigen expression in spleen, liver, or duodenum.

Virus Titers in Nasal Swabs and Organs

Influenza virus was isolated from nasal swabs of all eight ferrets at 2, 4, and 6 dpi and in three out of seven ferrets at 7 dpi. Mean virus titers were 4.9 (SD = 0.7) at 2 dpi, 4.7 (SD = 0.6) at 4 dpi, 3.1 (SD = 1.4) at 6 dpi, and 0.6 (SD = 0.2) at 7 dpi. Influenza virus was isolated from lung from two of seven ferrets at 7 dpi (Table 1), but not from cerebrum, spleen, or duodenum of any ferrets. The discrepancy between virological results for the cerebrum and IHC results for this tissue (see Table 2, expression of viral antigen) may be because samples for virus isolation were collected from the parietal lobe, in which virus antigen expression was not observed.

Comparison of Pulmonary Disease in Intranasally and Intratracheally Inoculated Ferrets

From 3 dpi onward, intratracheally inoculated ferrets showed severe clinical signs, including breathing difficulties, lethargy, decreased appetite, and weight loss, but no specific neurological signs.¹⁶ At necropsy, all six intratracheally inoculated ferrets had multifocal or diffuse dark red areas of consolidation in the lungs, affecting a larger area of lung tissue and associated with a higher relative lung weight than in intranasally inoculated ferrets (Table 1). On histological examination, all six intratracheally inoculated ferrets had severe multifocal or extensive bronchointerstitial pneumonia (Figure 1, D and E), with abundant virus antigen expression in alveolar epithelial cells, mainly type II pneumocytes (Figure 1F). The histo-

Figure 2. Histological lesions and viral antigen expression in the CNS of ferrets inoculated intranasally with influenza virus A/Indonesia/5/2005 (H5N1). **A:** Leptomeninges of the cerebrum infiltrated with many inflammatory cells, mainly macrophages. **B and C:** Influenza viral antigen in mesothelial cells of the leptomeninges. **D:** Infiltration of mainly macrophages in the connective tissue of the choroid plexus, and segmental dissociation of the epithelial layer. **E and F:** Influenza viral antigen in epithelial cells of the choroid plexus. **G:** Segmental loss of the epithelial layer of the ependyma, and infiltration of mainly macrophages. **H and I:** Influenza viral antigen in epithelial cells of the ependyma. **J:** Infiltration of inflammatory cells, vacuolation, and neuronal necrosis in the cerebrum. **K and L:** Influenza viral antigen in neurons and glial cells of the cerebrum. **M:** Infiltration of inflammatory cells, vacuolation, and neuronal necrosis in the olfactory bulb. **N and O:** Influenza viral antigen in glial cells and neuronal cells of the olfactory bulb. Original magnification: $\times 400$ (**A, B, D, E, G, H, J, K, M, and N**); $\times 1000$ (**C, F, I, L, and O**).

logical lesions were similar in character to those in intranasally inoculated ferrets, except that there were more inflammatory cells in alveolar, bronchiolar, and bronchial lumina and walls, and more extensive epithelial necrosis in alveolar, bronchiolar, and bronchial walls. From semi-

quantitative analysis, the mean histopathology score for the intratracheally inoculated ferrets was more than four times higher than that for the intranasally inoculated ferrets (Table 1). From virological analysis, infectious virus was detected in all six intratracheally inoculated ferrets at

a mean virus titer approximately five times higher than that in the intranasally inoculated ferrets (Table 1).

Discussion

In the present study, all the ferrets inoculated intranasally with this influenza A/H5N1 virus developed widespread and often fatal CNS disease, characterized by nonsuppurative meningoencephalitis, choroiditis, and ependymitis. Surprisingly for a highly pathogenic respiratory virus inoculated into the respiratory tract, bronchointerstitial pneumonia developed in only a minor proportion of ferrets, and was milder than the CNS disease.

Comparison of our results with those from other influenza A/H5N1 virus infections in experimental animals points to the importance of route of inoculation for primary disease presentation and character of CNS lesions.

First, route of inoculation is important for primary disease presentation. Intranasal inoculation in our ferrets resulted in primary disease of the CNS rather than of the respiratory tract. These findings are consistent with those of previous studies,^{21,22} in which intranasal inoculation of influenza A/Vietnam/1203/04 (H5N1) in ferrets resulted in death from severe neurological disease but caused only mild lung lesions. The authors suggested that these results were likely due to the low virus inoculation dose used [10 or 10² median egg infectious dose (EID₅₀) per ferret], and indicated that higher doses (ie, 10⁶ or 10⁷ EID₅₀) led to more severe pulmonary lesions,⁶ as others have also found.^{23,24} However, in the present study, we used a relatively high dose (5 × 10⁶ TCID₅₀) without inducing severe pulmonary lesions in any ferrets. A possible explanation is that intranasal inoculation results in virus deposition in the lower respiratory tract of some ferrets but not others. In contrast, intratracheal inoculation of a 50-fold lower dose (1 × 10⁵ TCID₅₀) of exactly the same virus stock¹⁶ resulted in severe bronchointerstitial pneumonia associated with influenza virus infection in all ferrets (Figure 1, D–F; Table 1), which had to be euthanized at 4 dpi because of severe respiratory distress. CNS disease was not evaluated pathologically, but was not considered significant at that time point, based on lack of specific neurological signs.

Second, route of inoculation is important for the character of CNS lesions. In our intranasally inoculated ferrets, CNS lesions were centered on tissues adjacent to cerebrospinal fluid in the subarachnoid space and ventricular system (ie, leptomeninges, choroid plexi, and ependyma) and were characterized by prominent infiltration of mononuclear cells; there was lesser involvement of adjacent brain parenchyma. Other studies also have identified the infiltration of mononuclear cells in the meninges of intranasally inoculated ferrets.^{22,23} In contrast, intratracheal inoculation of influenza A/H5N1 virus in cats resulted in randomly distributed foci of necrosis and inflammation in the brain parenchyma, with minor involvement of leptomeninges, choroid plexi, and ependyma.⁵

A possible explanation for these disparities in pathological changes between routes of inoculation is a different route of virus entry into the CNS. As has been sug-

gested previously, intranasal inoculation of influenza A/H5N1 virus in ferrets may lead to direct spread of virus from the nasal cavity, via olfactory nerves through the ethmoidal plate, to the olfactory bulb.⁶ This route of entry has been shown for influenza A/H5N1 virus in mice^{3,25} and for canine distemper virus in ferrets.²⁶ Because the barrier between olfactory bulb and cerebrospinal fluid is weak,²⁷ virus would have easy and rapid access to tissues around the subarachnoid space and ventricular system. This route of entry would explain the prominent involvement of the olfactory bulb, leptomeninges, choroid plexi, and ependyma in our intranasally inoculated ferrets. In contrast, there was evidence of bloodborne spread of influenza A/H5N1 virus in intratracheally inoculated cats,⁵ which might explain the random distribution of lesions in the brain parenchyma and the lesser involvement of tissues around the subarachnoid space and ventricular system in those animals.

In conclusion, we have shown that choice of route of inoculation is critical in designing ferret models to study the pathogenesis of influenza A/H5N1 virus infection in humans. Based on our results with different routes of inoculation of influenza virus A/Indonesia/5/2005 (H5N1) in ferrets, intranasal inoculation would be the route of choice to study influenza A/H5N1-virus-induced CNS disease, whereas intratracheal inoculation may be more appropriate to study influenza A/H5N1-virus-induced lower respiratory tract disease.

Acknowledgments

We thank Peter van Run and animal care takers of the Dutch Vaccine Institute for excellent technical assistance and Debby van Riel and Judith van den Brand for stimulating discussions.

References

1. Writing Committee of the Second World Health Organization Consultation on Clinical Aspects of Human Infection with Avian Influenza A Virus, Abdel-Ghaffar AN, Chotpitayasunondh T, Gao Z, Hayden FG, Nguyen DH, de Jong MD, Naghdaliyev A, Peiris JS, Shindo N, Soerose S, Uyeki TM: Update on avian influenza A (H5N1) virus infection in humans. *N Engl J Med* 2008, 358:261–273
2. Gambotto A, Barratt-Boyes SM, de Jong MD, Neumann G, Kawaoka Y: Human infection with highly pathogenic H5N1 influenza virus. *Lancet* 2008, 371:1464–1475
3. Tanaka H, Park CH, Ninomiya A, Ozaki H, Takada A, Umemura T, Kida H: Neurotropism of the 1997 Hong Kong H5N1 influenza virus in mice. *Vet Microbiol* 2003, 95:1–13
4. Lu X, Tumpey TM, Morken T, Zaki SR, Cox NJ, Katz JM: A mouse model for the evaluation of pathogenesis and immunity to influenza A (H5N1) viruses isolated from humans. *J Virol* 1999, 73:5903–5911
5. Rimmelzwaan GF, van Riel D, Baars M, Bestebroer TM, van Amerongen G, Fouchier RA, Osterhaus AD, Kuiken T: Influenza A virus (H5N1) infection in cats causes systemic disease with potential novel routes of virus spread within and between hosts. *Am J Pathol* 2006, 168:176–183; quiz 364
6. Govorkova EA, Rehg JE, Krauss S, Yen HL, Guan Y, Peiris M, Nguyen TD, Hanh TH, Puthavathana P, Long HT, Buranathai C, Lim W, Webster RG, Hoffmann E: Lethality to ferrets of H5N1 influenza viruses isolated from humans and poultry in 2004 [Erratum appeared in *J Virol* 2006, 80:6195]. *J Virol* 2005, 79:2191–2198

7. Zitzow LA, Rowe T, Morken T, Shieh WJ, Zaki S, Katz JM: Pathogenesis of avian influenza A (H5N1) viruses in ferrets. *J Virol* 2002, 76:4420–4429
8. Gao R, Dong L, Dong J, Wen L, Zhang Y, Yu H, Feng Z, Chen M, Tan Y, Mo Z, Liu H, Fan Y, Li K, Li CK, Li D, Yang W, Shu Y: A systematic molecular pathology study of a laboratory confirmed H5N1 human case. *PLoS One* 2010, 5:e13315
9. de Jong MD, Bach VC, Phan TQ, Vo MH, Tran TT, Nguyen BH, Beld M, Le TP, Truong HK, Nguyen VV, Tran TH, Do QH, Farrar J: Fatal avian influenza A (H5N1) in a child presenting with diarrhea followed by coma. *N Engl J Med* 2005, 352:686–691
10. Rimmelzwaan GF, Kuiken T, van Amerongen G, Bestebroer TM, Fouchier RA, Osterhaus AD: Pathogenesis of influenza A (H5N1) virus infection in a primate model. *J Virol* 2001, 75:6687–6691
11. Bodewes R, Rimmelzwaan GF, Osterhaus AD: Animal models for the preclinical evaluation of candidate influenza vaccines. *Expert Rev Vaccines* 2010, 9:59–72
12. Kuiken T, van den Brand J, van Riel D, Pantin-Jackwood M, Swayne DE: Comparative pathology of select agent influenza A virus infections. *Vet Pathol* 2010, 47:893–914
13. van der Laan JW, Herberts C, Lambkin-Williams R, Boyers A, Mann AJ, Oxford J: Animal models in influenza vaccine testing. *Expert Rev Vaccines* 2008, 7:783–793
14. van Riel D, Munster VJ, de Wit E, Rimmelzwaan GF, Fouchier RA, Osterhaus AD, Kuiken T: Human and avian influenza viruses target different cells in the lower respiratory tract of humans and other mammals. *Am J Pathol* 2007, 171:1215–1223
15. Imai H, Shinya K, Takano R, Kiso M, Muramoto Y, Sakabe S, Ito S, Murakami M, Yamada S, Le MT, Nidom CA, Sakai-Tagawa Y, Takahashi K, Omori Y, Noda T, Shimajima M, Kakugawa S, Goto H, Iwatsuki-Horimoto K, Horimoto T, Kawaoka Y: The HA and NS genes of human H5N1 influenza A virus contribute to high virulence in ferrets. *PLoS Pathog* 2010, 6:e1001106
16. Bodewes R, Kreijtz JH, van Amerongen G, Geelhoed-Mieras MM, Verburgh RJ, Heldens JG, Bedwell J, van den Brand JM, Kuiken T, van Baalen CA, Fouchier RA, Osterhaus AD, Rimmelzwaan GF: A single immunization with CoVaccine HT-adjuvanted H5N1 influenza virus vaccine induces protective cellular and humoral immune responses in ferrets. *J Virol* 2010, 84:7943–7952
17. Rimmelzwaan GF, Baars M, Claas EC, Osterhaus AD: Comparison of RNA hybridization, hemagglutination assay, titration of infectious virus and immunofluorescence as methods for monitoring influenza virus replication in vitro. *J Virol Methods* 1998, 74:57–66
18. Karber G: Contribution to collective treatment of pharmacological experiments. *Naunyn Schmiedebergs Arch Exp Pathol Pharmacol* 1931, 162:480–483
19. van den Brand JM, Stittelaar KJ, van Amerongen G, Rimmelzwaan GF, Simon J, de Wit E, Munster V, Bestebroer T, Fouchier RA, Kuiken T, Osterhaus AD: Severity of pneumonia due to new H1N1 influenza virus in ferrets is intermediate between that due to seasonal H1N1 virus and highly pathogenic avian influenza H5N1 virus. *J Infect Dis* 2010, 201:993–999
20. Haagmans BL, Kuiken T, Martina BE, Fouchier RA, Rimmelzwaan GF, van Amerongen G, van Riel D, de Jong T, Itamura S, Chan KH, Tashiro M, Osterhaus AD: Pegylated interferon-alpha protects type 1 pneumocytes against SARS coronavirus infection in macaques. *Nat Med* 2004, 10:290–293
21. Govorkova EA, Ilyushina NA, Boltz DA, Douglas A, Yilmaz N, Webster RG: Efficacy of oseltamivir therapy in ferrets inoculated with different clades of H5N1 influenza virus. *Antimicrob Agents Chemother* 2007, 51:1414–1424
22. Boltz DA, Rehg JE, McClaren J, Webster RG, Govorkova EA: Oseltamivir prophylactic regimens prevent H5N1 influenza morbidity and mortality in a ferret model. *J Infect Dis* 2008, 197:1315–1323
23. Maines TR, Lu XH, Erb SM, Edwards L, Guarner J, Greer PW, Nguyen DC, Szretter KJ, Chen LM, Thawatsupha P, Chittaganpitch M, Waicharoen S, Nguyen DT, Nguyen T, Nguyen HH, Kim JH, Hoang LT, Kang C, Phuong LS, Lim W, Zaki S, Donis RO, Cox NJ, Katz JM, Tumpey TM: Avian influenza (H5N1) viruses isolated from humans in Asia in 2004 exhibit increased virulence in mammals. *J Virol* 2005, 79:11788–11800
24. Lipatov AS, Kwon YK, Pantin-Jackwood MJ, Swayne DE: Pathogenesis of H5N1 influenza virus infections in mice and ferret models differs according to respiratory tract or digestive system exposure. *J Infect Dis* 2009, 199:717–725
25. Park CH, Ishinaka M, Takada A, Kida H, Kimura T, Ochiai K, Umemura T: The invasion routes of neurovirulent A/Hong Kong/483/97 (H5N1) influenza virus into the central nervous system after respiratory infection in mice. *Arch Virol* 2002, 147:1425–1436
26. Rudd PA, Cattaneo R, von Messling V: Canine distemper virus uses both the anterograde and the hematogenous pathway for neuroinvasion [Erratum appeared in *J Virol* 2006, 80:11416]. *J Virol* 2006, 80:9361–9370
27. Johnston M, Zakharov A, Papaiconomou C, Salmasi G, Armstrong D: Evidence of connections between cerebrospinal fluid and nasal lymphatic vessels in humans, non-human primates and other mammalian species. *Cerebrospinal Fluid Res* 2004, 1:2