High Pathogenicity of Influenza A (H10N8) Virus in Mice

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Abstract. Three human cases of H10N8 virus infections were initially reported in China in late 2013 and early 2014, two of which were fatal. This was the first time the H10N8 subtype has been detected in humans, and the pathogenicity of this virus remains under characterized. We first assessed its pathogenicity by infecting BALB/c mice with two H10N8 isolates, A/Jiangxi-Donghu/346-1/2013 and A/Chicken/Jiangxi/102/2013. The human isolate (H346-1) demonstrated stronger capability of replication and induced higher cytokine response *in vivo* than the chicken isolate (C102). In addition, H346-1 was fatal to mice, while all mice ($N = 14$) in C102-infected group survived during the infection course without weight loss. We hypothesized that the 627K mutation in the *PB2* gene (PB2-K627) in H346-1 was associated with high pathogenicity in mice. Taken together, this study based on mouse model provides some insight into understanding the pathogenicity of the emerging viruses in mammals.

Avian influenza viruses (AIV) of different subtypes sporadically infect people and cause a wide range of clinical outcomes, from asymptomatic infections to fatal pneumonia.¹ AIV is generally considered species specific and rarely crosses the species barrier to infect mammals and humans.² However, genetic reassortment with different subtypes enables the viruses to infect humans. H6N1, H7N2, H7N3, H7N7, H9N2, and H10N7 viruses have caused conjunctivitis or mild respiratory symptoms, or both, in people, although some severe cases have been reported.^{3–6} Human infections with avian influenza A H5N1 and H7N9 viruses are more commonly detected and can result in fatal pneumonia.⁷

Human infections with novel avian influenza A (H10N8) viruses were initially reported in China in December 2013. The infections coincided with the second wave of H7N9 outbreak in mainland China, causing three human cases of infection in Nanchang city with two being fatal.⁸ Although the phylogeny and clinical characteristics of H10N8 viruses are clear, the underlying molecular mechanisms for their high pathogenicity in humans remain largely unknown.^{8,9} Thus far, no assessment of pathogenicity of H10N8 was reported in any animal model. Here we evaluated this virus's pathogenicity and found that this virus was highly pathogenic in mice.

Two H10N8 isolates, human isolate A/Jiangxi-Donghu/346-1/2013 (GISAID accession nos: EPI530523-EPI530530) isolated from the first human case, and chicken isolate A/Chicken/Jiangxi/102/2013 (EPI530539-EPI530546) from chicken in a live poultry market (LPM) that the patient visited, were propagated from 9-day-old specific pathogen-free embryonated chicken eggs. The virus titers and infectivity were determined by EID₅₀ chicken embryos. In brief, 10-fold serial dilutions of rescued stock viruses were used to inoculate chicken embryos at 37°C for 72 hours. The EID₅₀ values were calculated by the method of Reed and Muench.¹⁰ Fourteen 4-week-old (~12 g) female BALB/c mice of each group were anesthetized with CO₂ and inoculated intranasally with 50 μ L of 10⁶

EID₅₀ of virus diluted in phosphate-buffered saline (PBS). Mock-infected control mice were inoculated with 50 μ L PBS. Studies with H10N8 viruses were conducted in a biosecurity level 3 laboratory approved by College of Veterinary Medicine, South China Agricultural University. All animal studies were approved by the Review Board of College of Veterinary Medicine, South China Agricultural University.

Three mice from each group were euthanized at 3 and 5 days postinfection (dpi), and their lungs were collected and homogenized using a QIAGEN TissueLyser II machine (Qiagen, Hilden, Germany) (30 cycles/s, 5 minutes) in 1 mL of cold PBS under sterile conditions. Then, solid debris was pelleted by centrifugation at 5,000 $\times$ g for 10 minutes, and the homogenates were used for virus titrations in 9-day-old embryonated chicken eggs and for cytokine detection by the cytometric bead array method using a Mouse Inflammation Kit (BD), respectively. In addition, all of the mice were monitored daily for general behavior and clinical signs, including food intake, body weight, inactivity, and mortality for 10 days. Animals that lost more than 25% of their initial body weight were scored dead. The H346-1-infected mice presented severe clinical manifestation, including inappetence, inactivity, and conspicuous body weight loss (Figure 1A). Five of eight mice in H346-1-infected group died at 3 dpi, and then two and one died at 4 and 5 dpi, respectively (Figure 1B). In contrast, mice in C102-infected group survived infection and did not show any appreciable weight loss compared with mock-infected group (Figure 1A and B). Apparently, H346-1 showed extremely high pathogenicity in mice.

The replication capability of the two isolates *in vivo* was evaluated by lung virus titers (LVTs). As shown in Table 1, both H346-1- and C102-infected mice presented measurable LVTs over the course of the infection. Compared with C102-infected group, the H346-1-infected mice presented higher

TABLE 1
Lung virus titers of mice infected with H10N8 viruses

Strain	Titers (mean log ₁₀ EID ₅₀ /mL)	
	Day 3	Day 5
A/Jiangxi-Donghu/346-1/2013	6.3	7.4
A/Chicken/Jiangxi/102/2013	5.7	5.3

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FIGURE 1. Body weight change (A) and percent survival (B) of mice infected with H10N8 viruses.

LVTs over the course of the infection. To analyze the relationship between cytokines and the virulence of influenza A (H10N8) viruses in mice, as well as to explore the dynamics of cytokine secretion after H10N8 virus infection, the levels of inflammatory cytokines were measured at two time points based on the progression of the disease after infection. As shown in Figure 2, both the human and chicken isolates provoked cytokine response during infection. The interferon-gamma levels were significantly enhanced as soon as 3 dpi and persisting at 5 dpi in both groups. The interleukin-6 (IL-6), interferon gamma-

induced protein 10 (IP-10), monocyte chemotactic protein-1 (MCP-1), and MCP-3 values in H346-1-infected groups were significantly enhanced at 3 dpi, with IP-10, MCP-1 and MCP-3 persisting while IL-6 decreasing at 5 dpi. In C102-infected groups, these four cytokines were moderately enhanced at 5 dpi. The values of RANTES (regulated on activation, normal T cell expressed and secreted) and macrophage inflammatory protein-1 alpha were also moderately enhanced in both groups at 5 dpi. However, tumor necrosis factor-alpha (TNF- α) level was not induced during infection course in both groups.

FIGURE 2. Cytokines in mouse evoked by the H10N8 viruses. The concentrations of the cytokines interferon-gamma (IFN- γ) (A), IP-10 (B), interleukin-6 (IL-6) (C), macrophage inflammatory protein-1 alpha (MIP-1 α) (D), monocyte chemotactic protein-1 (MCP-1) (E), MCP-3 (F), keratinocyte-derived chemokine (KC) (G), RANTES (regulated on activation, normal T cell expressed and secreted) (H), and tumor necrosis factor-alpha (TNF- α) (I) were measured using cytometric bead array assays, according to the manufacturers' instructions. The graph was constructed by GraphPad Prism 6 Demo software package (GraphPad Software). Statistically significant differences were determined using student's *t* test and analysis of variance. *P* values < 0.05 were considered statistically significant. **P* < 0.05; ***P* < 0.01; ****P* < 0.001.

FIGURE 3. Pathological changes of mouse lung infected by the H10N8 viruses. (A) and (B) are Hematoxylin and eosin (H and E)-stained lung sections (200×) at 5 days postinfection (dpi). of H346-1 and C102 infection groups, respectively. (A) ↑: Mild acute bronchiolitis, →: mild emphysema and pulmonary alveolar expansion, ←: pulmonary septal narrowing. (B) →: Focal acute interstitial pneumonia, ←: mild emphysema.

Besides, keratinocyte-derived chemokine that can be induced by TNF- α showed a mild increase in both groups. In brief, the human isolate provoked faster infection and higher cytokine expression than the chicken isolate did with equivalent dose of infection in mice.

In addition, pathological sections suggested that lung infections were caused by both isolates as Figure 3 showed. The H346-1-infected group displayed mild-to-moderate focal acute interstitial pneumonia and relevant symptoms, whereas C102-infected group displayed slight emphysema during the infection.

Our results revealed different level of pathogenicity between these two H10N8 isolates, of them H346-1 showed high fatality in mice rather than C102. In addition, H346-1 displayed higher replication capability in vivo as the LVTs indicated. Our recent study revealed that these two isolates share a > 99.3% sequence identity in six genes, except for the *PB1* (89.4%) and *PB2* (93.3%) genes. The HA genes of these two isolates shared 99.9% identity and only one basic amino acid (arginine, R) was noted at the HA cleavage site. In addition, the amino acid residues 226Q and 228G (H3 numbering) of HA protein in both isolates indicated an avian-like receptor-binding preference.⁹ H346-1, rather than C102, has the E627K substitution in *PB2* gene (PB2-E627K), which has been associated with the increased virulence of AIV in mammals, such as H5N1, H9N2, and H7N9.^{11–13} These results suggest that PB2-E627K in H10N8 virus is critical for the high pathogenicity in mammals. The H10N8 virus, as a novel avian-origin influenza A virus, demonstrated higher virulence than the reported H10 subtype influenza viruses as H346-1 was proved to be fatal in human and mouse.^{14,15} In addition, phylogenetic analysis indicated that H10N8 viruses shared 97–98% HA gene sequence identity with H10N7 virus isolated from infected seals in Europe in 2014, and the predicted amino acid sequence of the HA cleavage site of all the A (H10N7) seal viruses were unique (PELVQGR*GLF) and contained only one basic amino acid, which indicated low pathogenicity.¹⁶

Avian flu patients often die of acute respiratory distress syndrome caused by a cytokine storm after virus infection. The recent study revealed that cytokines were strongly induced in H10N8 infection cases, especially in the fatal cases.¹⁷ And this study also showed the human isolate of H10N8 virus provoked acute and persisting cytokine expression in mice, indicating this virus can cause a cytokine storm in mammals.

In summary, relying on the mouse model of infection with H10N8 viruses, this study indicated that the novel H10N8 viruses replicated well in mice's lungs and induced high cytokine response. And the PB2-E627K substitution was hypothesized to be associated with this virus's high fatality in mice, which needs further confirmation. Nevertheless, new H10N8 human infections are likely to reemerge as high detection rate of AIVs and mixed infection of different HA subtypes were observed in LPMs of Nanchang City.¹⁸ Therefore, it is vital to monitor the circulation of H10N8 viruses and their key amino acid substitutions associating with high pathogenicity in mammals.

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REFERENCES

- García-Sastre A, Schmolke M, 2014. Avian influenza A H10N8—a virus on the verge? *Lancet* 383: 676–677.
- Kuiken T, Holmes EC, McCauley J, Rimmelzwaan GF, Williams CS, Grenfell BT, 2006. Host species barriers to influenza virus infections. *Science* 312: 394–397.
- Webster RG, Peiris M, Chen H, Guan Y, 2006. H5N1 outbreaks and enzootic influenza. *Emerg Infect Dis* 12: 3–8.
- Wei SH, Yang JR, Wu HS, Chang MC, Lin JS, Lin CY, Liu YL, Lo YC, Yang CH, Chuang JH, Lin MC, Chung WC, Liao CH, Lee MS, Huang WT, Chen PJ, Liu MT, Chang FY, 2013. Human infection with avian influenza A H6N1 virus: an epidemiological analysis. *Lancet Respir Med* 1: 771–778.
- Peiris M, Yuen KY, Leung CW, Chan KH, Ip PL, Lai RW, Orr WK, Shortridge KF, 1999. Human infection with influenza H9N2. *Lancet* 354: 916–917.
- Belser JA, Bridges CB, Katz JM, Tumpey TM, 2009. Past, present, and possible future human infection with influenza virus A subtype H7. *Emerg Infect Dis* 15: 859–865.
- To KK, Tsang AK, Chan JF, Cheng VC, Chen H, Yuen KY, 2014. Emergence in China of human disease due to avian influenza A (H10N8)—cause for concern? *J Infect* 68: 205–215.
- Chen H, Yuan H, Gao R, Zhang J, Wang D, Xiong Y, Fan G, Yang F, Li X, Zhou J, Zou S, Yang L, Chen T, Dong L, Bo H, Zhao X, Zhang Y, Lan Y, Bai T, Dong J, Li Q, Wang S, Zhang Y, Li H, Gong T, Shi Y, Ni X, Li J, Zhou J, Fan J, Wu J, Zhou X, Hu M, Wan J, Yang W, Li D, Wu G, Feng Z, Gao GF, Wang Y, Jin Q, Liu M, Shu Y, 2014. Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: a descriptive study. *Lancet* 383: 714–721.

9. Qi W, Zhou X, Shi W, Huang L, Xia W, Liu D, Li H, Chen S, Lei F, Cao L, Wu J, He F, Song W, Li Q, Li H, Liao M, Liu M, 2014. Genesis of the novel human-infecting influenza A (H10N8) virus and potential genetic diversity of the virus in poultry, China. *Euro Surveill* 19: 20841.
10. Reed LJ, Muench H, 1938. A simple method of estimating fifty percent endpoints. *Am J Hyg* 27: 493–497.
11. Hatta M, Gao P, Halfmann P, Kawaoka Y, 2001. Molecular basis for high virulence of Hong Kong H5N1 influenza A viruses. *Science* 293: 1840–1842.
12. Li X, Qi W, He J, Ning Z, Hu Y, Tian J, Jiao P, Xu C, Chen J, Richt J, Ma W, Liao M, 2012. Molecular basis of efficient replication and pathogenicity of H9N2 avian influenza viruses in mice. *PLoS One* 7: e40118.
13. Mok CK, Lee HH, Lestra M, Nicholls JM, Chan MC, Sia SF, Zhu H, Poon LL, Guan Y, Peiris JS, 2014. Amino acid substitutions in polymerase basic protein 2 gene contributes to the pathogenicity of the novel A/H7N9 influenza virus in mammalian hosts. *J Virol* 88: 3568–3576.
14. Arzey GG, Kirkland PD, Arzey KE, Frost M, Maywood P, Conaty S, Hurt AC, Deng YM, Iannello P, Barr I, Dwyer DE, Ratnamohan M, McPhie K, Selleck P, 2012. Influenza virus A (H10N7) in chickens and poultry abattoir workers, Australia. *Emerg Infect Dis* 18: 814–816.
15. Beare AS, Webster RG, 1991. Replication of avian influenza viruses in humans. *Arch Virol* 119: 37–42.
16. Zohari S, Neimanis A, Härkönen T, Moraeus C, Valarcher JF, 2014. Avian influenza A (H10N7) virus involvement in mass mortality of harbour seals (*Phoca vitulina*) in Sweden, March through October 2014. *Euro Surveill* 19: 20967.
17. Liu M, Li X, Yuan H, Zhou J, Wu J, Bo H, Xia W, Xiong Y, Yang L, Gao R, Guo J, Huang W, Zhang Y, Zhao X, Zou X, Chen T, Wang D, Li Q, Wang S, Chen S, Hu M, Ni X, Gong T, Shi Y, Li J, Zhou J, Cai J, Xiao Z, Zhang W, Sun J, Li D, Wu G, Feng Z, Wang Y, Chen H, Shu Y, 2015. Genetic diversity of avian influenza A (H10N8) virus in live poultry markets and its association with human infections in China. *Sci Rep* 5: 7632.
18. Ni X, He F, Hu M, Zhou X, Wang B, Feng C, Wu Y, Li Y, Tu J, Li H, Liu M, Chen H, Chen S, 2015. Investigation of avian influenza virus in poultry and wild birds due to novel avian-origin influenza A (H10N8) in Nanchang City, China. *Microbes Infect* 17: 48–53.