Predicting Disease Severity and Viral Spread of H5N1 Influenza Virus in Ferrets in the Context of Natural Exposure Routes

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ABSTRACT

Although avian H5N1 influenza virus has yet to develop the capacity for human-to-human spread, the severity of the rare cases of human infection has warranted intensive follow-up of potentially exposed individuals that may require antiviral prophylaxis. For countries where antiviral drugs are limited, the World Health Organization (WHO) has developed a risk categorization for different levels of exposure to environmental, poultry, or human sources of infection. While these take into account the infection source, they do not account for the likely mode of virus entry that the individual may have experienced from that source and how this could affect the disease outcome. Knowledge of the kinetics and spread of virus after natural routes of exposure may further inform the risk of infection, as well as the likely disease severity. Using the ferret model of H5N1 infection, we compared the commonly used but artificial inoculation method that saturates the total respiratory tract (TRT) with virus to upper respiratory tract (URT) and oral routes of delivery, those likely to be encountered by humans in nature. We show that there was no statistically significant difference in survival rate with the different routes of infection, but the disease characteristics were somewhat different. Following URT infection, viral spread to systemic organs was comparatively delayed and more focal than after TRT infection. By both routes, severe disease was associated with early viremia and central nervous system infection. After oral exposure to the virus, mild infections were common suggesting consumption of virus-contaminated liquids may be associated with seroconversion in the absence of severe disease.

IMPORTANCE

Risks for human H5N1 infection include direct contact with infected birds and frequenting contaminated environments. We used H5N1 ferret infection models to show that breathing in the virus was more likely to produce clinical infection than swallowing contaminated liquid. We also showed that virus could spread from the respiratory tract to the brain, which was associated with end-stage disease, and very early viremia provided a marker for this. With upper respiratory tract exposure, infection of the brain was common but hard to detect, suggesting that human neurological infections might be typically undetected at autopsy. However, viral spread to systemic sites was slower after exposure to virus by this route than when virus was additionally delivered to the lungs, providing a better therapeutic window. In addition to exposure history, early parameters of infection, such as viremia, could help prioritize antiviral treatments for patients most at risk of succumbing to infection.

Since the emergence of highly pathogenic avian influenza H5N1 in humans in 1997, outbreaks have occurred sporadically, primarily in populations associated with direct contact with sick birds (1–4). Currently, outbreaks occur in regions where H5N1 is endemic in migratory birds and where a large proportion of the population rear backyard poultry (5) or visit live-bird markets (4). Transmission to humans can occur through multiple exposure pathways (6), with the potential for infection to be established via aerosol, droplets, or ingestion (5, 7). Currently, it is unclear whether and how exposure routes influence the disease severity of H5N1 infection for people.

In humans, infection with H5N1 has a high case fatality rate of 53% (8), indicating that the pathogenesis differs from that associated with human seasonal influenza viruses. Fatal infection with H5N1 has been associated with acute respiratory distress syndrome (ARDS) (9–11), multiple organ failure (12–18), and encephalitis (19), but overall, few human autopsies have been conducted and it is difficult to determine the contribution of systemic infection to case fatality rates. Some autopsy reports indicate that infection was limited to the respiratory and gastrointestinal tracts (9, 20), while others have isolated virus from the brain and gastrointestinal tract (21) or serum and cerebrospinal fluid (CSF) (22–24) or detected viral mRNA in the brain (21, 25). Measurements from blood samples taken early during the infectious period link severe disease outcomes with lymphopenia and hypercytokinemia (2, 10, 14, 26), while the presence of high loads of negative-sense...
viral RNA (vRNA) in plasma is indicative of systemic infection (24).

In an attempt to prevent severe disease following H5N1 exposure, clinicians are recommended by the World Health Organization (WHO) to treat patients that have confirmed or suspected H5N1 infection with oseltamivir (27). During an H5N1 outbreak, when the supply of oseltamivir may be limited, its prescription may be prioritized to individuals in high-risk exposure groups; for example, people who have had close contact with H5N1 virus-infected birds or patients (28). For a better-informed decision when prioritizing oseltamivir treatment, a more precise understanding of what constitutes high-risk groups is required, because for natural exposure, the source of virus is ambiguous, usually based on an anecdote provided by the patient or the patient’s family (26). Furthermore, the characterization of high-risk exposure is based on the assumption that all mucosal sites of initial viral deposition are equally permissive to infection; however, differences between mucosal sites, such as receptor distribution, temperature, and pH, may contradict this assumption. To identify predictors of lethal infection and understand whether virus-exposure routes influence disease outcome, it may be more precise to utilize animal infection models that are relevant to human H5N1 exposure.

Ferrets develop disease signs similar to those in humans when infected with human isolates of H5N1 virus (29, 30). The hematological, metabolic, and physiological changes detected in ferrets over the course of infection and changes found in laboratory samples from infected patients are alike (31). However, some differences between the two species in response to H5N1 have been noted. Infected humans frequently show progression to ARDS, but neurological disease is rarely manifested (2, 13, 14, 19, 21, 26, 32, 33), while detection of virus in the brain and neurological disease are common in ferrets experimentally infected by the total respiratory tract (TRT) route (34, 35). H5N1 virus spread outside the respiratory tract in ferrets has enabled this model to be employed as a means to study the anatomical pathways of viral dissemination. In the ferret, H5N1 virus can spread via cranial nerves (36, 37) into the olfactory pole (38), a region within the central nervous system (CNS), and likely via the bloodstream (hematogenous route) (39) and lymphatic system (40). These anatomical pathways for dissemination into systemic organs are potentially influenced by the route of initial infection (36). However, current inoculation methods used to infect ferrets do not accurately reflect natural exposure of humans to the virus (6) because the animals are anesthetized prior to inoculation and are infected with large volumes of viral suspension delivered into the nares (29, 30, 35, 39, 41) or directly into the trachea (36). Following these delivery procedures, the inoculum deposits in peripheral lung tissue and, hence, does not model exposure to droplets, which would predominantly deposit in the upper respiratory tract (URT), including the trachea (42). Recent studies employed alternate exposure routes as a means to further characterize viral disease in ferrets (36, 40, 43, 44). One study demonstrated that infection methods using smaller, 0.2-ml volumes can limit the inoculum to the URT and esophagus in the first instance and reduce disease morbidity, virus lung loads, and pulmonary tissue damage for 2009 pandemic H1N1 (pdmH1N1109) and H3N2 variant (H3N2v) viruses compared to these disease parameters with 0.5- to 1.0-ml volumes (44). In the present study, we found that the inoculation route had a negligible impact on mortality rates but modulated the kinetics and locations of H5N1 spread throughout the body, and these data have identified virological markers detectable during a systemic infection that are predictive of severe disease outcomes.

MATERIALS AND METHODS

Virus. A/Vietnam/1203/2004 (H5N1) was propagated in embryonated eggs as previously described (41). Experiments were conducted under biosafety level 3 (BSL3) conditions at Commonwealth Scientific and Industrial Research Organization’s (CSIRO) Australian Animal Health Laboratory (AAHL) at Geelong, Victoria, Australia. Additional experiments were performed using the seasonal H3N2 virus A/Port Chalmers/1/73 virus under physical containment level 2 (PC2) conditions.

Ferrets. All animal experiments were carried out according to the Australian Code of Practice for the Care and Use of Animals for Scientific Purposes with the approval of the CSIRO AAHL Animal Ethics Committee. Female (F) and male (M) ferrets were supplied by the Institute of Medical and Veterinary Science, Adelaide, South Australia, Australia, and were used in this study when 10 to 12 months old and weighing 650 to 1,800 g. Ferrets in each individual experiment were of the same age and sourced from a single cohort.

Ferrets were housed in pairs in Perspex-and-wire cages containing a carpet, polar fleece bedding material, and toys for environmental enrichment. Pre-exposure and terminal sera were collected to determine H5-specific antibody titers, which were measured by hemagglutination inhibition (HI) assay as described previously (45). Ferrets were shown to be seronegative for the currently circulating seasonal strains (H1N1, H3N2, and B viruses) prior to the commencement of the study.

Viral challenge. Ferrets were exposed to H5N1 via different inoculation methods. The inoculation routes were chosen to mimic natural exposure to H5N1 virus that may be encountered by humans during interspecies transmission scenarios. To depict fluid dispersion that may occur during real-life viral exposure, we did not aim to deliver inoculum so that it would be entirely restricted to a specific site but anticipated that some of the inoculum would seep into anatomically linked sites. Viral deposition imparted via upper respiratory tract (URT) and total respiratory tract (TRT) exposure routes was indicated by delivery of Evans blue dye to animals and examination of the dispersion of dye following euthanasia.

URT infections were performed by administering 500 µl of phosphate-buffered saline (PBS) containing 10^8 50% egg infectious doses (EID50) to the nares of sedated ferrets in a dropwise manner as previously described (41). Administering Evans blue dye in this manner demonstrated that the TRT infection method dispered the inoculum throughout the nasal and oral cavities, the trachea, the lungs, and the esophagus. URT infections were performed by administering 50 µl of PBS containing 10^6 EID50 to the nares of conscious ferrets by using a 1-ml syringe. Administering Evans blue dye via this route demonstrated that the inoculum was mainly delivered to the nasal and oral cavities, as well as the pharynx; however, lung access was prevented by throat reflex. To limit experimental variation between animals exposed to virus via the URT route, each ferret’s head was steadied by restraining the animal during infection and the animal handlers performing infections were kept constant across different experiments. The oral route was not only intended to deliver inoculum directly to the stomach but to reflect swallowing of contaminated liquid that would include exposure of the pharynx to the inoculum. To deliver virus orally, 500 µl of PBS containing 10^6 EID50 was placed toward the back of the oral cavity by being slowly dispensed from a 1-ml syringe and was swallowed by the ferrets.

An additional experiment infecting ferrets with 10^4.5 50% tissue culture infective doses (TCID50) of A/Port Chalmers/1/73 virus as a TRT infection was also performed.

Animal monitoring. All ferrets were monitored daily for signs of disease and twice daily if individual animals had decreased activity. Activity in response to play stimulus was scored in gradations of 0.5 between 0 (playful) and 3 (not alert). The disease signs measured included neurological signs, such as prominent hind limb weakness, loss of balance, and
changes in gait, and systemic disease signs, such as hunching and abdominal discomfort. Body weight and rectal temperatures were also measured on days 1, 3, 5, and 7 postinfection. Ferrets were euthanized at a predetermined humane endpoint, which was specified as a greater than 10% loss in body weight and/or the development of signs of neurological or systemic disease that are known to precede fatal disease. The use of this experimental endpoint method, involving the assessment of ferrets at similar disease stages, clarified associations between disease signs, viral infection, and tissue pathology. As part of the study, we also analyzed the effects of preinfection weight and gender, both of which were found to have no effect on time to euthanasia (not shown). Following infection, the majority of ferrets that developed significant illness progressed to the humane endpoint within 0 to 7 days after infection; this factor was used to define two populations of ferrets, referred to as survivors and nonsurvivors.

Sample collection. Ferrets were sedated with ketamine-metomidine (50:50) at 0.1 ml/kg of body weight, delivered by intramuscular injection. Body weights and rectal temperatures were measured, and nasal washes were collected by rinsing the nasal cavity with 1 ml of PBS. Approximately 1 ml of blood was taken from the axillary vein into EDTA or serum separator Vacutainers (BD, Franklin Lakes, NJ, USA). Following sample collection, sedation was reversed with atipemazole, and the ferrets were kept warm to aid recovery. For euthanasia, ferrets were sedated and administered 150-mg/kg pentobarbitone by intracardiac injection. At autopsy, cerebrospinal fluid (CSF) was collected via cisternal puncture. Tissue samples (1 g) were collected from various organs into tubes containing stainless steel beads and 1 ml of PBS with antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, and 30 µg/ml gentamicin). Samples were stored at −80°C. A range of tissue samples were fixed in 10% neutral buffered formalin.

Virus isolation. Virus was freed from tissues by homogenization in a mini-bead beater (FastPrep-24; MP Biomedicals) for 30 s at 4 m/s. The titers of infectious virus in the supernatants of homogenized tissues, nasal washes, and CSF samples were determined by a quantal assay in Vero cells. Samples were serially diluted 10-fold in PBS-azide (PBSA), and each dilution added in quadruplicate to flat-bottom 96-well plates (Nunc-Thermo Fisher Scientific, Australia) containing a monolayer of Vero cells. Plates were incubated for 5 days at 37°C, 5% CO₂, and viral titers determined by scoring each well for cytopathic effect (CPE). The TCID₅₀ was determined by the method of Reed and Muench (46).

Virus in plasma and serum was assayed in a similar manner except that the samples were removed and replaced with fresh medium after 1 h of incubation on the monolayers. Following 5 days of incubation at 37°C, 5% CO₂, the TCID₅₀ was determined as described above. To confirm the presence of infectious virus in serum and plasma, 10-day-old embryo-embryonated hen’s eggs were inoculated with a 1/5 dilution of the axillary vein into EDTA or serum separator Vacutainers (BD, Franklin Lakes, NJ, USA). Following sample collection, sedation was reversed with atipemazole, and the ferrets were kept warm to aid recovery. For euthanasia, ferrets were sedated and administered 150-mg/kg pentobarbitone by intracardiac injection. At autopsy, cerebrospinal fluid (CSF) was collected via cisternal puncture. Tissue samples (1 g) were collected from various organs into tubes containing stainless steel beads and 1 ml of PBS with antibiotics (100 IU/ml penicillin, 100 µg/ml streptomycin, and 30 µg/ml gentamicin). Samples were stored at −80°C. A range of tissue samples were fixed in 10% neutral buffered formalin.

Histology and immunohistochemistry (IHC). Tissue pieces from brain, nasal turbinates, trachea, lung, lymph nodes, spleen, heart, pancreas, liver, and small intestine were fixed in neutral buffered formalin. A single slice of each tissue was processed, with the exception of the URT infection experiments, in which the brain was sectioned into 12 parts in a coronal manner and lung sections were sampled for each lobe. Tissue slices were embedded in paraffin wax, sectioned, and stained as previously described (47). These were examined by light microscopy for the presence of lesions and viral antigen.

Gene quantification by real-time PCR. Viral RNA was extracted from 140 µl of plasma by using the QIAamp viral RNA kit (Qiagen, Hilden, Germany), and the copies of the M gene were quantified using a one-step reverse transcription-PCR kit (Applied Biosystems, USA). This involved the addition of 2 µl of extracted RNA to 6.125 µl of nuclease-free water, 12.5 µl of TaqMan universal PCR master mix, No AmpErase UNG, and 0.625 µl of MultiScribe RNase inhibitor mix. Reverse transcription of negative-sense RNA was performed in the presence of 900 nM forward primer (5′-AGATGAGTCTCTTAACCGAGTTGC-3′) with incubation at 48°C for 30 min. Real-time PCR was conducted by the addition of 900 nM reverse primer (5′-TGAAGACATCTTTGTTTGTG-3′) and 250 nM probe (5′FAM-TCAGGCCCCTCTAAAGCCGA-TAMRA3′ [FAM, 6-carboxyfluorescein; TAMRA, 6-carboxytetramethylrhodamine] (Applied Biosystems). Hot-start Taq polymerase was activated at 95°C for 10 min, and PCR conducted by running 45 cycles of 15 s at 95°C and 1 min at 60°C on the Applied Biosystems 7500 Fast cycler. Tenfold dilutions of A/Vietnam/1203/2004 matrix plasmid were run in each real-time PCR assay in triplicate, and amplification was used to generate standard curves, which were used to quantify the copy number as previously described (48).

Statistical analysis. Statistical analysis was conducted with the software package Prism (version 5.0a) from GraphPad Software, Inc. One-way analysis of variance (1WANOVA) or two-way analysis of variance (2WANOVA) with a 95% confidence interval was used to determine the significance of data. Where appropriate, a repeated-measures (RM) analysis was used. The P values shown were obtained using a Bonferroni or Newman-Keuls posttest. To compare survival curves, the log-rank (Mantel-Cox) test was employed.

RESULTS
Severe disease develops in H5N1 virus-infected ferrets irrespective of exposure route. To examine whether exposure to H5N1 virus via natural routes affected disease severity, ferrets 1 to 8 (4 M and 4 F) were inoculated by seeding a small, 50-µl volume of virus into the upper respiratory tract (URT) to mimic contact-dependent exposure. Viral inocula were also administered orally to ferrets 9 to 16 (4 M and 4 F) to represent the ingestion of H5N1-contaminated food or liquid. For comparison, the same dose of virus was also delivered in a large, 500-µl volume of inoculum to the nares of sedated animals (ferrets 17 to 24; 6 M and 2 F) to infect the total respiratory tract (TRT), as this method is routinely performed in H5N1 pathogenesis studies (29, 30, 36). Following inoculation, temperature, weight loss, and activity scores were assessed periodically as an indication of disease severity, and ferrets were euthanized at the predetermined humane endpoint.

Postchallenge, the URT- and orally infected ferrets exhibited changes in rectal temperatures (Fig. 1A), percentages of body weight loss (Fig. 1B), and activity scores (Fig. 1C) that were comparable to those of the TRT-infected ferrets, suggesting that the severity of induced disease was not dependent upon the exposure route. Although more ferrets reached humane endpoints following TRT infection (6/8 ferrets) than after oral (2/8 ferrets) and URT (4/8 ferrets) exposure, the difference in survival was not statistically significant, even considering the comparison between oral and TRT routes (n = 0.25, Log-rank test) (Fig. 1D).

Viral load in nasal wash of infected ferrets and induction of strain-specific antibodies. The route of exposure had a significant effect on nasal wash viral loads; all ferrets inoculated orally had low titers at 3 and 5 days postinfection (dpi) in comparison to ferrets with direct exposure of the nasal cavity via either URT or TRT routes (P < 0.01) (Fig. 2A). Thus, exposure of the oropharynx as virus is swallowed is insufficient to allow a similar level of infection to spread to the nasal cavity. In addition, by 7 dpi, the majority of URT-infected ferrets had cleared virus from the nose, while TRT-infected ferrets still had viral loads of >2 log₁₀ URT (P < 0.05, 2WANOVA). To further confirm infection had occurred in ferrets that were not euthanized following oral and URT inoculation, sera were assayed for H5N1-specific antibodies. In 11 of 12 survivors, HI titers of 32 or 64 (Fig. 2B) were detected; this is consistent with postinfection antibody at levels considered protective against developing severe disease upon reinfection (41).

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The remaining animal, ferret 15 (oral exposure group), had a lower HI titer of 8 at 14 dpi.

**Pathways involved in systemic infection following oral, URT, and TRT exposure.** To determine whether infection via the different exposure routes resulted in dissemination to systemic sites, we measured viral loads in the organs of ferrets that had reached the humane endpoint. Various tissues were sampled, including those from the upper (nasal turbinates and pharynx) and lower (trachea and lung) respiratory tract, the intestinal tract, and systemic organs comprising the pancreas, spleen, and liver. Lymph nodes, including the pharyngeal, gastropancreatic, and bronchial lymph nodes, were also sampled, as was one section of the central nervous system (CNS), the cerebrum.

Infection of at least one systemic organ beyond the inoculation site was commonly detected in the nonsurvivors regardless of the exposure route (Fig. 2C). The two nonsurviving ferrets in the orally infected group displayed severe neurological disease signs (Fig. 2D) and had significantly higher viral loads in the CNS than those infected via the URT or TRT routes ($P < 0.01$, RM-2WANOVA) (Fig. 2C), although this comparison may be biased by the small number of ferrets that had to be euthanized in the orally infected group. Nonsurviving orally infected ferrets had low viral loads in their nasal washes (Fig. 2A) and also had no detectable viral antigen in the olfactory epithelium and olfactory pole (data not shown). This suggested that viral spread to the CNS occurred via alternative anatomical pathways, such as hematogenously or via the vestibulocochlear nerve, rather than via olfactory receptor neurons as previously described (49).

In contrast, virus was not reisolated from the CNS of URT-inoculated ferrets that developed fatal disease (Fig. 2C), although some ferrets had developed clinical signs suggestive of encephalitis attributable to influenza infection (Fig. 2D). It was unclear whether the absence of reisolation was due to a lack of neuroinvasion following URT infection or, alternatively, was due to sampling only a single site of the brain, the cerebrum, for virus isolation.

**Physiological parameters of severe disease.** The URT route of virus delivery in ferrets appeared most relevant to the study of human infection, it being a plausible natural route of exposure in people. Accordingly, we infected a further 12 female animals (ferrets 25 and 36) via the URT route to more closely investigate disease signs associated with severe disease outcome and clarify whether neuroinvasion did indeed occur following inoculation by this route. After URT exposure to virus, ferrets 29, 30, 32, 33, 35, and 36 were euthanized at the humane endpoint on days 3 to 5 postinfection, and ferrets 25, 26, and 31 were euthanized on day 7. These ferrets were included in the nonsurviving category. Three ferrets (ferrets 27, 28, and 34) were included in the surviving category, as they had only mild or subclinical infection and were
One limitation of this analysis is that data are compared between ferrets euthanized on different days. However, as ferrets were euthanized at similar stages of disease severity, i.e., at predetermined disease endpoints, the use of this experimental method clarified associations between end-stage disease and parameters of infection such as rectal temperatures, tissue damage, and the distribution and magnitude of viral loads.

The percentage of initial body weight was calculated and revealed that the nonsurvivor population had lost significantly more weight than the survivors at 3 to 7 dpi (Fig. 3B). In addition, the nonsurvivors developed significantly higher temperatures (P = 0.0030) (Fig. 3C) throughout infection (2WANOVA). Consistent with decisions around humane endpoint determinations, the activity scores of the nonsurviving population were higher, denoting less activity than in the survivor population (Fig. 3D).

Anatomical parameters of severe disease. The relevance of infection present at particular anatomical locations following URT exposure to virus was examined by measuring and comparing viral load, viral antigen, and lesions in mucosal and systemic sites in the nonsurvivors (ferrets 25, 26, 29 to 33, 35, and 36) and the survivors (ferrets 27, 28, and 34), which were electively euthanized on day 7 postinfection. Analysis of viral loads revealed that the nonsurvivor population of ferrets contained substantially higher virus levels than the survivor population (Fig. 4A, Table 1). This was most apparent in the URT and CNS (P < 0.05, Bonferroni posttest and RM-2WANOVA), but the viral loads in nasal washes (Fig. 4B), at other respiratory sites, and in some organs (Fig. 4A) also exhibited a similar trend that was not statistically supported. Interestingly, infection of the liver was observed in the survivor population and the nonsurvivor population (Fig. 4A), suggesting that involvement of some systemic organs may not always confer poor disease outcome. Unlike the tissue sampling method whose results are shown in Fig. 2, in which a single site of the cerebrum was collected for virus isolation, in this experiment,

killed on day 7 for the purposes of necropsy (Fig. 3A, Table 1). Disease signs, rectal temperatures, activity scores, and viral loads measured for nonsurviving ferrets on days 3 to 7 were compared with similar measurements from surviving ferrets, which did not develop severe disease but were electively euthanized on day 7.

![FIG 3 Disease signs associated with severe disease and poor outcome. Twelve ferrets were challenged via the URT route with 10^6 EID\textsubscript{50} of A/Vietnam/1203/2004. (A) The days postinfection when ferrets reached the humane endpoint and were euthanized are shown. (B and C) The nonsurvivors (ferrets 25, 26, 29 to 33, 35, and 36, dark symbols) and survivors (ferrets 27, 28, and 34, white symbols) were compared for percentages of weight loss (B) and rectal temperatures (C). The mean values (symbols) and SEM (error bars) are shown, and the populations were compared by 2WANOVA. (D) Each ferret was scored for activity in response to play on a scale of 0 (playful) to 3 (not alert).](http://jvi.asm.org/content/90/4/1892)

### TABLE 1 Viral loads of tissues from ferrets infected with H5N1 via the URT route

<table>
<thead>
<tr>
<th>Tissue</th>
<th>Non-survivor (day\textsuperscript{a})</th>
<th>Survivor (day)</th>
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<tr>
<td></td>
<td>30 (3)</td>
<td>32 (3)</td>
</tr>
<tr>
<td>NT</td>
<td>7.50</td>
<td>5.00</td>
</tr>
<tr>
<td>PLN</td>
<td>4.00</td>
<td>1.75</td>
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<tr>
<td>Trachea</td>
<td>&gt;</td>
<td>3.50</td>
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<td>BLN</td>
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<td>Lung</td>
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<td>Liver</td>
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<tr>
<td>Spleen</td>
<td>1.75</td>
<td>&lt;</td>
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<tr>
<td>Pancreas</td>
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</tr>
<tr>
<td>SI</td>
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<tr>
<td>OP</td>
<td>3.75</td>
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<td>Brain\textsuperscript{b}</td>
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<td>CSF</td>
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\textsuperscript{a} NT, nasal turbinates; PLN, pharyngeal lymph node; BLN, bronchial lymph node; SI, small intestine; OP, olfactory pole; CSF, cerebrospinal fluid.

\textsuperscript{b} Euthanasia criteria included >10% weight loss (W) and abdominal (A), neurological (N), and respiratory (R) disease signs.

\textsuperscript{c} Day of humane endpoint or the termination of the experiment on day 7.

\textsuperscript{d} Titer was less than 0.75 log\textsubscript{10} TCID\textsubscript{50}/100 μl, the detection limit of the assay.

\textsuperscript{e} Data are the average log\textsubscript{10} TCID\textsubscript{50}/100 μl values of >10 brain sections.

\textsuperscript{f} Virus growth was also confirmed by inoculation of 0.1 ml CSF into 10-day-old embryonated hens’ eggs.
neurological infection was thoroughly characterized by sampling 12 brain sections from individual ferrets. Sampling in this manner facilitated the detection of infectious virus in the CNS, and surprisingly, the detection of CNS infection was always associated with lethality (Fig. 4A).

To understand the potential pathways of viral invasion into the CNS in the nonsurvivors that developed neurological infection after challenge, viral loads were measured in sequential coronal sections of a hemisphere of the brain. The sections, shown in Fig. 4C as A to L, spanned the olfactory pole, cerebrum, brainstem, and cerebellum. The viral loads in each section are shown irrespective of location for each individual ferret in Fig. 4D. Virus was not reisolated from the brains of just two nonsurviving animals, ferrets 30 and 32 (Table 1), underscoring the frequency of viral spread into the brain following H5N1 infection and that neurological infection was likely to be associated with severe disease. Ferrets 26, 31, and 33 had virus throughout all of the brain sections, with significantly more sites infected than were seen in ferrets 25, 29, 35, and 36 (P < 0.05, RM-1WANOVA and Bonferroni multiple-comparison test). In ferrets 26, 31, and 33, high viral loads were isolated from more than 10 brain sections spanning the olfactory pole, cerebrum, and cerebellum, suggesting that viral invasion into the brain could have occurred via olfactory, vestibulocochlear, or hematogenous pathways. The distribution of viral antigens in the brains of these ferrets was also shown to be widespread by immunohistochemistry, with antigen detected in multiple sections of the cerebrum and choroid plexus (Fig. 4Eii and iv) and associated with encephalitis (4Eiii). Ferrets 26, 31, and 33 also had infectious virus in the cerebrospinal fluid (Table 1), which corresponded to the presence of viral antigens in ependymal cells (Fig. 4Eiv and v) and lesions in the parenchyma adjacent to the 4th ventricle. In contrast to H5N1 infection, infectious virus was not detected by assay of TCID50 in ferret sequential coronal brain sections collected following infection with a seasonal H3N2 influenza A virus, A/Port Chalmers/73 (data not shown), indicating that productive viral infection in brain tissue is not a typical sequela of ferret influenza infection.

Ferrets 25, 29, 35, and 36 contained significantly fewer sites with virus in their brains (P < 0.05, RM-1WANOVA) (Fig. 4D). Where present, viral antigen was sparse in the brain parenchyma and olfactory tracts of these ferrets (Fig. 4Ei), and encephalitis and mild neurological signs were only observed for ferret 36 (Table 1). While ferrets 25, 29, and 35 had relatively mild CNS infection, they reached the humane endpoint on days 3 to 7 postinfection on the basis of >10% weight loss and inactivity. Confirmation of mild focal brain infection in the absence of neurological signs indicates the need for rigorous sampling of the ferret CNS in pathogenicity studies involving exposure via the URT route. In essence, this finding suggests that spread of H5N1 virus into the CNS following URT infection may go undetected, which could also occur in human H5N1 cases that have not been similarly examined.

Following the observation of viral antigen in the choroid plexus, a common site of hematogenous viral spread into the brain, in ferrets 26, 31, and 33, we investigated the viral loads in plasma of ferrets at 1, 3, 5, and 7 dpi. We postulated that ferrets with neurological infection were also likely to contain virus in their bloodstream. Virus was not detected in the plasma of ferrets in the survivors (ferrets 27, 28, and 34) as determined by the absence of matrix vRNA or infectious virus in plasma (Table 2). In contrast, 7/9 animals (ferrets 26, 31, 33, 30, 29, 35, and 36) within the nonsurviving population contained infectious virus in their plasma at some time point after infection, suggesting an association between the presence of infectious virus in plasma and poor disease outcome. Ferrets that had severe neurological infection (ferrets 26, 31, and 33) also contained significantly higher copy numbers of matrix vRNA in plasma than other ferrets without neurological infection (ferrets 30 and 32) and surviving ferrets (P < 0.05, 2WANOVA) (Table 2). The presence of vRNA and...
Viremia precedes systemic and neurological infection. In nonsurviving ferrets, the viremia was detected as early as 1 dpi (Table 2), although it was unclear how the kinetics of viremia related to H5N1 infection of the respiratory tract and the timing of viral spread to systemic organs, such as the spleen, pancreas, and liver. To examine the relationship between respiratory infection, viremia, and systemic spread, we infected an additional eight ferrets (4 M and 4 F) by the URT or the TRT route and measured viral loads at 24 and 72 h postinfection (hpi), time points previously associated with the onset of severe disease (Fig. 1 and 3). The differences between infection profiles at these time points as a consequence of infection route enabled us to investigate the virological events contributing to systemic infection.

Following URT and TRT infection, only very low levels of virus, if any, were evident at 24 hpi in systemic organs and the brain (Fig. 5A and B). Nevertheless, infectious virus was demonstrable in the serum of these ferrets at this early time point (Fig. 5C). At 72 hpi, the viral loads in systemic organs and the brain were increased only in those ferrets infected via the TRT route ($P < 0.01$), whereas the titers in URT-infected ferrets remained low, suggesting that the progression to systemic infection was delayed in ferrets infected via this route (RM-2WANOVA) (Fig. 5A and B).

The viral loads in the respiratory tract may have facilitated systemic spread in ferrets inoculated via the TRT route, as they contained significantly higher viral titers in the lung than did URT-infected ferrets at 24 and 72 hpi ($P < 0.05$ RM-2WANOVA) (Fig. 5D) and three of four ferrets infected via the TRT route had developed necrotizing pneumonia by 24 hpi, which was also a feature in the lungs of TRT-infected ferrets at 72 hpi (data not shown). Another factor contributing to exacerbated systemic infection in ferrets infected via the TRT route could have been higher viral loads in their sera at 24 hpi, although these were not significantly higher than the titers of virus in the sera of URT-infected ferrets (Fig. 5C).

TABLE 2 Viral loads in plasma of H5N1-infected ferrets

<table>
<thead>
<tr>
<th>Population</th>
<th>Ferrets</th>
<th>CNS infection</th>
<th>Copy no.</th>
<th>+ve</th>
<th>Copy no.</th>
<th>+ve</th>
</tr>
</thead>
<tbody>
<tr>
<td>Nonsurvivors</td>
<td>26, 31, 33</td>
<td>$++++^a$</td>
<td>5.03 ± 3.06</td>
<td>2/3</td>
<td>4.76 ± 1.84</td>
<td>2/3</td>
</tr>
<tr>
<td></td>
<td>25, 29, 35</td>
<td>$++$</td>
<td>1.99 ± 0.06</td>
<td>2/4</td>
<td>3.96</td>
<td>1/4</td>
</tr>
<tr>
<td></td>
<td>36</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td></td>
</tr>
<tr>
<td>Survivors</td>
<td>27, 28, 34</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
<td>$-$</td>
</tr>
</tbody>
</table>

$^a$ Number of vRNA copies of the matrix gene, determined by real-time PCR.

$^b$ Number of ferrets positive (+ve) for infectious virus/total number in indicated group.

$^c$ Virus was isolated in 10-day-old embryonated hens’ eggs and in cell culture by TCID$_{50}$ assay.

$^d$ $- -$, severe CNS infection (mean titer of >4 log$_{10}$TCID$_{50}$/100 µl); $+$, mild CNS infection (mean titer of <2 log$_{10}$TCID$_{50}$/10 µl); $-$, no CNS infection.

$^e$ $- - -$, severe systemic infection (mean titer of >6 log$_{10}$TCID$_{50}$/ml of plasma) ± SD or no. of ferrets positive for infectious virus in plasma of ferrets in the nonsurviving population suggested that viremia played an important role in viral spread.

**DISCUSSION**

The ferret infection model is used extensively as a research tool in influenza virus studies, and hence, investigating the experimental factors that could influence the disease course in the ferret is valuable. Recently, one study found that for pdmH1N109 and H3N2v influenza viruses, the use of 0.5- to 1.0-ml volumes for ferret infection, in comparison to 0.2-ml volumes, results in consistent lung infection and uniform disease profiles within a cohort of animals (44). Consistency within these parameters may be important for experimental reproducibility; however, reductionist inoculation methods may not enable the development of the full spectrum of respiratory, neurological, and systemic disease that presents for H5N1-infected humans (33). Therefore, in this study, we have employed the conscious ferret model to develop natural infection routes, such as oral or URT exposure to virus, and we have compared the kinetics of viral spread and the severity of disease with the results of infection via traditional TRT infection methods. Regardless of the exposure route, each ferret infected with H5N1 developed individual disease profiles, characteristic of out-bred animals, including neurological, respiratory, or abdominal disease signs observed between days 3 to 7 postinfection.

For a detailed study of viral pathogenesis, we assessed tissue damage and measured viral loads at predetermined humane endpoints; however, humane endpoints occurred on different days postinfection. Hence, a limitation of our analysis was the comparison of viral titers between ferrets euthanized on different days. In contrast, other researchers study viral pathogenesis by serially sacrificing ferrets on days 1, 3, 5, and 7 and analyze viral loads and tissue pathology in groups of animals that are at different stages of disease (49). Analysis of data from serial euthanasia experiments does not account for the disease status of each ferret, and therefore, the data analyzed are likely to have high standard deviations due to grouping of diseased ferrets, with high viral loads and tissue damage, with nondiseased ferrets, which have undetectable virus loads. Furthermore, this kind of analysis does not enable associations to be made between viral infection and disease, as ferrets euthanized on days 1 and 3 are unlikely to display disease signs.

We regularly observed neurological and systemic disease signs and detected virus in organs outside the respiratory tracts of ferrets irrespective of their inoculation route. These data suggested that humans would also become infected if exposed to H5N1 orally, via direct contact, or via aerosols. This finding corresponds with human epidemiological studies that have identified a variety of risk activities for infection (1, 5, 6). Ferrets exposed to H5N1 via

![FIG 5 Early events of infection predictive of severe disease outcomes.](http://jvi.asm.org/ Downloaded from http://jvi.asm.org/ on August 19, 2019 at MONASH UNIVERSITY)
the oral route showed a trend toward less severe disease outcomes. The majority of orally infected ferrets developed subclinical infections characterized by seroconversion and low levels of viral shedding from the nasopharynx. This finding contrasts with the earlier studies where oral delivery of H5N1-contaminated liquid inocula did not readily establish infection in the ferret unless it was mixed with meat and consumed within a capsule (40) or delivered intragastrically via a feeding tube (43). However, in these early studies, the liquid inocula were delivered directly into the stomach, bypassing the oropharynx. Our findings of subclinical infection in ferrets following oral exposure to H5N1 does, however, correspond with human epidemiological studies that have found a higher prevalence of seropositivity to H5N1 in rural areas that frequently use outdoor reservoirs as a source of water (50).

Our study highlighted that multiple foci of infection develop in ferrets exposed to H5N1 virus via the TRT route, including those that are dispersed throughout the brain and associated with neurological disease signs. This is not a common feature of ferrets exposed to H5N1 virus via the URT route, including those post mortems of H5N1-infected patients (9, 12, 19). During human CNS infection is not consistently detected, as shown by various studies where oral delivery of H5N1-contaminated liquid inocula did not readily establish infection in the ferret unless it was mixed with meat and consumed within a capsule (40) or delivered intragastrically via a feeding tube (43). However, in these early studies, the liquid inocula were delivered directly into the stomach, bypassing the oropharynx. Our findings of subclinical infection in ferrets following oral exposure to H5N1 does, however, correspond with human epidemiological studies that have found a higher prevalence of seropositivity to H5N1 in rural areas that frequently use outdoor reservoirs as a source of water (50).

In addition to the overt neurological disease and readily detectable CNS infection common for ferrets infected via the TRT route, we found that focal CNS infection was common for ferrets inoculated via the URT route, which could easily be overlooked unless several sections spanning multiple anatomic regions of the brain were sampled for infectious virus and viral antigen. In humans, CNS infection is not consistently detected, as shown by various post mortems of H5N1-infected patients (9, 12, 19). During human CNS infection, however, 1 or 2 sites of the CNS are usually sampled for detection of infectious virus (9, 17), which is similar to the sampling procedure used during our initial ferret experiment, whose results are presented in Fig. 2, where only the cerebrum was examined. Our findings support the idea that thorough sampling of the brain lobes, brainstem, and cerebellum is important to understand the complete extent of human CNS infection (21). In the absence of thorough sampling, CNS infection in humans exposed to virus via the URT route may also remain undetected, and neurological infection may, perhaps inappropriately, be considered a rare outcome of H5N1.

In addition, CNS infection was detected in the cerebrum of 2 of 8 ferrets that were infected orally. In these two animals, virus was detected at very high loads in the CNS, which indicated neurological infections that were greater in severity than those in TRT-inoculated ferrets; however, this finding may be influenced by only including viral titers from ferrets that reached the humane endpoint, which would limit the standard deviations. The absence of viral antigens and lesions in the olfactory epithelium and bulb of these two orally infected ferrets suggested that neuroinvasion did not occur via spread of virus along the olfactory tract but by alternative anatomical pathways, such as the vestibulocochlear nerve (38) or even the hematogenous route (40). Viremia may have occurred following oral infection but, as this was not directly tested in this experiment, involvement of this pathway for H5N1 neuroinvasion remains unclear.

Many studies examining H5N1 infection of ferrets (39) and humans (19, 22, 24, 53) have attempted to delineate the association between hematogenous spread of the virus and severe disease outcomes. These studies have not measured infectious viral loads in blood but, rather, have quantified vRNA copy numbers by real-time PCR. However, this method does not discriminate between noninfectious particles shed into the bloodstream from distal sites of infection and infectious viral particles. Here, we have shown that the presence of low levels of infectious virus in plasma of ferrets was associated with a poor disease outcome, in support of previous studies that have also shown an association between the presence of vRNA in blood and lethal disease outcomes (39). Furthermore, the detection of vRNA and virus in plasma as early as 24 hpi suggests that viremia is key to early dissemination of virus and that this event could be utilized as an early warning sign of poor disease outcome and might provide the impetus to increase the dose of oseltamivir used (28), administer combined antiviral therapies, or even deliver passive antibodies (33) in order to reduce systemic viral loads.

Due to the contribution of viremia to viral spread and severe disease outcomes, it is not surprising that different routes of infection will lead to systemic infections that can spread to the CNS. In ferrets inoculated by the URT route, however, systemic spread was delayed, and this factor may provide a longer therapeutic window for effective oseltamivir treatment. An additional application of the URT infection route, due to focal replication within the low-pH conditions of the mammalian nasal passage (54), includes growth characterization and selection of H5N1 viruses with acid-stable hemagglutinins, such as those mutants identified in previous transmission studies (55) or mutants isolated from infected patients (56). This study suggests that exposure route is an important factor that needs to be taken into consideration when evaluating antiviral regimes and investigating antiviral and vaccine efficacy in the ferret model.

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38. Gustin, Wang


