Research Article

Pathogenicity of Duck-Originated H9N2 Influenza Viruses on Chickens

Abstract

Background: The spreading of H9N2 avian influenza viruses in poultry in Eurasia and Africa accompanied with the great economic losses to poultry industry in past decades has attracted the great attention of whole world. Domestic ducks play a critical role in the ecology of avian influenza viruses.

Methods: In this study, 6 strains of H9N2 viruses were isolated from ducks and were evaluated for the pathogenicity on chickens. The infected chickens were observed for 10 days and tracheal and cloacal swabs were collected for virus shedding detection.

Results: All 6 isolates showed low pathogenicity to chickens. Clinical signs were not observed during 10 days in any of the infected chickens. While viruses were recovered from most of the infected chickens, and at least 4/5 chickens in each group shed virus even at 7 days post infection.

Conclusion: Chickens infected with duck-originated H9N2 avian influenza viruses shed viruses for at least 7 days which provides a wide window period for virus transmission.

Material and Methods

Viruses and animals

Six H9N2 AIVs were isolated from domestic ducks. The H9N2 subtype was confirmed by HA/H1 test and HA/NA gene sequencing. The detailed information about these viruses is listed in Table 1. Each virus was amplified in 10-day-old specific pathogen free (SPF) embryonated chicken eggs and virus titer (EID50) was determined in SPF embryonated chicken eggs and calculated by Karber method based on the HA assay of allantoic fluid of eggs inoculated with 10-fold serial dilutions of viruses. SPF Lehighern chickens were purchased from Beijing Merial Vital Laboratory Animal Technology Co., Ltd., and raised in high-efficiency particulate air-filtered negative-pressure isolators with ad libitum access to feed during the experimental stage. All animal experiments were approved by the Institutional Animal Care and Use Committee at National Research Center for Veterinary Medicine and conventional animal welfare regulations and standards were taken into account.

Pathogenicity experiment

To investigate the pathogenicity of isolates in chickens, 35 six-

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Table 1: Information about H9N2 viruses used in this study.

| H9N2 Isolates | Abbreviation | Isolation Province | virus titer (lgEID50/0.1ml) |
|---------------|--------------|--------------------|---------------------------|
| A/duck/Jiangsu/YZG4/2009 | YZG4 | Jiangsu | 8.0 |
| A/duck/Shandong/SD01/2009 | SD01 | Shandong | 7.8 |
| A/duck/Liaoning/SL/2009 | DL | Liaoning | 7.8 |
| A/duck/Jiangsu/S1/2008 | D1 | Jiangsu | 8.4 |
| A/duck/Anhui/D2/2009 | D2 | Anhui | 7.8 |
| A/duck/Anhui/D3/2009 | D3 | Anhui | 8.0 |
week-old SPF chickens were divided into 7 groups with 5 chickens in each group. Chickens were inoculated intravenously (i.v.) with 200µl of the virus positive allantoic fluid diluted in phosphate buffered saline (PBS) to yield a 107.0EID50. The chickens in the last group worked as negative control and were inoculated i.v. with 200µl of an equivalent dilution of noninfectious allantoic fluid. All chickens were monitored daily for clinical signs for up to 10 days, and tracheal and cloacal swabs were collected on days 2, 3, 5, and 7 days post infection (dpi) and resuspended in 1ml PBS (2000U penicillin G, 200µg streptomycin). The samples were used to inoculate SPF embryonated chicken eggs and passaged twice to isolate virus.

**Results**

**Clinical manifestation of infected chickens**

Six viruses were inoculated into chickens in different groups to test their pathogenicity. Virus-infected chickens did not show any explicit clinical symptoms throughout the study, which is consistent with previous studies [9]. All chickens were euthanized at 10 dpi. There was no gross pathology observed on different tissues of infected and negative control chickens at 10 dpi.

**Virus shedding from tracheal swabs**

Tracheal and cloacal swabs were collected to monitor virus shedding by chicken embryo inoculation at designated days. As shown in Table 2, the virus could be detected at 2 dpi from tracheal swabs in SD01-, D1- and D2-infected groups, while the positive rate was only one to two out of five chickens. Virus shedding detected in tracheal swabs increased rapidly and peaked at 5-7 dpi. All chickens shed virus from respiratory tracts except one chicken in DL group in 5 dpi and 7 dpi.

**Virus shedding from cloacal swabs**

As for cloacal swabs, virus shedding was similar to that of tracheal swabs (Table 3). Only two cloacal samples with one from SD01 group and another from D1 group were identified positive. As for 3 dpi, virus shedding from cloaca was significantly lower than that from tracheal swabs. Virus shedding detected in cloacal swabs peaked at 5 dpi and decreased slightly at 7 dpi.

**Discussion**

Previous studies have showed that wild waterfowls are carriers of almost all variety of subtypes of AIVs, and constitute the reservoir of the virus [10]. Indeed, wild waterfowls usually shed the virus in their faces while remains asymptomatic. Among the bird population, peak excretion titers of up to 108.7EID50 per gram feces have been measured [11]. The excretion of the virus by the fecal route results in the contamination of the environment and keeps the infection cycle going. Domestic ducks plan an important role in the transmission of AIVs from wild waterfowls to land poultry because of the numerous ducks and chickens raised in China and domestic ducks have the chance to contact closely with wild birds and land poultry simultaneously.

AIVs can be divided into two forms of viruses known as highly pathogenic avian influenza viruses (HPAIVs) and low pathogenic avian influenza viruses (LPAIVs). As of now, all HPAIVs belonged to H5 or H7 subtype except for little isolates belonged to subtype H10 [3,12,13]. Although much scientific and public health interest has focused on the H5 and H7 influenza viruses, the H9N2 AIVs are also considered having significant impact on poultry industry and public health. H9N2 AIVs are considered enzootic in poultry in some Asian and Middle Eastern countries, and caused disease in poultry. Also, they denoted internal genes to the HPAIVs H5N1, and H7N9, which has become one of the most severe zoonotic infection from AIV causing high morbidity and case fatality in humans [14,15].

Therefore, surveillance on the epidemiology and pathogenicity of LPAIVs such as H9N2 influenza virus from waterfowl such as ducks is important to prevent and control the diseases effectively. In this study, we explored the pathogenicity of 6 strains of H9N2 AIVs obtained from ducks. These 6 isolates showed low pathogenicity when the ducks were experimentally challenged with 107.0EID50 with each virus (unpublished data). Chickens infected with different strains of H9N2 AIVs did not show any clinical symptoms in the experiments which consistent with previous findings. We euthanized chickens at 10dpi, and there was no gross pathology observed in the tissues of infected chickens.

Virus shedding works as a crucial parameter to characterize H9N2 AIVs since there was no obvious clinical symptoms and gross pathological changes in infected chickens. The shedding virus could be detected at 2 dpi and increased gradually, then peaked at 5 dpi and 7 dpi (Table 1,2). However, the ratio of shedding virus in tracheal samples was higher than that in cloacal swabs at 3dpi, which indicated higher copy numbers and speed of proliferating viruses in trachea. However, quantification of shedding virus in tracheal and cloacal swabs was necessary and need to be performed to support the above conclusion.

Previous studies have showed that most chicken-origin H9N2 viruses induced no clinical signs or deaths in chickens, although only

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**Table 2:** Tracheal Virus shedding of chickens after infection with different strains of H9N2 virus.

| Virus strain | 2dpi | 3dpi | 5dpi | 7dpi |
|--------------|------|------|------|------|
| YZG4         | 0/5  | 3/5  | 5/5  | 5/5  |
| SD01         | 2/5  | 4/5  | 5/5  | 5/5  |
| DL           | 0/5  | 4/5  | 4/5  | 4/5  |
| D1           | 1/5  | 3/5  | 5/5  | 5/5  |
| D2           | 1/5  | 3/5  | 5/5  | 5/5  |
| D3           | 0/5  | 3/5  | 5/5  | 5/5  |

**Table 3:** Cloacal Virus shedding of chickens after infection with different strains of H9N2 virus.

| Virus strain | 2dpi | 3dpi | 5dpi | 7dpi |
|--------------|------|------|------|------|
| YZG4         | 0/5  | 1/5  | 5/5  | 5/5  |
| SD01         | 1/5  | 3/5  | 5/5  | 5/5  |
| DL           | 0/5  | 1/5  | 5/5  | 4/5  |
| D1           | 1/5  | 2/5  | 5/5  | 5/5  |
| D2           | 0/5  | 2/5  | 4/5  | 5/5  |
| D3           | 0/5  | 3/5  | 5/5  | 3/5  |

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a few isolates showed pathogenicity to chickens, and almost all those inoculated chickens shed viruses from tracheas or cloacal samples [9,16-18]. These findings demonstrated that the disease or death in the poultry farms where these H9N2 viruses were isolated may not be caused by H9N2 viruses alone and may be a result of co-infection with other pathogens.

Our studies also indicated that H9N2 viruses in ducks can transmitted to chickens, and chickens infected can shed viruses for a long time, which caused circulation of transmission of viruses among ducks and chickens. As the important reservoir hosts of AIVs, genetic recombination can occur when ducks infected two or more different subtypes of viruses simultaneously, which lead to new viruses, and some viruses among these have been proved to acquire the ability to infect humans [3]. Thus, continuous monitoring in poultry is important to prevent the emergence of H9 viruses.

Conclusion

To conclude, 6 strains of H9N2 influenza viruses obtained from ducks were tested for the pathogenicity on chickens. The results showed these viruses were low pathogenic to chickens with no obvious clinical symptoms, while shed viruses from respiratory tracts and cloaca.

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References

1. Tong S, Li Y, Rivailier P, Conrardy C, Castillo DA, et al. (2012) A distinct lineage of influenza A virus from bats. Proc Natl Acad Sci U S A 109: 4269-4274.
2. Tong S, Zhu X, Li Y, Shi M, Zhang J, et al. (2013) New world bats harbor diverse influenza A viruses. PLoS Pathog 9: e1003657.
3. Joseph U, Su YC, Vijaykrishna D, Smith GJ (2016) The ecology and adaptive evolution of influenza A interspecies transmission. Influenza Other Respir Viruses.
4. Liu H, Liu X, Cheng J, Peng D, Jia L, et al. (2003) Phylogenetic analysis of the hemagglutinin genes of twenty-six avian influenza viruses of subtype H9N2 isolated from chickens in China during 1996-2001. Avian Dis 47: 116-127.
5. Butt KM, Smith GJ, Chen H, Zhang LJ, Leung YH, et al. (2005) Human infection with an avian H9N2 influenza A virus in Hong Kong in 2003. J Clin Microbiol 43: 5760-5767.
6. Cong YL, Pu J, Liu QF, Wang S, Zhang GZ, et al. (2007) Antigenic and genetic characterization of H9N2 swine influenza viruses in China. J Gen Virol 88: 2035-2041.
7. Zhang H, Xu B, Chen Q, Chen Z (2011) Characterization of H9N2 influenza viruses isolated from Dongting Lake wetland in 2007. Arch Virol 156: 95-105.
8. Chen LJ, Lin XD, Guo WP, Tian JH, Wang W, et al. (2016) Diversity and evolution of avian influenza viruses in live poultry markets, free-range poultry and wild wetland birds in China. J Gen Virol 97: 844-854.
9. Zhu Y, Yang Y, Liu W, Liu X, Yang D, et al. (2015) Comparison of biological characteristics of H9N2 avian influenza viruses isolated from different hosts. Arch Virol 160: 917-927.
10. Medina RA, Garcia-Sastre A (2011) Influenza A viruses: new research developments. Nat Rev Microbiol 9: 590-603.
11. Webster RG, Yakhno M, Hinshaw VS, Bean WJ, Murti KG (1978) Intestinal influenza: replication and characterization of influenza viruses in ducks. Virology 84: 268-278.
12. Wood GW, Banks J, Strong I, Parsons G, Alexander DJ (1996) An avian influenza virus of H10 subtype that is highly pathogenic for chickens, but lacks multiple basic amino acids at the haemagglutinin cleavage site. Avian Pathol 25: 799-806.
13. Su S, Bi Y, Wong G, Gray GC, Gao GF, et al. (2015) Epidemiology, Evolution, and Recent Outbreaks of Avian Influenza Virus in China. J Virol 89: 8671-8676.
14. Guan Y, Shortridge KF, Krauss S, Chin PS, Dyrling KC, et al. (2000) H9N2 influenza viruses possessing H5N1-like internal genes continue to circulate in poultry in southeastern China. J Virol 74: 9372-9380.
15. Yu X, Jin T, Cui Y, Pu X, Li J, et al. (2014) Influenza H7N9 and H9N2 viruses: coexistence in poultry linked to human H7N9 infection and genome characteristics. J Virol 88: 3423-3431.
16. Li C, Yu K, Tian G, Yu D, Liu L, et al. (2005) Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology 340: 70-83.
17. Pawar SD, Kale SD, Rawankar AS, Koratkar SS, Raut CG, et al. (2012) Avian influenza surveillance reveals presence of low pathogenic avian influenza viruses in poultry during 2009-2011 in the West Bengal State, India. Virology 419: 151.
18. Qi X, Tan D, Wu C, Tang C, Li T, et al. (2016) Deterioration of eggshell quality in laying hens experimentally infected with H9N2 avian influenza virus. Vet Res 47: 35.