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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.

Abbreviations

AIV: Avian Influenza Virus; SPF: Specific Pathogen Free; DPI: Days Post Inoculation; EID_{50}: 50% Embryo Infections Dose

Introduction

Influenza A viruses are members of the family *orthomyxovirudae*, which are categorized into 18 hemagglutinin (HA) subtypes and 11 neuraminidase (NA) subtypes, with H17N10 and H18N11 subtypes were discovered in bats, according to the antigenicity of the surface glycoproteins HA and NA [1-3]. Among these, the H9N2 subtype is of great concern, as it has been well established in land-based poultry, and caused economic losses to the poultry industry across Eurasia and Middle East, and has occasionally been transmitted to mammalian species, including humans and pigs [4-6]. Usually, chickens infected with H9N2 viruses did not show any clinical symptoms under laboratory condition, while infections with H9N2 AIVs in commercial poultry may result in respiratory disease, drop in egg production and increased mortality because of the secondary bacterial infections [7].

As an important reservoir of AIVs, domestic ducks play an important role in the evolution, and spread of many subtypes of AIVs. It was found that H9N2 AIVs were prevalent in domestic ducks from farms and live bird markets in China [8]. While the pathogenicity of duck-originated H9N2 AIVs to chickens was seldom explored. In this study, we isolated 6 H9N2 viruses from domestic ducks, and these viruses were used to infect chickens to evaluate their pathogenicity.

Material and Methods

Viruses and animals

Six H9N2 AIVs were isolated from domestic ducks. The H9N2 subtype was confirmed by HA/HI 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 (EID_{50}) 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 Lehgorn 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-

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

H9N2 Isolates	Abbreviation	Isolation Province	virus titer ($lgEID_{50}$/0.1ml)
A/duck/Jiangsu/YZG4/2009	YZG4	Jiangsu	8.0
A/duck/Shandong/SD01/2009	SD01	Shandong	7.8
A/duck/Liaoning/DL/2009	DL	Liaoning	7.8
A/duck/Jiangsu/D1/2008	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.

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

Virus strain	Virus shedding (Positive/Total number)			
	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	Virus shedding (Positive/Total number)			
	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
v	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

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 (Tables 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

a few isolates showed pathogenicity to chickens, and almost all those inoculated chickens shed viruses from tracheas or clocal 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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