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# Pathogenesis of novel reassortant avian influenza virus A (H5N8) Isolates in the ferret ☆

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ABSTRACT

Outbreaks of avian influenza virus H5N8 first occurred in 2014, and spread to poultry farms in Korea. Although there was no report of human infection by this subtype, it has the potential to threaten human public health. Therefore, we evaluated the pathogenesis of H5N8 viruses in ferrets. Two representative Korean H5N8 strains did not induce mortality and significant respiratory signs after an intranasal challenge in ferrets. However, ferrets intratracheally infected with A/broiler duck/Korea/Buan2/2014 virus showed dose-dependent mortality. Although the Korean H5N8 strains were classified as the HPAI virus, possessing multiple basic amino acids in the cleavage site of the hemagglutinin sequence, they did not produce pathogenesis in ferrets challenged intranasally, similar to the natural infection route. These results could be useful for public health by providing the pathogenic characterization of H5N8 viruses.


Influenza viruses have a broad host range including birds and mammals. Highly pathogenic avian influenza virus (HPAI) is very contagious and induces multiorgan systemic disease in poultry leading to mortality (Swayne and Suarez, 2000). According to the World Health Organization (WHO), human infections by the H5N1 strain of HPAI have increased and show a high mortality of up to 59% (WHO/GIP, 2014). Most human cases were attributed to direct contact with H5N1 from infected poultry. Although human-to-human transmission has been very rarely reported, the H5 varieties of HPAI are still considered to be the most likely candidates for human pandemics (Shinya et al., 2006). In Korea, outbreaks of HPAI H5N1 have been reported four times in poultry farms since 2003 and have caused great concern for public health and the economy (Kim Hye-Ryoung et al., 2012). However, to date, there has been no human case of avian influenza including H5N1 in Korea.

Some of the H5 and H7 influenza viruses have multiple basic amino acids in the hemagglutinin (HA) cleavage site, which is a genetic feature of HPAI (Senne et al., 1996). Most of the HPAI strains produced considerable mortality in chickens with an avian receptor ($\alpha$2,3-linked sialic acid) distribution, predominantly in the respiratory tract (Salomon et al., 2006). Ferrets and mice are widely used to characterize the pathology of influenza viruses (Belser et al., 2007). In particular, ferrets are known to be susceptible to influenza virus infection and show fever and clinical respiratory signs such as nasal discharge, sneezing, and coughing, like humans (Maher and Joanne, 2004). Therefore, the ferret is the most critical animal model to evaluate the pathogenicity of newly emerging influenza viruses. The recent H5N1 strain has produced high mortality in human because most individuals do not have any T cell memory against it, unlike seasonal influenza viruses. Furthermore, the H5 varieties of HPAI are antigenically evolved to interact with the human ($\alpha$2,6-linked sialic acid) receptors without the need for an antigenic shift in an intermediate host (Herfst et al., 2012).

Amid general concern about potential pandemics caused by H5N1, a novel reassortant H5N8 HPAI infection occurred in a duck breeding farm in Gochang province, in the southern part in Korea, and spread throughout the country in 2014 (Lee et al., 2014). The first H5N8 outbreak was reported in Ireland in 1983 (Swayne, 2008) where turkeys were the most susceptible hosts to H5N8 infection but ducks could not produce the virus. Following an H5N8 outbreak in China in 2010, it was reported that novel H5N8 viruses arose by continuous genetic reassortment and evolution (Zhao et al., 2013).

To evaluate the risk of human infection by H5N8, it is important to understand the characteristics of the virus and prepare con trol measures against the next round of pandemics. Therefore, we evaluated pathogenesis in ferret model of the novel reassortant HPAI H5N8 viruses isolated from poultry in Korea in 2014.

☆Two H5N8 Korean isolates had low pathogenesis in intranasally challenged ferrets.

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## Results

### *Pathogenicity of Korean H5N8 isolates in ferrets*

When ferrets were challenged intranasally with H5N8 viruses, they did not show any mortality in either group. Half of ferrets among 8 numbers of Buan2-infected started to show mild respiratory signs such as sneezing and clear nasal discharges after 3 dpi, whereas the Gochang1-infected ferrets showed no clinical respiratory signs. The highest body weight losses were similar in both Buan2-infected ferrets (8.3% at 10 dpi.) and Gochang1-infected ferrets (7.5% at 10 dpi.). Fever was higher in the Buan2-infected ferrets (1.6 °C at 5 dpi.) than in the Gochang1-infected ones (0.7 °C at 5 dpi.) (Table 1). Buan2 virus infected ferrets maintained fever for 7days and Gochang1 virus infected one returned to normal temperature at 6 dpi. Viruses were shed in nasal washes for 5 dpi in Buan2-infected ferrets but only at 1 dpi in Gochang1-infected ferrets (Table 2). Viruses were detected in the nasal turbinate of Buan2-infected ferrets for 5 days with a decreased viral titer from $10^3$ $EID_{50}$/ml at 1 dpi to $10^{2.5}$ $EID_{50}$/ml at 5 dpi, suggesting that the virus did not get through the lower respiratory tract to reach the lung. In contrast, viruses from the nasal turbinate in Gochang1-infected ferrets were only secreted until 3 dpi. However, Gochang1 was detected only up to 1 dpi in the nasal washes (Table 2). These viruses were not detected in other organs including respiratory ones. To induce more pathogenesis in ferrets, we applied a more aggressive intratracheal challenge with the two types of viruses. There was no mortality and significant clinical signs in ferrets intratracheally challenged with $10^7$ $TCID_{50}$/ml of Gochang1, while ferrets similarly infected with Buan2 showed 100% mortality at a dose of $10^7$ $TCID_{50}$/ml and 50% mortality at doses of both $10^6$ $TCID_{50}$/ml and $10^5$ $TCID_{50}$/ml. Most of the ferrets challenged with the Buan2 virus showed diarrhea and severe body weight loss before death. In the infected lung tissues, Buan2 was detected until 7 dpi but Gochang1 had cleared after 5 dpi. An intranasal challenge had no impact on lungs for either viral strain, whereas an intratracheal challenge with Buan2 induced swelling and edema finally resulting in severe pneumonia. The ferrets challenged with Gochang1 via the trachea showed mild signs of pneumonia until 5 dpi but recovered after that (data not shown) (Table 3).

In terms of histopathology, the ferrets infected intranasally showed mild inflammation in alveoli up to 5 dpi for Buan2 and up to 3 dpi for Gochang1. In the alveoli of ferrets challenged intratracheally with Buan2, severe inflammation was induced after 3 dpi and fibrous pneumonia was observed at 5 and 7 dpi. Intermediate inflammation was observed at 5 dpi in the alveoli of Gochang1-infected ferrets but the alveoli recovered at 7 dpi with only mild inflammation (Fig. 1).

Immunohistochemistry results were consistent with the viral titers in tissues. Only the intratracheally challenged ferrets showed immunostaining for viral antigens in their alveoli: up to 7 dpi for Buan2 and 5 dpi for Gochang1 (Fig. 2).

Both intranasal and intratracheal challenges with these viruses induced weak HI titers against each strain and cross-reactivity between Buan2 and Gochang1 viruses was confirmed (Table 4).

## Discussion

Outbreaks of the HPAI H5N8 in poultry farms were reported in Ireland in 1983 (Swayne, 2008) and in China throughout 2009 and 2010 (Zhao et al., 2013). Pathogenesis of H5N8 has been demonstrated only for birds such as chickens, turkeys, and ducks, but no human infection has been reported. The Korean H5N8 viruses isolated in 2014 were classified as highly pathogenic, possessing multiple basic amino acids at the cleavage site of the HA sequence: Gochang1 has LREKRRKR/GLF motifs and Buan2 has LRERRRKR/GLF motif (Lee et al., 2014) which showed L at position 9 with a deletion at position 4 of the HA cleavage site (Kang, 2015). Kang et al reported that Korean isolated H5N8 viruses were moderately pathogenic in wild mallard ducks and did not cause severe illness or death. However viral replication and shedding were greater in H5N8 infected mallards than in H5N1 infected mallards (Kang, 2015).

**Table 1**
Clinical signs of Korean H5 isolates-infected ferrets.

| Virus | Subtype | Body weight | Temperature | Survival[b] | Respiratory[b] | Lethargy |
|---|---|---|---|---|---|---|
| | | Loss (%) | Rise (°C) | | | |
| A/broiler duck/Korea/Buan2/2014 | H5N8 | 8.3 | 1.6 | 8/8 | 4/8 | Not severe |
| A/breeder duck/Korea/Gochang1/2014 | | 7.5 | 0.7 | 8/8 | 0/8 | None |
| A/chicken/Korea/ES/2003[a] | H5N1 | 5 | NA | 3/3 | 0/3 | Not severe |
| A/Vietnam/1204/2004[a] | | 22 | NA | 1/3 | 2/3 | Severe |

NA – not available.
[a] This experiment was conducted by CDC, USA (Kwon et al., 2014).
[b] No. of animal/total.

**Table 2**
Viral titration of nasal washes in Korean H5N8 isolates-infected ferrets by challenge routes.

| Virus | Route | Viral titer (log $EID_{50}$/ml)[a] | | | | |
|---|---|---|---|---|---|---|
| | | 1 dpi | 3 dpi | 5 dpi | 7 dpi | 9 dpi |
| A/broiler duck/Korea/Buan2/2014 | IN | $3 \pm 0.5$ | $2.5 \pm 0.0$ | $2.5 \pm 0.5$ | ND | ND |
| | IT | ND | ND | ND | ND | ND |
| A/breeder duck/Korea/Gochang1/2014 | IN | 2.5 | ND | ND | ND | ND |
| | IT | ND | ND | ND | ND | ND |
| A/chicken/Korea/ES/2003[b] | IN | 5.2 | 4.4 | 3.6 | ND | NA |
| A/Vietnam/1204/2004[b] | | 5.4 | 5.2 | 4.2 | 2.8 | NA |

dpi – days post infection, IN – intranasal, IT – intratracheal, ND – not detected, NA – not available.
[a] Data are represented as mean ± SD.
[b] This experiment was conducted by CDC, USA (Kwon et al., 2014).