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Brief Original Article

A small-scale study on airborne transmission of H9N2 avian influenza virus under field conditions

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Abstract

Introduction: H9N2 avian influenza viruses (AIV) can transmit in chicken flocks through direct contact and aerosols. Nevertheless, data on airborne transmission of AIV is very limited, especially under field conditions. To fill this literature gap, this study was designed to investigate airborne transmission of H9N2 AIV originating from infected chicken flocks under field conditions, with the aim to further characterize the airborne transmission of H9N2 AIV.

Methodology: Oropharyngeal swabs were collected from different diseased chickens to confirm H9N2 AIV infection. All glass impingers 30 (AGI-30) were used to collect indoor, upwind and downwind air samples for three chicken houses with H9N2 AIV infected chickens. Swabs and air samples were tested for H9N2 AIV using a real-time reverse transcription polymerase chain reaction (RRT-PCR). H9N2 AIV was isolated in embryonated chicken eggs and hemagglutinin (HA) gene sequence similarity of the isolated AIV was compared.

Results: The results showed that indoor air samples were all RRT-PCR positive for H9N2 AIV. Downwind air samples collected between 10 m and 1.5 km away from the chicken houses were also found positive with an average load 2.62-5.21×10^3 RNA copies/m^3. However, upwind air samples were all negative for H9N2 AIV. In addition, H9N2 AIV was isolated from swabs and indoor air samples.

Conclusion: In summary, this study provides insights into the airborne transmission of H9N2 AIV under field conditions.

Key words: AIV; Airborne transmission; RRT-PCR; H9N2; field conditions.

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Introduction

H9N2 avian influenza virus (AIV) was first isolated from turkeys in North America in 1966, and it has since become most prevalent in poultry and causes enormous economic losses to poultry industry worldwide [1-3]. Although H9N2 AIV belongs to the low pathogenic avian influenza (LPAI) virus, it increases morbidity and mortality rates when poultry are co-infected with other pathogens, such as *Escherichia coli* and *Staphylococcus aureus* [4,5]. In China, since H9N2 AIV was first isolated from diseased chickens in Guangdong Province in 1994, the virus has become the most prevalent type nationwide [6-11].

Importantly, H9N2 AIV can cross species barriers to infect mammals, such as human beings and pigs [12-14]. For example, in Hong Kong H9N2 AIV was first confirmed in domestic pigs [12], before the virus was isolated from humans in 1999 [13]. The wide prevalence of H9N2 AIV in poultry flocks, together with their ability to infect mammals, has raised concerns about their potential role in a possible influenza pandemic [14]. Therefore, an in-depth understanding of the transmission characteristics of H9N2 AIV is of utmost importance.

In general, H9N2 AIV can transmit through direct contact and aerosols [15,16], but data on airborne transmission routes of H9N2 AIV are very limited, especially under field conditions. To fill the literature gap, this study was designed to investigate airborne transmission of H9N2 AIV originating from infected chicken flocks under field conditions, with the aim to further understand airborne transmission characteristics of H9N2 AIV.

Methodology

Selection of chicken farms

When veterinarians of chicken farms reported to the Animal Disease Control Center of Tai'an City that chicken flocks were showing influenza-like symptoms, such as widespread dyspnea, rhinorrhea, anorexia and

lethargy, investigators visited the chicken farms within 2-3 days to carry out sampling.

Collection of the samples

Ten oropharyngeal swab samples were collected from different chickens in each chicken house to confirm that chicken flocks were infected by H9N2 AIV. All-glass impingers (AGI-30) (Ace Glass Inc., Vineland, USA) were used to collect indoor and outdoor air samples. Six air samples were collected from each sampling site. Briefly, AGI-30 impingers were placed near the middle of the chicken house to collect indoor air samples. In parallel, AGI-30 samplers were used to collect outdoor air samples at different sites upwind (10 m and 100 m away) and downwind (100 m, 1.0 km and 1.5 km away) from the chicken houses. During the sampling, the AGI-30 samplers, containing 20 mL phosphate buffered saline solution, were placed 1.5 m above the ground and run for 30 min to collect air samples with air flow rate 12.5 L/min [15,17]. Once sampling was completed, samples were stored on ice and transported within 12 hours to our laboratory for further processing.

Detection of H9N2 AIV

In accordance with previously published references [18-20], all samples were treated and inoculated into 10-day-old embryonated chicken eggs through the allantoic route. Embryonated chicken eggs were incubated at 35 ℃ for 72 hours, and then allantoic liquid was collected under routine conditions. Viral isolates were identified using hemagglutination inhibition assays (HAI) and neuraminidase inhibition tests using a panel of reference sera. At the same time, samples were tested for H9N2 AIV by a real-time reverse transcription polymerase chain reaction (RRT-PCR) targeting the hemagglutinin gene (primers: HA-F, 5'-AAGCTGGAATCTGAAGGAACTTACA-3'; HA-R, 5'-ATTGGACATGGCCCAGAACA-3'; Probe: 5'-FAM-ACCATTTATTCGACTGTCGCCTCATCTCTTG-TAMRA-3'). Samples that produced a cycle threshold (ct) value below 32 were considered positive, and those that produced a ct value above 32 were considered negative [15].

Quantification of H9N2 AIV

RRT-PCR positive samples were further subjected to quantitative RRT-PCR of H9N2 AIV, as previously described [15]. Briefly, each primer and probe were used at concentrations of 0.8 μM and 0.4 μM, respectively. The reaction system contained 10 μL 2X Premix Ex Taq, 0.4 μL 50X ROX Reference Dye, 2.0 μL complementary DNA (cDNA) sample and sterile distilled water, added to give a final volume of 20 μL. The RRT-PCR was performed on a standard 7500 Real-Time PCR System (Applied Biosystems, Foster, USA) according to the following cycling protocol: an initial denaturation step of 30 s at 95℃, 40 cycles for 10 s at 95℃, and 34 s at 60℃.

HA sequencing and alignment

H9N2 AIV RNA was transcribed to cDNA using the Uni12-primer (AGCAAAAGCAGG) and amplified following the instructions of the RNA PCR Kit (AMV) Ver. 3.0 (TaKaRa Biotech Co. Ltd., Dalian, China). Amplification of the HA gene was carried out by using pairs of specific primers (HA-F: 5'-AGCAAAAGCAGGGGAATTTCAC-3', HA-R: 5'-AGTAGAAACAAGGGTGTTTTTGC- 3') [15]. HA fragments were sequenced using an ABI 3730 automated DNA sequencer (Applied Biosystems, Foster, USA). Nucleotide sequences were edited and aligned using DNASTAR software

Statistical analyses

T-test was used in this study to compare the amount of H9N2 AIV RNA copies between indoor air and outdoor air samples. When *P* value was less than 0.05, differences were regarded as statistically significant. Statistical analyses were conducted using SAS 9.2 (SAS Institute Inc., Cary, USA).

Results

Isolation and sequencing of H9N2 AIV

Between November and December 2015, three chicken farms (A, B, and C) in different regions were visited to conduct sampling. During the sampling, the size of chicken flock and meteorological conditions, including temperature, relative humidity and wind speed, were recorded (Table 1). In chicken farm A, 10 H9N2 AIV isolates were obtained from 7 swab samples (7/10, 70.0%) and 3 inside air samples (3/6, 50.0%). In chicken farm B, 8 H9N2 AIV isolates were isolated from 6 swabs (6/10, 60.0%) and 2 inside air samples (2/6, 33.3%). In chicken farm C, 5 H9N2 AIV isolates were isolated from 4 swab samples (4/10, 40.0%) and 1 inside air samples (1/6, 16.7%) (Table 2). Of note, these viruses shared 100% HA sequence similarity, and these sequences are all identical with the HA sequence of H9N2 isolate from diseased chicken in Shandong Province, China (access No. JN683647).

Table 1. Description of the three chicken flocks and meteorological conditions.

Farm	Layout	Age (wks)	N	Ventilation system	Indoor			Outdoor			Sample time
					T(℃)	RH(%)	WS(m/s)	T(℃)	RH(%)	WS(m/s)	
A	Half-closed	7	500	Natural	17	72	0.3-1.0	-5	68	1.7-4.7	Nov. 2015
B	Half-closed	4	4500	Natural	16	71	0.5-1.2	-8	65	1.6-5.0	Dec. 2015
C	Half-closed	5	5000	Natural	15	74	0.8-1.3	-12	68	1.6-6.4	Dec. 2015

N, number of chickens; T, temperature; RH, relative humidity; WS, wind speed.

Table 2. Positive results of H9N2 AIV of swab and indoor air samples.

Farm	Swabs		Indoor air samples	
	RRT-PCR (%)	Culture (%)	RRT-PCR (%)	Culture (%)
A	10/10 (100.0)	7/10 (70.0)	6/6 (100.0)	3/6 (50.0)
B	10/10 (100.0)	6/10 (60.0)	6/6 (100.0)	2/6 (33.3)
C	10/10 (100.0)	4/10 (40.0)	6/6 (100.0)	1/6 (16.7)

RRT-PCR, real-time reverse transcription polymerase chain reaction.

Table 3. Average H9N2 AIV loads and standard deviation (SD) values for indoor air samples.

Farm	Inside Air (10^5 RNA copies/m^3)	
	Mean	SD
A	4.36	2.32
B	5.29	2.65
C	6.72	3.27

Table 4. Number of H9N2 AIV positive samples, and average RNA copies/m^3 and standard deviation (SD) values of downwind air samples.

Farm	Distance	Positive samples (%)	10^3 RNA copies/m^3 of air	
			Mean	SD
A	100 m	6/6 (100.0)	4.32	1.78
A	1.0 km	3/6 (50.0)	2.88	1.48
A	1.5 km	0/6 (0.0)	--	--
B	100 m	6/6 (100.0)	4.73	2.01
B	1.0 km	6/6 (100.0)	3.89	1.49
B	1.5 km	4/6 (66.7)	2.62	1.14
C	100 m	6/6 (100.0)	5.21	1.32
C	1.0 km	6/6 (100.0)	3.93	1.45
C	1.5 km	3/6 (50.0)	2.72	1.58

Air samples inside chicken houses and swab samples

The RRT-PCR detection showed that all oropharyngeal swab and indoor air samples from the three chicken houses were positive for H9N2 AIV (Table 2), and the average viral concentrations of indoor air samples was 4.36-6.72×10^5 RNA copies/m^3 (Table 3).

Air samples outside chicken houses

RRT-PCR detection showed that positive results of H9N2 AIV of downwind air samples collected between 100 m and 1.5 km away from the chicken houses B and C were found, with an average load 2.62-5.21×10^3 RNA copies/m^3. The RRT-PCR positive results of H9N2 AIV of outdoor air samples collected between 100 m and 1.0 km away from the chicken house A were also found, with an average load 2.88-4.73×10^3 RNA copies/m^3 (Table 4). RRT-PCR detection showed that the 1.5 km downwind air samples of chicken house A and the upwind air samples of three chicken houses were all negative for H9N2 AIV. Additionally, the average viral concentrations differed significantly (P<0.05) between inside air and downwind air samples.

Discussion

Airborne transmission of H9N2 AIV is poorly understood under field conditions, but infected chickens may pose a serious risk to other chicken flocks and humans, so understanding airborne transmission of H9N2 AIV is of significance [21-24]. In the present study, H9N2 AIV was tested positive in swabs, indoor and downwind air samples, but no virus was detected in upwind air samples. These results showed that the chicken flocks infected by H9N2 AIV form and transmit viral aerosols. In addition, the results support previously published papers where exposure to H9N2 AIV aerosols is considered an important route of airborne transmission within chicken flocks [25-27].

In this study, the indoor air samples were RRT-PCR positive with a mean viral concentration 4.36-6.72×10^5 RNA copies/m^3. Downwind air samples collected between 10 m and 1.5 km away from the chicken houses were also RRT-PCR positive with an average viral load 2.62-5.21×10^3 RNA copies/m^3. These differences in airborne loads of H9N2 AIV may be due to the airborne spread of influenza viruses being associated with wind speed, temperature, relative humidity and the size of chicken flocks. The correlation between the spread of H9N2 AIV and meteorological parameters was not analyzed due to the small size of sampling data in this study.

Conclusions

In summary, this study can provide insights into further understanding the spread characteristics of H9N2 AIV aerosol via air exchange under field conditions.

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