Novel Avian-Origin Human Influenza A(H7N9) Can Be Transmitted Between Ferrets via Respiratory Droplets

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The outbreak of human infections caused by novel avian-origin influenza A(H7N9) in China since March 2013 underscores the need to better understand the pathogenicity and transmissibility of these viruses in mammals. In a ferret model, the pathogenicity of influenza A(H7N9) was found to be less than that of an influenza A(H5N1) strain but comparable to that of 2009 pandemic influenza A(H1N1), based on the clinical signs, mortality, virus dissemination, and results of histopathologic analyses. Influenza A(H7N9) could replicate in the upper and lower respiratory tract, the heart, the liver, and the olfactory bulb. It is worth noting that influenza A(H7N9) exhibited a low level of transmission between ferrets via respiratory droplets. There were 4 mutations in the virus isolated from the contact ferret: D678Y in the gene encoding PB2, R157K in the gene encoding hemagglutinin (H3 numbering), I109T in the gene encoding nucleoprotein, and T10I in the gene encoding neuraminidase. These data emphasized that avian-origin influenza A(H7N9) can be transmitted between mammals, highlighting its potential for human-to-human transmissibility.

Keywords. H7N9 influenza virus; pathogenicity; transmissibility; animal models.

In March 2013, a novel avian-origin influenza A(H7N9) strain was recognized as the causative agent of influenza-like illnesses in humans in eastern China [1]. Most patients presented with respiratory infections that progressed to severe pneumonia and dyspnea [1]. The majority of the patients had a history of contact with live poultry, and cross-species poultry-to-person transmission of this virus has been proven [2]. There is a great concern that this virus may evolve to become transmissible among humans and lead to another influenza pandemic. Limited person-to-person transmissions were observed in the outbreak of influenza A(H7N7) infection in the Netherlands in 2003 [3]. Three suspected family clusters of cases of influenza A(H7N9) infection had been reported in China [4], which may indicate the possibility of limited human-to-human transmission of this influenza A virus subtype.

Sequencing analyses in both recent studies revealed a substitution of Q226L (H3 numbering) at the 210 loop in the influenza A(H7N9) hemagglutinin (HA) gene in all patients except the first patient with laboratory-confirmed infection [1, 2]. This site increases or decreases the affinity for human-type receptor and might increase the ability of the virus to be more transmissible by airborne routes [5, 6]. The first 4 influenza A(H7N9) isolates from humans contained either PB2-627K or 701N, which are important for the efficient replication of avian influenza A(H5N1) and influenza A(H7N7) in mammals [7]. These findings indicated that influenza A(H7N9) strains possess some properties of human-adapted influenza viruses [8]. Therefore, it is important to evaluate the pathogenic properties and potential of transmissibility of this virus via respiratory droplets in animal models.

In this study, a ferret model (Mustela putorius furo) was used to study the pathogenesis (including clinical signs, virus shedding, tissue dissemination, and pathology) of influenza A (H7N9), compared with that of a highly pathogenic avian influenza A(H5N1) strain and 2009 pandemic influenza A(H1N1) (hereafter, “influenza A[H1N1]pdm09”). Airborne transmissibility of influenza A(H7N9) was also evaluated.

METHODS AND RESULTS

Four groups of 3 ferrets were inoculated intranasally either with 10^6 median tissue culture infective doses (TCID50) of influenza...
A/Anhui/1/2013(H7N9), 10⁶ TCID₅₀ of influenza A/Anhui/1/2013(H7N9), 10⁶ TCID₅₀ of influenza A/California/07/2009 (H1N1), or 10⁴ TCID₅₀ of influenza A/Vietnam/1203/2004 (H5N1). The animals were observed for clinical signs and weighed daily as an indicator of disease. All 3 viruses caused sneezing, ruffled fur, lethargy, and decreased appetite for food in ferrets. One ferret inoculated with influenza A(H5N1) died on day 4 after inoculation, and no ferrets inoculated with influenza A(H7N9) or influenza A(H1N1)pdm09 died. The age of ferrets challenged with influenza A(H5N1) were a few months older than others, which may resulting in decreased susceptibility to influenza virus infection and lower mortality, compared with younger ferrets. The mean maximum weight loss during the 14-day period after inoculation was 10.9% and 9.2% for animals inoculated with 10⁶ or 10⁵ TCID₅₀, respectively, of influenza A(H7N9), 8.2% for those inoculated with influenza A(H1N1)pdm09, and 13.0% for those inoculated with influenza A(H5N1) (Table 1 and Figure 1A). The clinical condition of the animals infected with influenza A(H7N9) or influenza A(H5N1) improved beginning 5 days after inoculation. In comparison, for ferrets inoculated with influenza A(H1N1)pdm09, their clinical conditions showed improvement beginning 4 days after inoculation. Furthermore, chest radiography was performed by micro–computed tomography for all 3 animals inoculated with 10⁸ TCID₅₀ of influenza A(H7N9), and results showed that there were inflammatory lesions (mild to bilateral ground-glass opacity) in the left superior lobe and the right superior and middle lobes from days 6–14 after inoculation (Figure 1C).

Nasal and throat swab specimens were collected from inoculated animals on days 1, 3, 5, 7, and 9 after inoculation, and virus titers were determined by end point titration in Madin-Darby canine kidney (MDCK) cells. Virus shedding was observed to start on day 1 after inoculation for all viruses and continued until day 7 after inoculation for influenza A(H7N9)–infected ferrets. Virus shedding in nasal swab specimens from the upper respiratory tract of animals inoculated with influenza A(H1N1)pdm09 were generally higher than those from animals inoculated with influenza A(H7N9) or influenza A(H5N1) (P < .05, by the t test), with the exception for the nasal swab specimens collected on day 5 after inoculation, in which shedding was comparable for all 3 viruses (P > .05, by the t test). Meanwhile, for throat swab specimens, virus shedding in animals inoculated with influenza A(H1N1)pdm09 were higher than those from animals inoculated with influenza A(H7N9) or influenza A(H5N1) (P < .05, by the t test; Figure 1B).

For animals inoculated with 10⁶ TCID₅₀ of influenza A(H7N9), 2 randomly selected animals were euthanized, one on days 3 and the other on day 7 after inoculation, and for animals inoculated with influenza A(H1N1)pdm09 or influenza A

<table>
<thead>
<tr>
<th>Virus Subtype</th>
<th>Clinical Sign</th>
<th>Weight Lossa</th>
<th>Sneezingb</th>
<th>Lethalityc</th>
<th>Time to Euthanization, dd</th>
<th>Virus Titer (Log₁₀ TCID₅₀/g Tissue)</th>
<th>Trachea Lung Heart Brain Spleen Kidney Intestine Olfactory Bulb</th>
</tr>
</thead>
<tbody>
<tr>
<td>A(H7N9)</td>
<td></td>
<td>6/6 (10.9/9.2)</td>
<td>3 6/6</td>
<td>0/3 3</td>
<td>4.54 2.37 Neg 3.19 Neg</td>
<td>A(H7N9)</td>
<td>Neg 3.19 Neg Neg Neg Neg Neg Neg Neg Neg Neg Neg 5.41</td>
</tr>
<tr>
<td>A(H1N1)pdm09</td>
<td></td>
<td>3/3 (8.2)</td>
<td>2/3 0/3 5</td>
<td>3.92 2.65 Neg</td>
<td>1.91 1.51 Neg Neg Neg ND Neg</td>
<td>A(H1N1)pdm09</td>
<td>Neg 1.91 Neg Neg Neg Neg Neg Neg Neg Neg Neg Neg 1.87</td>
</tr>
<tr>
<td>A(H5N1)</td>
<td></td>
<td>3/3 (13.0)</td>
<td>3/3 1/3 5</td>
<td>5.17 3.83 4.92 1.80 Neg</td>
<td>3.81 1.30 1.30 1.82 ND ND</td>
<td>A(H5N1)</td>
<td>Neg 3.81 Neg Neg Neg Neg Neg Neg Neg Neg Neg Neg 1.82</td>
</tr>
</tbody>
</table>

Abbreviations: A(H1N1)pdm09, 2009 pandemic influenza A(H1N1); Neg, negative; ND, not done; TCID₅₀, median tissue culture infective dose.

a Data are no. in which weight loss was observed/total no. observed (% mean maximum weight loss after inoculation).
b Data are no. in which sneezing was observed after inoculation/total no. observed.
c Data are no. euthanized after reaching a clinical end point before the end of the 14-day experimental period/total no. observed.

A value of 10.9% was observed for animals inoculated with 10⁶ TCID₅₀ of A(H7N9), and 9.2% was observed for animals inoculated with 10⁵ TCID₅₀ of A(H7N9), and 8.2% for those inoculated with influenza A(H1N1)pdm09, and 13.0% for those inoculated with influenza A(H5N1) (Table 1 and Figure 1A). The clinical condition of the animals infected with influenza A(H7N9) or influenza A(H5N1) improved beginning 5 days after inoculation. In comparison, for ferrets inoculated with influenza A(H1N1)pdm09, their clinical conditions showed improvement beginning 4 days after inoculation. Furthermore, chest radiography was performed by micro–computed tomography for all 3 animals inoculated with 10⁸ TCID₅₀ of influenza A(H7N9), and results showed that there were inflammatory lesions (mild to bilateral ground-glass opacity) in the left superior lobe and the right superior and middle lobes from days 6–14 after inoculation (Figure 1C).
Figure 1. Pathogenesis and transmission of influenza A(H7N9) in ferrets. A, Weight loss of individual inoculated ferrets. Four groups of 3 ferrets were inoculated intranasally either with $10^6$ or $10^8$ median tissue culture infective doses (TCID$_{50}$) of influenza A/Anhui/1/2013(H7N9), $10^6$ TCID$_{50}$ of influenza A/California/07/2009(H1N1), or $10^4$ TCID$_{50}$ of influenza A/Vietnam/1203/2004(H5N1). Body weight of individual ferrets is indicated as a percentage of their weight at the start of the experiment. For animals inoculated with $10^6$ TCID$_{50}$ of influenza A(H7N9), one randomly selected animal was euthanized separately on days 3 and 7 after inoculation, and for animals inoculated with 2009 pandemic influenza A(H1N1) (hereafter, “influenza A[H1N1]pdm09”) or...
(H5N1), all surviving animals were euthanized on day 5 after inoculation and used for pathologic and virologic examination of the trachea, lung, brain, heart, liver, spleen, kidney, stomach, intestine, and olfactory bulb.

Histopathologic analyses revealed that, on day 3 after inoculation, lung tissue from ferrets inoculated with 10^6 TCID_{50} of influenza A(H7N9) had a multifocal mild or moderate interstitial inflammatory hyperemia and exudative pathologic changes, and bronchitis could been seen in part of lung. Analyses of specimens from day 7 after inoculation revealed larger lung tissue lesions, and fusing of multiple patchy lesions was observed (Figure 1D). The ferrets inoculated with influenza A(H1N1)pdm09 displayed interstitial pneumonia similar to that of ferrets inoculated with influenza A(H7N9). However, ferrets inoculated with influenza A(H5N1) exhibited severe exudative necrotizing pneumonia. Inflammatory hyperemia, hemorrhage, edema, and necrotic pathologic changes could be observed in the lung tissues (Figure 1E). Impaired and necrotic pathologic changes were also observed in the tissues of liver, kidney, brain, and spleen from influenza A(H5N1)–infected ferrets. Immunohistochemical analysis was also performed to assess the presence of influenza A(H7N9)–infected cells in tissues, including bronchial epithelial cells and alveolar epithelial cells, from infected animals (Figure 1D). In ferrets inoculated with influenza A(H7N9), infected cells were in the same area of the lung as cells infected with influenza A(H1N1)pdm09. By contrast, profound numbers of infected cells were detected in lung tissues of ferrets that were inoculated with influenza A(H5N1) (Figure 1E).

Parts of the tissues described above were homogenized, and virus titers were determined by end point titration in MDCK cells. For animals inoculated with influenza A(H7N9) and euthanized on day 3 after inoculation, virus could be detected in the lung, trachea, heart, and olfactory bulb. On day 7 after inoculation, influenza A(H7N9) could be isolated from the trachea, lung, and liver. In comparison, influenza A(H1N1)pdm09 could be detected in the lung and intestine on day 5 after inoculation, whereas influenza A(H5N1) was detected in the trachea, lung, brain, heart, kidney, and intestine on day 5 after inoculation (Table 1).

Transmissibility of influenza A(H7N9) and influenza A (H5N1) via respiratory droplets was tested in ferrets. Transmission experiments were conducted in cages designed to prevent direct contact between animals but allow airflow between an inoculated ferret and a neighboring influenza virus–naïve ferret (Figure 1F). Three ferrets each in 2 groups were inoculated with 10^6 TCID_{50} of influenza A/Anhui/1/2013(H7N9) or 10^4 TCID_{50} of influenza A/Vietnam/1203/2004(H5N1). Twenty-four hours
after inoculation, each animal was housed individually with a naive ferret in a transmission cage to test the transmissibility of virus. Nasal and throat swab specimens were collected from inoculated and naive animals on days 1, 3, 5, 7, 9, 11, and 13 after inoculation or exposure. Shedding of influenza A(H7N9) was detected beginning on day 1 after inoculation in nasal and throat swab specimens from inoculated ferrets, with virus titers of up to $10^{3.94}$ TCID$_{50}$/mL on day 5 after inoculation (Supplementary Table 1). Influenza A(H7N9) was no longer detectable in nasal and throat swab specimens beginning on day 9 after inoculation (Figure 1G). Notably, influenza A(H7N9) was transmitted from the inoculated to naive ferrets in 1 of 3 transmission experiments. Transmission was detected 7–11 days after placing the naive ferret in the cage adjacent to the inoculated ferret (Figure 1G). Peak virus shedding reached $10^{4.01}$ TCID$_{50}$/mL (on day 9 after exposure), and sneezing, high body temperature (40.4°C on day 9 after exposure), and significant weight loss (12.9% on day 13 after exposure) were observed in a previously naive ferret that acquired infection (Figure 1H). Meanwhile, titers of influenza A (H7N9)–specific antibodies in convalescent-phase serum specimens from an infected, previously naive ferret reached 640 by the hemagglutination inhibition (HI) assay, while the HI titer was only 20 for 2 uninoculated naive ferrets (Supplementary Table 1). By contrast, no evidence of influenza A(H5N1) transmission was observed in naive ferrets, although shedding of influenza A(H5N1) was detected beginning on day 1 after inoculation in nasal and throat swab specimens from inoculated ferrets, with virus titers of up to $10^{3.88}$ TCID$_{50}$/mL on day 5 after inoculation (Figure 1I and Supplementary Table 1).

Sequencing analyses showed that there were 4 amino acid substitutions in the virus isolated from the airborne contact ferret: R148K in the HA gene, T101I in the neuraminidase gene, I109T in the nucleoprotein gene, and D678Y in the PB2 gene (Supplementary Table 2). A series of recombinant viruses should be constructed by reverse genetics to confirm whether any of these substitutions may be critical to the transmissibility of influenza A(H7N9) in mammals.

**DISCUSSION**

Cross-species transmission of influenza A(H7N9) from poultry to persons has been proven in a recent study [2]. It is noticeable that only a portion of the patients had a history of contact with live poultry. Furthermore, 3 suspected family clusters of human cases had been reported. Thus, there is great concern about the possibility of human-to-human transmission of avian-origin influenza A(H7N9). Our results indicated that the novel avian-origin influenza A(H7N9) strain could replicate efficiently in the upper and lower respiratory tracts of infected ferrets and could be transmitted between ferrets via respiratory droplets, although the transmissibility potential is low, with transmission occurring in only one-third of tested animals.

The incubation period for the naive ferret that was infected with influenza A(H7N9) via respiratory droplets lasted 7 days after exposure, which correlated with clinical observations that the time between the onset of symptoms and initial exposure was generally >6 days [1, 2]. A recently published study by Zhu et al also showed that one-third of naive ferrets could be infected with influenza A/Shanghai/2/2013(H7N9) via airborne transmission; however, the incubation period in naive ferrets was increased, from 3 to 8 days after exposure [10]. More recently, Zhang et al reported that virus could be detected in 3 exposed ferrets that also had been inoculated with influenza A/Anhui/1/2013(H7N9), although the exposed ferrets did not show marked changes in body temperature and weight, with no detectable amino acid changes [11]. The discrepant results may be attributable to the different strains used in each study and to individual differences among the ferrets.

Compared with other zoonotic influenza viruses, such as influenza A(H5N1), that cause disease that regularly results in severe and often fatal outcomes in the ferret model, influenza A (H7N9) caused less severe disease without mortality in our ferret model. However, our data indicated that it had comparable pathogenicity to influenza A(H1N1)pdm09 in ferrets. This study provided important evidence of the pathogenic properties and transmissibility of influenza A(H7N9) in the ferret model.

**Supplementary Data**

Supplementary materials are available at The Journal of Infectious Diseases online (http://jid.oxfordjournals.org/). Supplementary materials consist of data provided by the author that are published to benefit the reader. The posted materials are not copyedited. The contents of all supplementary data are the sole responsibility of the authors. Questions or messages regarding errors should be addressed to the author.

**Notes**

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**Potential conflicts of interest.** All authors: No reported conflicts.

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**References**


