Brief Original Article

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

Song Li¹, Yufa Zhou², Shujuan Gao², Quanhai Pang³, Zengmin Miao⁴

¹ College of Basic Medicine, Taishan Medical University, Tai’an, China
² Center for Disease Control, Daiyue Bureau of Animal Husbandry, Tai’an, China
³ College of Animal Science and Technology, Shanxi Agricultural University, Taigu, China
⁴ College of Life Sciences, Taishan Medical University, Tai’an, China

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 2.62-5.21×10³ RNA copies/m³. 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 originating from infected chicken flocks under field conditions.

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

J Infect Dev Ctries 2017; 11(12):962-966. doi:10.3855/jidc.9013

(Received 20 June 2016 – Accepted 09 November 2016)

Copyright © 2017 Li et al. This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

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 °C for 72 hours, and then allantoic liquid was collected under routine conditions. Viral isolates were identified using haemagglutination inhibition assays (HAI) and neuraminidase inhibition tests using a panel of reference sera. Viral isolates were identified using haemagglutination 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 (RT-PCR) targeting the haemagglutinin gene (primers: HA-F, 5'-AAGCTGGAATCTGAAGGAACTTACA-3'; HA-R, 5'-ATGGACATGCCCAGAAC-3'; Probe: 5'-FAM-ACCATTATGCCTGTGCGCTATCTCTTTG-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°C, 40 cycles for 10 s at 95°C, and 34 s at 60°C.

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’-AGTAGAAACAAGGGTGTTTGTGC- 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 swath 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 swath 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 |
|------|------------|-----------|-----|--------------------|---------|----------|-------------|
| A    | Half-closed| 7         | 500 | Natural            | 17      | 0.3-1.0  | 68          | 1.7-4.7     | Nov. 2015   |
| B    | Half-closed| 4         | 4500| Natural            | 16      | 0.5-1.2  | 65          | 1.6-5.0     | Dec. 2015   |
| C    | Half-closed| 5         | 5000| Natural            | 15      | 0.8-1.3  | 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 (%) | $10^5$ 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 2.62-5.21×10^5 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 2.88-4.73×10^5 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 2.62-5.21×10^5 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.

Acknowledgements

This study was supported by the National Natural Science Grant of China (81501357) and the College Students' Innovation and Entrepreneurship Training Program of China (201610439269 and 201710439327).

References

1. Alexander DJ (2000) A review of avian influenza in different bird species. Vet Microbiol 74: 3-13.
2. Brown IH, Banks J, Manvell RJ, Essen SC, Shell W, Slomka M, Londt B, Alexander DJ (2006) Recent epidemiology and ecology of influenza A viruses in avian species in Europe and the Middle East. Dev Biol 124: 45-50.
3. Lee YJ, Shin JY, Song MS, Lee YM, Choi JG, Lee EK, Jeong OM, Sung HW, Kim JH, Kwon YK, Kwon JH, Kim CJ, Webby RJ, Webster RG, Choi YK (2007) Continuing evolution of H9 influenza viruses in Korean poultry. Virology 359: 313-323.
4. Barbour EK, Mastori FA, Abdel Nour AM, Shaib HA, Jaber LS, Yaghi RH, Sabra A, Sleiman FT, Sawaya RK, Niedzwieck A, Tayeb IT, Kassabiy ZZ, Rath M, Harakeh S, Barbour KE (2009) Standardisation of a new model of H9N2/Escherichia coli challenge in broilers in the Lebanon. Vet Ital 45: 317-322.
5. Kishida N, Sakoda Y, Eto M, Sunaga Y, Kida H (2004) Co-infection of Staphylococcus aureus or Haemophilus paragallinarum exacerbates H9N2 influenza A virus infection in chickens. Arch Virol 149: 2095-2104.
6. Chen BL, Zhang Z, Chen WB (1994) The study of avian influenza: I. The isolation and preliminary serological identification of avian influenza virus in chicken. Chin J Vet Med 20: 3-5
7. Li C, Yu K, Tian G, Yu D, Liu L, Jing B, Ping J, Chen H (2005) Evolution of H9N2 influenza viruses from domestic poultry in Mainland China. Virology 340: 70-83.
8. Xu KM, Smith GJD, Bahl J, Duan L, Tai H, Vijaykrishna D, Wang J, Zhang JX, Li KS, Fan XH, Webster RG, Chen H, Peiris JSM, Guan Y (2007) The genesis and evolution of H9N2 influenza viruses in poultry from southern China, 2000 to 2005. J Virol 81: 10389-10401.
9. Ji K, Jiang WM, Liu S, Chen JM, Chen J, Hou GY, Li JP, Huang BX (2010) Characterization of the hemagglutinin gene of subtype H9 avian influenza viruses isolated in 2007-2009 in China. J Virol Methods 163: 186-189.
10. Zhou JP, Ge FF, Liu J, Ju HB, Yang DQ, Wang J, Zhang WY, Liu PH (2012) Epidemiological survey and genetic evolution of H9 subtype influenza viruses in Shanghai, China, from 2006 to 2010. Arch Virol 157: 1193-1198.
11. Su S, Bi Y, Wong G, Gray GC, Gao GF, Li S (2015) Epidemiology, evolution, and recent outbreaks of avian influenza virus in China. J Virol 89: 8671-8676.
12. Peiris JS, Guan Y, Markwell D, Ghose P, Webster RG, Shortridge KF (2001) Cocirculation of avian H9N2 and contemporary “human” H3N2 influenza A viruses in pigs in
southeastern China: potential for genetic reassortment? J Virol 75: 9679-9686.
13. Saito T, Lim W, Suzuki T, Suzuki Y, Kida H, Nishimura SI, Tashiro M (2001) Characterization of a human H9N2 influenza virus isolated in Hong Kong. Vaccine 20: 125-133.
14. Qi W, Shi W, Li W, Huang L, Li H, Wu Y, Yan J, Jiao P, Zhu B, Ma J, Gao GF, Liao M, Liu D (2014) Continuous reassortments with local chicken H9N2 virus underlie the human-infecting influenza A (H7N9) virus in the new influenza season, Guangdong, China. Protein Cell 5: 878-882.
15. Lv J, Wei B, Yang Y, Yao M, Cai Y, Gao Y, Xia X, Zhao X, Liu Z, Li X, Wang H, Yang H, Roesler U, Miao Z, Chai T (2012) Experimental transmission in guinea pigs of H9N2 avian influenza viruses from indoor air of chicken houses. Virus Res 170: 102-108.
16. Li X, Shi J, Guo J, Deng G, Zhang Q, Wang J, He X, Wang K, Chen J, Li Y, Fan J, Kong H, Gu C, Guan Y, Suzuki Y, Kawaoka Y, Liu L, Jiang Y, Tian G, Li Y, Bu Z, Chen H (2014) Genotypes, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 avian influenza viruses. PLoS Pathog 10:e1004508.
17. Corzo CA, Culhane M, Dee S, Morrison RB, Torremorell M (2013) Airborne detection and quantification of swine influenza A virus in air samples collected inside, outside and downwind from swine barns. PLoS ONE 8:e71444.
18. Swayne DE, Senne DA, Beard CW (1998) Avian influenza. In Swayne DE, Glisson JR, Jackwood MW, Pearson JE, Reed WM, editors. A laboratory manual for the isolation and identification of avian pathogens. Pennsylvania: American Association of Avian Pathologists Press. 150-155.
19. Xu C, Fan W, Wei R, Zhao H (2004) Isolation and identification of swine influenza recombinant A/Swine/Shandong/1/2003(H9N2) virus. Microbes Infect 6: 919-925.
20. Zhou J, Wu J, Zeng X, Huang G, Zou L, Song Y, Gopinath D, Zhang X, Kang M, Lin J, Cowling BJ, Lindsley WG, Ke C, Peiris, JSM, Yen HL (2016) Isolation of H5N6, H7N9 and H9N2 avian influenza A viruses from air sampled at live poultry markets in China, 2014 and 2015. Euro Surveill 21:30331.
21. Wan H, Sorrell EM, Song H, Hossain MJ, Ramirez-Nieto G, Monie I, Stevens J, Cattoli G, Capua I, Chen LM, Donis RO, Busch J, Paulson JC, Brockwell C, Webby R, Blanco J, Al-Natour MQ, Perez DR (2008) Replication and transmission of H9N2 influenza viruses in ferrets: evaluation of pandemic potential. PLoS ONE 3:e2923.
22. Shi H, Ashraf S, Gao S, Lu J, Liu X (2010) Evaluation of transmission route and replication efficiency of H9N2 avian influenza virus. Avian Dis 54: 22-27.
23. Sedlmair N, Hoppenheidt K, Krist H, Lehmann S, Lang H, Böttner M (2009) Generation of avian influenza virus (AIV) contaminated fecal fine particulate matter (PM_{2.5}): Genome and infectivity detection and calculation of immission. Vet Microbiol 139: 156-164.
24. Sang X, Wang A, Ding J, Kong H, Gao X, Li L, Chai T, Li Y, Zhang K, Wang C, Wan Z, Huang G, Wang T, Feng N, Zheng X, Wang H, Zhao Y, Yang S, Qian J, Hu G, Gao Y, Xia X (2015) Adaptation of H9N2 AIV in guinea pigs enables efficient transmission by direct contact and inefficient transmission by respiratory droplets. Sci Rep 5:15928.
25. Zhong L, Wang X, Li Q, Liu D, Chen H, Zhao M, Gu X, He L, Liu X, Gu M, Peng D, Liu X (2014) Molecular mechanism of the airborne transmissibility of H9N2 avian influenza A viruses in chickens. J Virol 88: 9568-9578.
26. Lv J, Wei L, Yang Y, Wang B, Liang W, Gao Y, Xia X, Gao L, Cai Y, Hou P, Yang H, Wang A, Huang R, Gao J, Chai T (2015) Amino acid substitutions in the neuraminidase protein of an H9N2 avian influenza virus affect its airborne transmission in chickens. Vet Res 46: 44.
27. Yao M, Zhang X, Gao J, Chai T, Miao Z, Ma W, Qin M, Li Q, Li X, Liu J, Zhang H (2011) The occurrence and transmission characteristics of airborne H9N2 avian influenza virus. Berl Munch Tierarztl Wochenschr 124: 136-141.

Corresponding author
Zengmin Miao
College of Life Sciences, Taishan Medical University
Changcheng Road 619, Tai’an 271000, China
Phone: +08605386236603
Fax: +08605386236603
E-mail: zengminmiao@126.com

Conflict of interests: No conflict of interests is declared.