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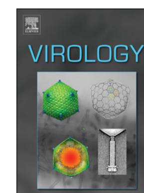

Transmissibility of novel H7N9 and H9N2 avian influenza viruses between chickens and ferrets

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ABSTRACT

Previous studies have shown that the H7N9 avian influenza virus cannot be transmitted efficiently between ferrets via respiratory droplets. Here, we studied the infectivity of the H7N9 avian influenza virus in chickens and its transmissibility from infected to naïve chickens and ferrets. The H7N9 virus (A/Anhui/1/2013) replicated poorly in chickens and could not be transmitted efficiently from infected chickens to naïve chickens and ferrets. H7N9 virus was shed from chicken tracheae for only 2 days after infection and from chicken cloacae for only 1 day after infection, while the H9N2 avian influenza virus, which is endemic in chickens in many Asian countries, was shed from tracheae and cloacae for 8 days after infection. Taken together, our results suggest that chickens may be a poor agent of transmission for the H7N9 virus to other chickens and to mammals, including humans.

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Introduction

In March 2013, it was reported that a novel H7N9 avian influenza virus had infected 3 humans in China (News. Xinhuanet, 2013; Gao et al., 2013). The hemagglutinin (HA) and neuraminidase genes of this virus are derived from Eurasian avian influenza viruses, and the remaining 6 genes are derived from the avian H9N2 influenza virus (Chen et al., 2013; Kageyama et al., 2013; Lam et al., 2013; Li et al., 2013). As of October 25, 2013, 137 humans had been infected with H7N9 avian influenza virus and 45 infected humans had died in China (Who.int, 2013). An epidemiological link between the patients infected with H7N9 avian influenza virus and exposure to poultry has been suggested (Chen et al., 2013; Gao et al., 2013; Han et al., 2013; Lee et al., 2013). Two previous studies reported that 6 out of 7H7N9 avian influenza virus-infected patients had a history of contact with chickens (Chen et al., 2013; Gao et al., 2013). In addition, an analysis of the association between the 12H7N9 avian influenza virus-infected patients and their exposure to poultry showed that all 12 patients had a history of poultry exposure before the onset of clinical signs (Han et al., 2013). Surveillance of the live poultry markets indicated that almost all positive samples of H7N9 avian influenza viruses were from chickens and ducks (Wang et al., 2013). However, a study of all the media-reported H7N9 avian influenza virus cases as of April 29, 2013, showed that, of 125H7N9 avian influenza virus-infected cases, 25 (25%) were associated with exposure to poultry on the farm, 12 (10%) cases had a history of poultry exposure in the live bird market, and the remaining 88 cases (75%) had no known history of poultry exposure (Lee et al., 2013). Surveillance of poultry in live bird markets and farms in Shanghai and Anhui in China revealed that 20 of the 280 samples from live bird markets were positive for H7N9 avian influenza virus and that none of 690 samples from the farm were positive (Lee et al., 2013).

The ferret (*Mustela putorius furo*) has been used as a model of influenza virus transmission in humans because it shows clinical signs of sneezing and fever that are similar to those shown by influenza virus-infected humans (Herfst et al., 2012; Imai et al., 2012; Kim et al., 2009, Belser et al., 2013). In other studies, H7N9 avian influenza viruses were easily transmitted to naïve ferrets through direct contact but, unlike the seasonal H3N2 influenza virus, were not transmitted well by respiratory droplets (Belser et al., 2013; Watanabe et al., 2013; Zhang et al., 2013).

In this study, we wanted to determine the role of chickens in transmitting H7N9 avian influenza virus both to chickens and to mammals. To do this, we studied the infectivity of the H7N9 avian

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influenza virus in chickens, and the transmissibility of the virus both between chickens and from chickens to ferrets. We also compared the infectivity and between-chicken transmissibility of the H7N9 avian influenza virus to that of the H9N2 avian influenza virus, which is endemic in farm chickens in many Asian countries, including China and Korea (Golender et al., 2008; Iqbal et al., 2009; Nagarajan et al., 2009; Nang et al., 2011; Negovetich et al., 2011; Xu et al., 2007).

Results

Infectivity and pathogenesis of H7N9 avian influenza virus in chickens

Chickens were intranasally (i.n.) and intratracheally (i.t.) infected with the H7N9 or H9N2 avian influenza virus and then subsequently checked for mortality and change in body weight (Fig. 1A and B). All chickens infected with the H7N9 avian influenza virus survived, but the mean mortality rate of chickens infected with the H9N2 avian influenza virus was 18.7% (Fig. 1A). The body-weight changes were greater in chickens infected with H9N2 avian influenza virus than in those infected with the H7N9 avian influenza virus. The peak loss of body weights of chickens infected with H7N9 avian influenza virus was 6.5% on 3 days post infection (p.i.), while that of chickens infected with H9N2 avian influenza virus was 8.0% on 7 days p.i. (Fig. 1B). Infected chickens were swabbed daily in tracheae and cloacae for 14 days p.i. to determine viral shedding from these areas, which can be potential sources of infection for humans. The H7N9 avian influenza virus did not shed well in the tracheae and cloacae of infected chickens as compared with the H9N2 avian influenza virus (Fig. 1C). The H7N9 avian influenza virus was shed in the tracheae of the infected chickens for only 2 days p.i., with mean viral titers as determined by 50% egg infectious dose (EID_{50}) of 2.5 EID_{50}/mL, and it was shed in the cloacae of the infected chickens for just 1 day p.i., with a mean viral titer of 3.0 EID_{50}/mL. All 16 inoculated chickens shed the H7N9 avian influenza virus, as demonstrated by the tracheal and cloacal swabs. However, the H9N2 avian influenza virus was shed in both tracheae and cloacae for 8 days p.i., with a range of mean viral titers from 2.5 to 5.0 EID_{50}/mL. All 16 inoculated chickens shed the H9N2 avian influenza virus until 5 days p.i., and 12 survived chickens shed the H9N2 avian influenza virus at 8 days p.i., as demonstrated by the tracheal and cloacal swabs (Fig. 1C). When we measured the mean viral titers in the lungs of infected chickens at 5 days p.i., a higher viral titer was detected in the lungs of chickens infected with the H9N2 avian influenza virus than in those of chickens infected with the H7N9 avian influenza virus, although the viral titers were similar on 3 days p.i. (Fig. 1D). The mean viral titer in the lungs of chickens infected with H9N2 avian influenza virus was 6.0 EID_{50}/mL at 5 days p.i., while that in the lungs of chickens infected with H7N9 avian influenza virus was 4.0 EID_{50}/mL. (Fig. 1D).

Histopathology and antigen detection in the lungs and large intestines of chickens infected with the H7N9 avian influenza virus

Lung tissue harvested on 3 and 5 days p.i. from chickens infected with either the H7N9 or H9N2 avian influenza virus was stained with hematoxylin and eosin (H&E) to determine pathological damage. The lung tissue from chickens infected with the H7N9 avian influenza virus shows mild interstitial pneumonia with a few infiltrations of lymphocytes (Fig. 2A and B), while that from chicken infected with the H9N2 avian influenza virus shows more severe interstitial pneumonia with infiltrations of lymphocytes (Fig. 2C and D). In contrast, the lung tissue from uninfected chickens does not show any sign of pneumonia (Fig. 2E).

The lung tissue was also stained with a mouse anti-influenza A nucleoprotein (NP) antibody to detect viral antigen. Lung tissue taken at 3 days p.i. from chickens infected with the H7N9 avian influenza virus shows positive staining in several cells (Fig. 2F), but

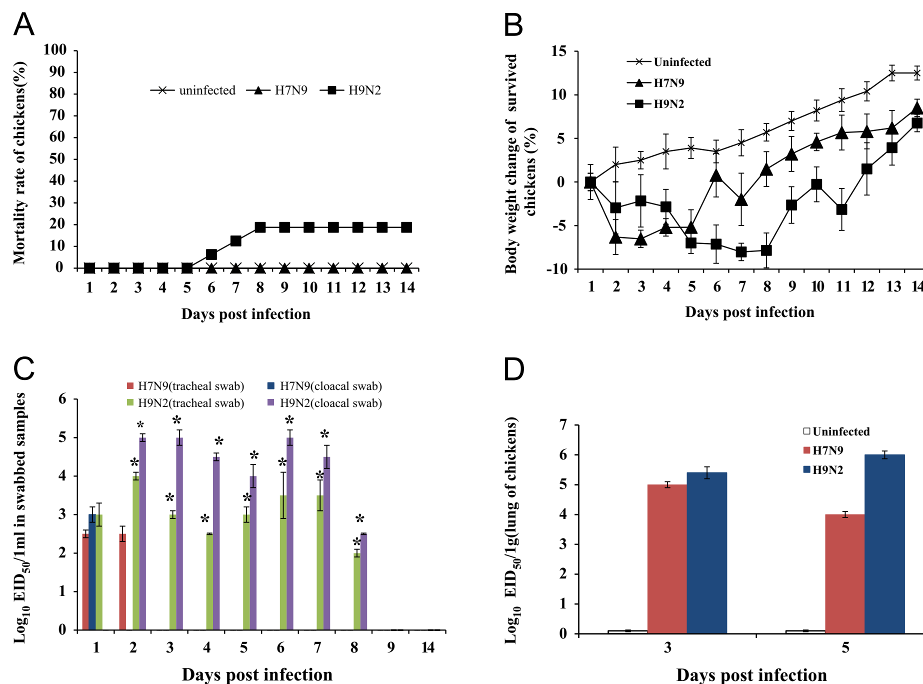

Fig. 1. Clinical signs and viral titers from swab and lung samples of chickens. Chickens ($n=16$ per group) were i.n. and i.t. infected with $10^{9.6}$ EID_{50} of the H7N9 or the H9N2 avian influenza virus. The survival rate (A), and change in body weight (B) were observed daily for 14 days p.i. Infected chickens ($n=16$ per group) were swabbed daily in their tracheae and cloacae with PBS (pH 7.4), and the viral titers were determined as $\log_{10}EID_{50}$/mL (C). At days 3 and 5 p.i., lung tissues (1 g) were harvested from euthanized chickens ($n=3$ per group) that had been i.n. and i.t. infected with $10^{9.6}$ EID_{50} of the H7N9 or H9N2 avian virus and were homogenized in 1 mL of isolation media (50% glycerol in PBS [pH 7.4], 0.5% gentamycin, and 1% mycostatin). The viral titers in the homogenized tissues were determined in eggs as $\log_{10}EID_{50}$/mL (D). Statistical analysis was performed by comparing H7N9 data to H9N2 data. $^{*}P < 0.05$.

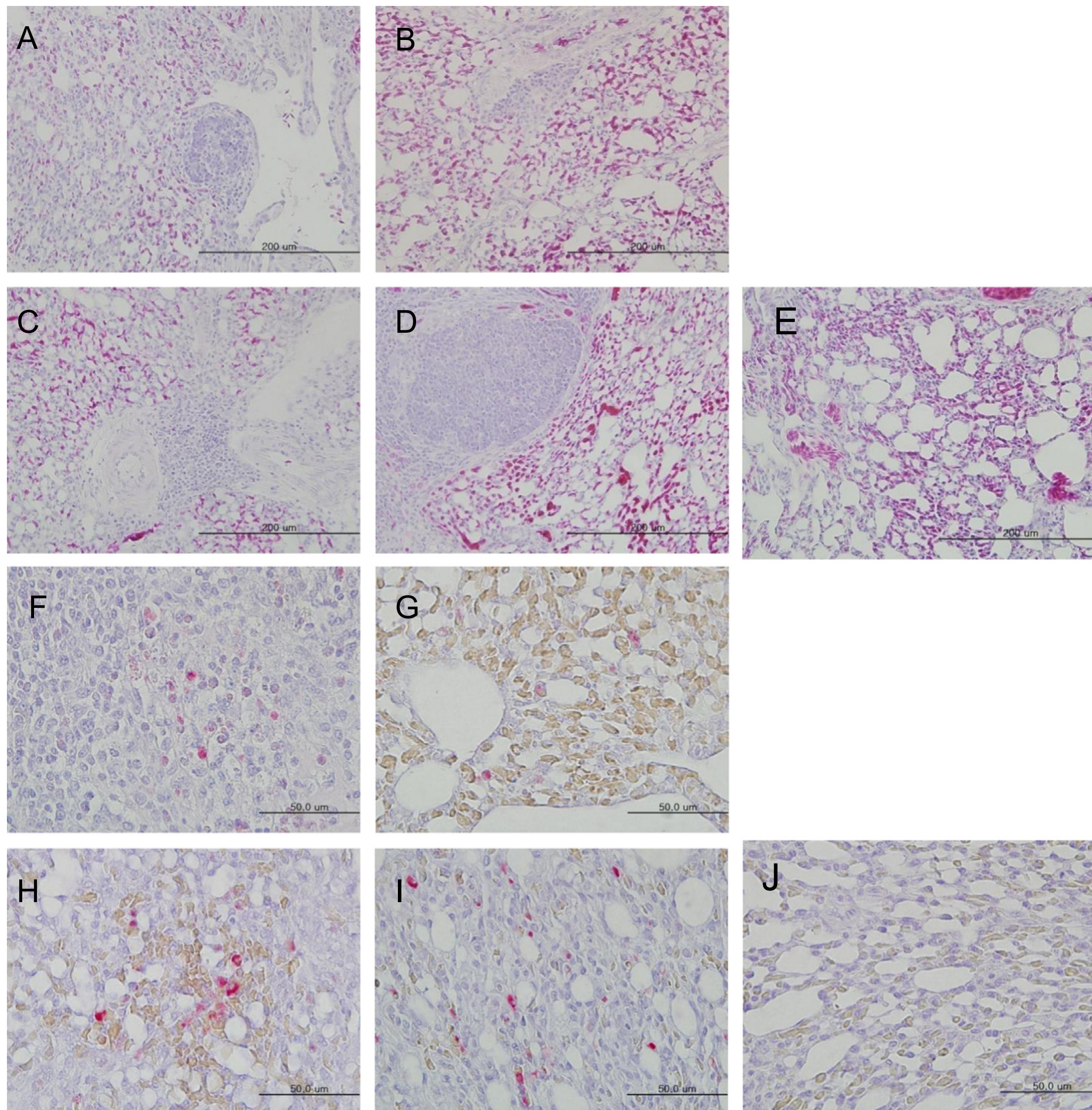

Fig. 2. Histopathology and immunohistochemistry in the lungs of chickens. Lung tissue harvested at 3 and 5 days p.i. from chickens ($n=3$ per group) that had been infected with $10^{9.6}$ EID_{50} of the H7N9 or the H9N2 avian influenza virus was stained with H&E (A–E) (400 × magnification) or with mouse anti-influenza A virus NP antibody, biotin-labeled goat anti-mouse immunoglobulin, and Vector red alkaline phosphatase substrate (F–J) (1000 × magnification). H&E-stained lung tissue taken at 3 days p.i. from a chicken infected with the H7N9 avian influenza virus. (B) H&E-stained lung tissue taken at 5 days p.i. from a chicken infected with the H7N9 avian influenza virus. (C) H&E-stained lung tissue taken at 3 days p.i. from a chicken infected with the H9N2 avian influenza virus. (D) H&E-stained lung tissue taken at 5 days p.i. from a chicken infected with the H9N2 avian influenza virus. (E) H&E-stained lung tissue from an uninfected control chicken. (F) IHC-stained lung tissue taken at 3 days p.i. from a chicken infected with the H7N9 avian influenza virus. (G) IHC-stained lung tissue taken at 5 days p.i. from a chicken infected with the H7N9 avian influenza virus (H) IHC-stained lung tissue taken at 3 days p.i. from a chicken infected with the H9N2 avian influenza virus. (I) IHC-stained lung tissue taken at 5 days p.i. from a chicken infected with the H9N2 avian influenza virus. (J) IHC-stained lung tissue of an uninfected control chicken. Similar results were obtained with 3 different chickens in each group.

very few cells in the lung tissue from chickens infected with the H7N9 avian influenza virus were positive at 5 days p.i. (Fig. 2G). In contrast, several cells in lung tissue taken 3 and 5 days p.i from chickens infected with the H9N2 avian influenza virus show positive staining (Fig. 2H and I). Lung tissue from uninfected chicken did not show any staining (Fig. 2J).

The large intestine tissue harvested on 3 and 5 days p.i. from chickens infected with either the H7N9 or H9N2 avian influenza virus was stained with H&E to determine pathological damage. The large intestine tissue of chickens infected with H7N9 avian influenza virus did not show pathological signs (Fig. 3A and B) like that of uninfected chicken (Fig. 3E), while that of chickens infected with H9N2 avian influenza virus had flattened villi and the infiltrations of inflammatory cells (Fig. 3C and D).

When the large intestine tissue was stained with a mouse anti-influenza A nucleoprotein (NP) antibody to detect viral antigen few positive staining were detected in the large intestine tissue of chickens infected with H7N9 avian influenza virus (Fig. 3F and G), but several positive staining was detected in the large intestine tissue of chickens infected with H7N9 avian influenza virus (Fig. 3H and I). No positive staining was detected in the large intestine tissue of uninfected chicken (Fig. 3J).

To determine the possible cause(s) of the histopathological lesions in the lungs of the infected chickens, we quantified the expression levels of various inflammatory cytokines (TGF-β3, TNF-α, IFN-α, IFN-β, IL-1β, IL-2, IL-4, IL-8, and IL-10) in lung tissue taken at 3 and 5 days p.i. from chickens infected with either the H7N9 or the H9N2 avian influenza virus. (Fig. 4A). The level of induction for these cytokines in the lungs of chickens infected with either the H7N9 or the H9N2 avian influenza virus was similar (Fig. 4A). We also quantified the expression levels of various Toll-like receptors (TLRs; TLR 1, 2, 3, 4, 5, 7, and 15) that

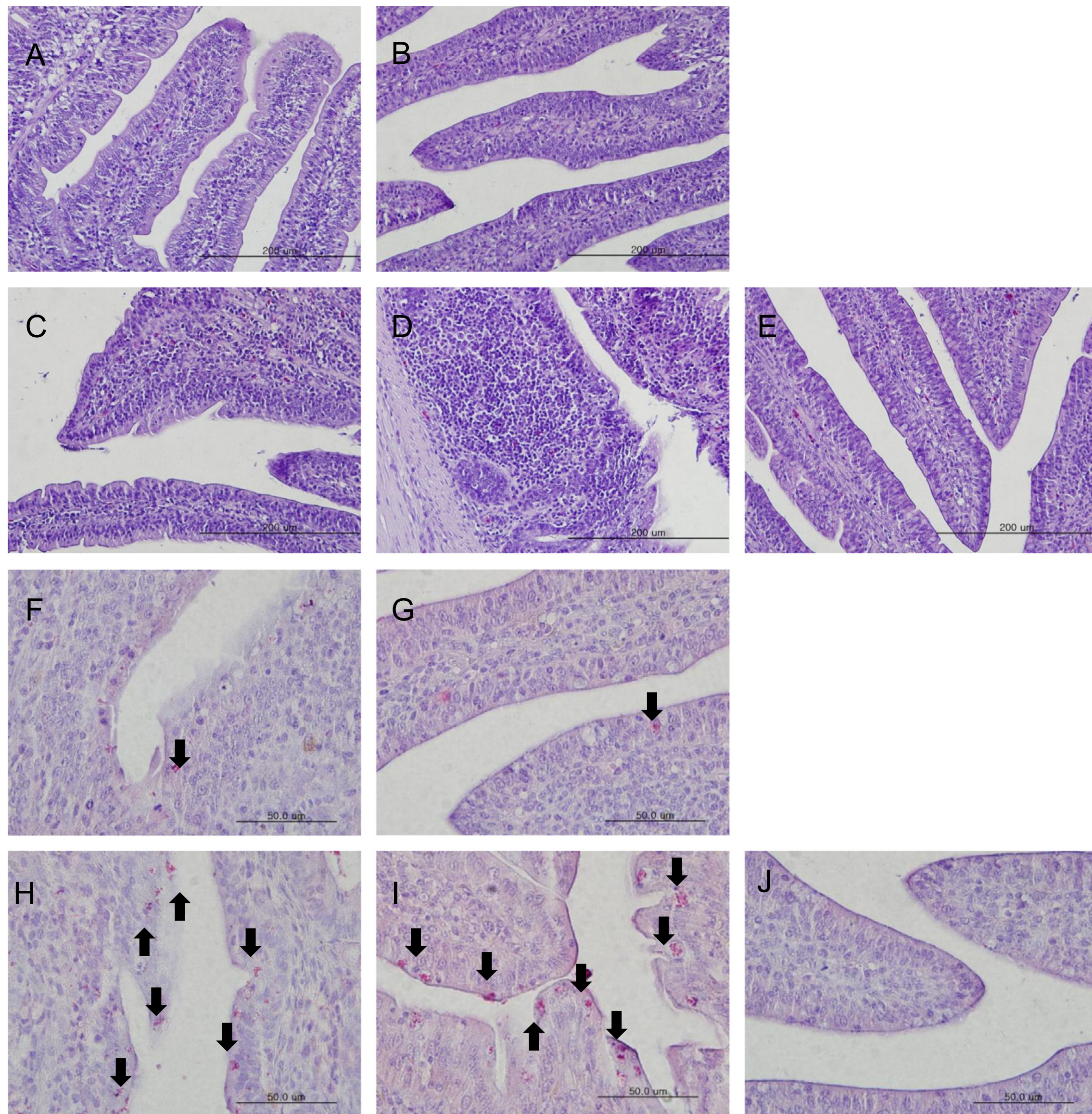

Fig. 3. Histopathology and immunohistochemistry in the large intestines of chickens. Large intestine tissue harvested at 3 and 5 days p.i. from chickens ($n=3$ per group) that had been infected with $10^{9.6}$ EID_{50} of the H7N9 or the H9N2 avian influenza virus was stained with H&E (A–E) (400 × magnification) or with mouse anti-influenza A virus NP antibody, biotin-labeled goat anti-mouse immunoglobulin, and Vector red alkaline phosphatase substrate (F–J) (1000 × magnification). (A) H&E-stained large intestine tissue taken at 3 days p.i. from a chicken infected with the H7N9 avian influenza virus. (B) H&E-stained large intestine tissue taken at 5 days p.i. from a chicken infected with the H7N9 avian influenza virus. (C) H&E-stained large intestine tissue taken at 3 days p.i. from a chicken infected with the H9N2 avian influenza virus. (D) H&E-stained large intestine tissue taken at 5 days p.i. from a chicken infected with the H9N2 avian influenza virus. (E) H&E-stained large intestine tissue from an uninfected control chicken. (F) IHC-stained large intestine tissue taken at 3 days p.i. from a chicken infected with the H7N9 avian influenza virus. (G) IHC-stained large intestine tissue taken at 5 days p.i. from a chicken infected with the H7N9 avian influenza virus H. IHC-stained large intestine tissue taken at 3 days p.i. from a chicken infected with the H9N2 avian influenza virus. (I) IHC-stained large intestine tissue taken at 5 days p.i. from a chicken infected with the H9N2 avian influenza virus. (J) IHC-stained large intestine tissue of an uninfected control chicken. Similar results were obtained with 3 different chickens in each group.

are involved in inducing innate immunity (Fig. 4B). TLR15 expression was significantly upregulated in the lungs of chickens infected with either the H7N9 or the H9N2 avian influenza virus (Fig. 4B). TLR15 expression in the lungs of chickens infected with the H7N9 avian influenza virus was increased by 14.22-fold at 5 days p.i. and by 30.7-fold in the lungs of chickens infected with the H9N2 avian influenza virus. (Fig. 4B).

Transmissibility of the H7N9 avian influenza virus between chickens and from chickens to ferrets

In order to determine the role of chickens in spreading the H7N9 avian influenza virus to chickens or to mammals (including humans), we examined transmission of the virus from infected chickens to naïve chickens or naïve ferrets (Table 1). Transmission of the H7N9 avian influenza virus from inoculated chickens to naïve chickens does not occur, and neither does aerosol transmission of the H7N9 avian influenza virus from inoculated chickens to naïve ferrets. Whereas the 3 chickens inoculated with H7N9 avian influenza virus shed virus in their tracheae for 2 days p.i. with a mean viral titer of 2.5 EID_{50}/mL, and in their cloacae for 1 day p.i. with a mean viral titer of 3.0 EID_{50}/mL, the 3 naïve chickens did not shed H7N9 avian influenza virus either in their tracheae or in their cloacae. In addition, although the 3 chickens inoculated with H7N9 avian influenza virus were serologically converted, with a mean hemagglutination-inhibition (HI) titer of 140, the 3 naïve chickens were not (Table 1). Moreover, looking at the air-borne transmission efficiency of the H7N9 avian influenza virus from

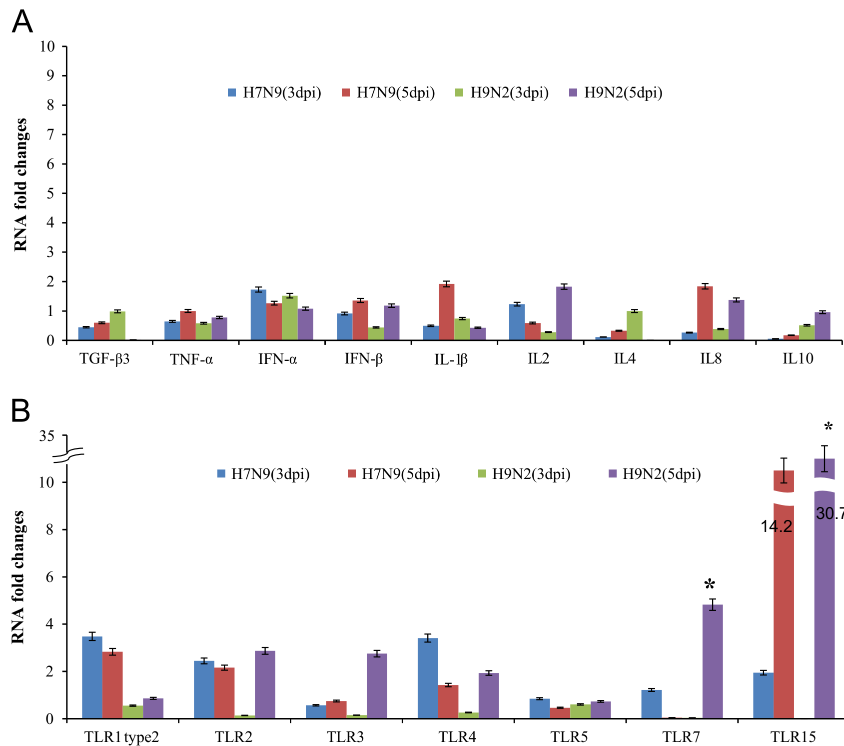

Fig. 4. Quantification of inflammatory cytokines and TLRs in chicken lungs. Total RNA was extracted from lung tissues (1 g) taken at 3 and 5 days p.i. from chickens ($n=3$ per group) that had been infected with $10^{9.6}$ EID_{50} of the H7N9 or the H9N2 avian influenza virus. Expression levels of cytokines and TLRs were quantified by SYBR Green-based real-time PCR using chicken-specific cytokine and TLR primers. Statistical analysis was performed by comparing H7N9 data to H9N2 data. *$P<0.05$. (A) Cytokines and (B) TLRs.

Table 1
Transmission of a novel H7N9 avian influenza virus between chickens, chickens to ferrets, and seroconversion.

Swab route	H7N9			H9N2	
	Chickens to chickens[a]		Chickens to ferret[b]	Chickens to chickens[a]	
	Inoculated No. of infected chickens/ no. of total chickens (The mean viral titer)	Contacted No. of infected chickens/ no. of total chickens (The mean viral titer)	Aerosol transmission No. of infected ferrets/ no. of total ferrets (The mean viral titer)	Inoculated No. of infected chickens/ no. of total chickens (The mean viral titer)	Contacted No. of infected chickens/no. of total chickens (The mean viral titer)
Tracheae	3/3 (2.5[c])	0/3 (< 1.0)	0/3 (< 1.0)	3/3 (3.28[d])	3/3 (2.41[e])
Cloacae	3/3 (3.0[f])	0/3 (< 1.0)	0/3 (< 1.0)	3/3 (4.14[d])	3/3 (3.43[e])
Seroconversion No. of positive animals/ no. of total animals (Mean HI titer[g])	3/3 (140)	0/3 (< 10)	0/3 (< 10)	3/3 (133)	3/3 (20)

[a] Chickens ($n=3$ per group) were i.n. & i.t. infected with $10^{9.6}$ EID50/mL of H7N9 or H9N2 avian influenza virus and were mixed with naïve 3 chickens in the same cage. The chickens were daily swabbed in the tracheae and cloacae in PBS (pH 7.4) for 14 days p.i.
[b] For the study of aerosol transmission of H7N9 avian influenza virus the cage containing the three infected chickens with H7N9 avian influenza virus was placed 5 cm apart from the cage containing naïve three ferrets. The inoculated chickens and naïve ferrets were daily swabbed in the trachea and cloacae for chickens (rectums for ferrets) for 14 days p.i. Viral titers in the swabbed samples were measured in eggs by $\log_{10}EID_{50}$/mL.
[c] Virus was detected in the swabbed samples untill 2 days 1 p.i., but no virus in the subsequent swabbed samples was detected from 3 days to 14 days p.i.
[d] Virus was detected in the swabbed samples until 8 days p.i.
[e] Virus was detected in the swabbed samples from 2 days p.i. to 7 days after contact.
[f] Virus was detected in the swabbed samples only on day 1 p.i., but no virus in the subsequent swabbed samples was detected from 2 days to 14 days p.i.
[g] Hemagglutination inhibition (HI) titer was determined in sera collected from animals on 21 days p.i.

inoculated chickens to naïve ferrets, we found that none of the 3 naïve ferrets become infected with H7N9 avian influenza virus or were serologically converted (Table 1).

In contrast, H9N2 avian influenza virus is readily transmitted to naïve chickens. The 3 contact naïve chickens were infected with the H9N2 avian influenza virus from 2 days after contact and shed virus from 2 to 7 days after contact through the tracheae and cloacae, with a range of mean viral titers from 2.41 to 3.43 EID_{50}/mL (Table 1). All 3 chickens inoculated with H9N2 avian influenza virus shed virus from 1 day p.i. to 8 days p.i., with a range of mean viral titers from 3.28 to 4.14 EID_{50}/mL (Table 1). All 3 chickens inoculated with the H9N2 avian influenza virus were serologically converted, with a mean HI titer of 133.0, and all 3 naïve chickens that had contact with chickens inoculated with the H9N2 avian influenza virus were serologically converted, with a mean HI titer of 20 (Table 1). No aerosol transmission of H9N2 avian influenza virus from the infected chickens to naïve ferrets occurred (data not shown).

Discussion

We studied the infectivity of the H7N9 avian influenza virus among chickens and the transmissibility of this virus from infected chickens to naïve chickens and ferrets. In contrast to chickens infected with the H9N2 avian influenza virus, which is endemic in chickens in many Asian countries, chickens infected with the H7N9 avian influenza virus did not shed virus well in their tracheae and cloacae, potential sources of virus in human infections. Moreover, aerosol transmission of H7N9 avian influenza virus from the infected chickens to naïve ferrets did not occur.

Our infectivity study showed that chickens infected with the H7N9 avian influenza virus shed virus in their tracheae and cloacae for 2 days p.i. and 1 day p.i., respectively; whereas chickens infected with the H9N2 avian influenza virus shed viruses in their tracheae and cloacae for 8 days p.i. These results suggest that, unlike the H9N2 avian influenza virus, the H7N9 avian influenza virus does not infect chickens well. The results from a previous genetic analysis of H7N9 avian influenza viruses support our observations (Kageyama et al., 2013). HA genes in H7N9 avian influenza viruses contain several mutations that may facilitate binding to human receptors (α-2,6-linked sialic acids) rather than to avian receptors (α-2,3-linked sialic acids). A/Shanghai/1/2013 (H7N9) has an A138S mutation in HA, and A/Shanghai/2/2013, A/Anhui/1/2013, the 2 avian viruses, and the virus from the environmental sample contain G186V and Q226L mutations in HA. All 3 mutations may increase the binding of the H7N9 avian influenza virus to human receptors (Srinivasan et al., 2013). Results from glycan receptor binding studies also show that the H7N9 avian influenza virus has a propensity to bind to human receptors (Tharakaraman et al., 2013; Xiong et al., 2013). In addition to increased binding to human receptors, the H7N9 avian influenza virus has a lysine at position 627 in the polymerase PB2, which may help avian influenza viruses to replicate in mammals (Hatta et al., 2001). The H9N2 avian influenza virus used in this study does not contain any mutations in either HA or PB2.

The H7N9 avian influenza virus was detected in the tracheae of infected chickens for only 2 days p.i. but was detected in the lungs of infected chickens at 3 and 5 days p.i. This result indicates that the H7N9 avian influenza virus seems to prefer infecting lungs to infecting tracheae in chickens.

Chickens infected with the H7N9 avian influenza virus did not show any mortality and only lost up to 6.5% body weight at 3 days p.i.; whereas chickens infected with the H9N2 avian influenza virus showed 18.7% mortality and lost up to 8.0% of their body weight by 7 days p.i. These results indicate that the H7N9 avian influenza causes much milder clinical signs in infected chickens than does the H9N2 avian influenza virus. Thus, it might be difficult for farmers to detect whether their chickens are infected with the H7N9 avian influenza virus.

H&E staining showed that lung tissue of chickens infected with both the H7N9 and the H9N2 avian influenza viruses had mild interstitial pneumonia, although the lung tissue from chickens infected with the H9N2 influenza virus was infiltrated with slightly more inflammatory cells. When we measured the expression levels of inflammatory cytokines and TLRs, which are known to be involved in causing pathogenesis in influenza infections (Nang et al., 2011), their levels of induction in the lungs was similar for both the H7N9 and the H9N2 influenza viruses.

Our results showed that aerosol transmission of the H7N9 avian influenza virus from infected chickens to naïve ferrets did not occur efficiently. This observation suggests that chickens may not transmit the H7N9 avian influenza virus efficiently to humans. The poor transmission from infected chickens to ferrets may be because of poor replication of the A/Anhui/1/2013 (H7N9) virus used in our study. We cannot rule out the possibility that other strains of H7N9 avian influenza viruses isolated in China can be transmitted from infected chickens to naïve ferrets, since the H7N9 avian influenza viruses isolated from chickens from live bird markets in China were genetically similar to the H7N9 avian influenza virus isolated from a human (Chen et al., 2013; Han et al., 2013; Kageyama et al., 2013).

Materials and methods

Viruses and animals

A novel H7N9 avian influenza virus (A/Anhui/1/2013) was kindly provided by the USA Centers for Disease Control and Prevention, which collaborates with the World Health Organization. The H9N2 avian influenza virus (A/chickens/Korea/SA1001/2013) was isolated from chickens on a farm in Korea in 2013. The H7N9 and H9N2 avian influenza viruses were grown in the amniotic cavities of 10-day-old hen eggs.

Four-week-old chickens were obtained from local live bird markets in Korea. The chickens were shown to be serologically negative to the H7N9 and H9N2 avian influenza viruses when their sera were tested using the HI assay with those viruses and 0.5% turkey red blood cells. The sera were also negative for other HA subtypes of avian influenza viruses (H1, H2, H3, H4, H5, H6, H8, H10, H11, H12, H13, and H14). Animal experiments were performed at an enhanced biological safety level 3 (BSL-3+) facility approved by the Korean government.

Ethics statement

This study was carried out in strict accordance with the recommendations in the Guide for the Care and Use of Laboratory Animals of the Korean Veterinary Quarantine and Service, and the protocol was approved by the Committee on the Ethics of Animal Experiments of Chungnam National University.

Infection of chickens with H7N9 and H9N2 avian influenza viruses

Chickens ($n=16$ per group) were i.n. and i.t. infected with $10^{9.6}$ EID_{50} of the H7N9 or H9N2 avian influenza virus, and their body weight and mortality were observed daily for 14 days p.i. The infected chickens were swabbed daily in their tracheae and cloacae for 14 days p.i. using 1 mL of isolation media (50% glycerol in PBS [pH 7.4], 0.5% gentamycin, and 1% mycostatin).

Contact and aerosol transmission of H7N9 avian influenza virus between chickens and from chickens to ferrets

Three chickens were i.n. and i.t. infected with $10^{9.6}$ EID_{50} of the H7N9 or the H9N2 avian influenza virus and then placed with 3 naïve chickens in the same cage to determine the contact transmission efficiency of H7N9 or H9N2 avian influenza virus. The chickens were swabbed daily in their tracheae and cloacae by using 1 mL of isolation media (50% glycerol in PBS [pH 7.4], 0.5% gentamycin, and 1% mycostatin) for 14 days p.i. and were bled at 21 days p.i.

To test the aerosol transmission of H7N9 avian influenza virus from chickens to ferrets, a cage containing 3 chickens i.n. and i.t. infected with $10^{9.6}$ EID_{50} of H7N9 avian influenza was placed 5 cm away from a cage containing 3 naïve ferrets. Animals were swabbed daily, in the tracheae and cloacae of the chickens or in the tracheae and rectums of the ferrets, using 1 mL of isolation media (50% glycerol in PBS (pH 7.4), 0.5% gentamycin, and 1% mycostatin) for 14 days p.i. and were bled on 21 days p.i.

Measurement of viral titers in swabbed samples and lungs of chickens

At days 3 and 5 p.i., lung tissue (1 g) was harvested from euthanized chickens ($n=3$ per group) that had been i.n. and i.t. infected with $10^{9.6}$ EID_{50} of the H7N9 or the H9N2 avian virus. The harvested lung tissues were frozen in liquid nitrogen, homogenized with a mortar and pestle, and then resuspended in 1 mL of PBS (pH 7.4) supplemented with a 2 × antibiotic–antimycotic solution (Sigma, St. Louis, MO, USA).

The swab and lung samples were diluted serially 10-fold in PBS (pH 7.4), and each diluted sample was inoculated into 10-day-old embryonated hens′ eggs. The presence of virus in the inoculated eggs was identified by the hemagglutination assay. Viral titers were calculated by $\log_{10}EID_{50}$/mL as previously described (Reed and Muench, 1938).

Measurement of antibody titers by the HI assay

Sera from chickens and ferrets were first treated with receptor destroying enzyme (RDE) (DENKA SEIKEN, Tokyo, Japan). The RDE-treated sera were diluted serially 2-fold in PBS (pH 7.4) in V-bottom 96-well plates. An equal amount of HA (8 units; 25 μL) from the H7N9 or H9N2 avian influenza virus was added. The plates were incubated for 15 min at room temperature, and then 50 μL of 0.5% turkey red blood cells were added. Plates were incubated at room temperature for 40 min. The HI titer was expressed as the reciprocal of the dilution that completely inhibited hemagglutination.

Histopathological and immunohistochemical staining of chicken lung and large intestine tissue

Lung and large intestine tissue from chickens ($n=3$ per group) that had been i.n. and i.t. infected with $10^{9.6}$ EID_{50} of the H7N9 or H9N2 avian influenza virus were fixed in 10% neutral buffered formalin and then embedded in paraffin. Five-micrometer sections were cut and stained with H&E as described (Bancrof and Stevens, 1996).

Five-micrometer-thick sections were stained with a mouse anti-influenza A virus NP antibody (Serotec, Oxford, United Kingdom). The tissue sections were deparaffinized, rehydrated in distilled water, and then fixed with 100% chilled acetone for 2 h. The endogenous peroxidase activity was blocked in 3% H_2O_2 for 15 min at 37 °C before the sections were blocked with 5% bovine serum albumin in PBS (pH 7.4) for 1 h. The blocked tissue sections were labeled with mouse anti-influenza A virus NP antibody (1:1000 dilution) by incubating at room temperature for 1 h. The labeled tissue sections were then stained with biotin-labeled goat anti-mouse immunoglobulin, VECTASTAIN ABC-AP, and Vector red alkaline phosphatase substrate (Vector Laboratories, Burlingame, CA, USA). The stained tissue sections were counterstained with hematoxylin QS (Vector Laboratories, Burlingame, CA, USA). The stained tissues were evaluated under an Olympus DP70 microscope (Olympus Corporation, Tokyo, Japan).

Quantification of inflammatory cytokines and TLRs in chicken lung tissue by quantitative real-time PCR

TRIzol reagent (Invitrogen, Carlsbad, CA, USA) was used to extract total RNA from the lung tissues (1 g) of chickens ($n=3$ per group) that had been infected with $10^{9.6}$ EID_{50} of the H7N9 or the H9N2 avian influenza virus. Then, the mRNAs of inflammatory cytokines and TLRs were quantified using quantitative real-time PCR. To synthesize the cDNA, 1 μL of oligo dT primers (0.5 pmole; Promega, Madison, WI, USA) was added to a total volume of 9 μL in a 0.05 mL tube. The mixture was denatured for 5 min at 70 °C prior to incubation for 5 min at 4 °C. Then, 4 μL of 25 mM $MgCl_2$, 4 μL of 5 × reverse transcriptase enzyme buffer, 1 μL of RNase inhibitor, 1 μL of reverse transcriptase, and 1 μL of dNTPs (10 mM) were added to each sample. Then, the samples were incubated for 5 min at 25 °C, 60 min at 42 °C, and 15 min at 70 °C. SYBR Green-based real-time PCR was performed using a Roto-Gene 6000 apparatus (Corbett, Mortlake, Australia) and SensiMix Plus SYBR (Quantace, London, UK) as per the manufacturers′ instructions. Samples were run in duplicate. A total volume of 20 μL containing 2 μL cDNA, 10 μL SYBR mixture, and inflammatory cytokine-specific or TLR-specific primers for chickens (Nang et al., 2011; 1 μL of forward primer [20 pmole] and 1 μL of reverse primer [20 pmole]) was used with 40 cycles of PCR under the following conditions: 5 s at 95 °C, 15 s at 60 °C, and 25 s at 72 °C. Cytokine and TLR mRNA expression levels in tissues were normalized to those of chicken glyceraldehyde-3-phosphate dehydrogenase (GAPDH). The results of real-time PCR were quantified by the comparative threshold method after subtracting the data from uninfected control chickens.

Statistical analysis

Statistical analysis was performed using the Statistical Product and Services Solutions (SPSS) package, version 10.0 (SPSS, Cary, NC, USA). The Student′s *t*-test was used. A P-value < 0.05 was considered to be significant.

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