Biological Characteristics of H9N2 Avian Influenza Viruses from Healthy Chickens in Shanghai, China

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Background: H9N2 avian influenza viruses that circulate in domestic poultry in eastern China pose challenges to human health. However, few studies have compared the biological characteristics of H9N2 viruses isolated from healthy chickens in Shanghai.

Material/Methods: Three H9N2 viruses – CK/SH/Y1/07, CK/SH/Y1/02, and CK/SH/23/13 – isolated from healthy chickens in Shanghai between 2002 and 2013, were selected and their biological characteristics were determined.

Results: All 3 H9N2 viruses showed a preference for both the avian- and human-like receptors, and they replicated well in MDCK and A549 cells. All H9N2 viruses were non-pathogenic to mini-pigs and were detected in the trachea and lung tissues. The CK/SH/Y1/07 and CK/SH/Y1/02 viruses were transmitted to mini-pigs through direct-contact or respiratory droplet exposure, but CK/SH/23/13 virus was not.

Conclusions: These results suggest that H9N2 viruses isolated from healthy chickens in Shanghai efficiently replicate and transmit among pigs and other mammals.

MeSH Keywords: Cultural Characteristics • Disease Transmission, Infectious • Influenza A Virus, H9N2 Subtype • Swine, Miniature • Virus Replication

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Background

Avian influenza viruses (AIVs) were previously considered to be species-specific, while wild aquatic birds and poultry are the primordial reservoirs. However, antigenic drifts and shifts, as well as genetic reassortment, of AIVs afford considerable opportunities to cross the species barrier to infect mammals. A newly emerged H7N9 AIV in China was reported in 18 provinces and caused at least 500 human infections, leading to 200 deaths by December 2015 (as reported by the National Health and Family Planning Commission of the People's Republic of China). Another novel H10N8 AIV caused 3 human infections in Jiangxi province during December 2013, and 2 of the cases died. Genetic analyses have demonstrated that the hemagglutinin (HA) gene and the neuraminidase (NA) gene of these 2 novel AIVs originated from wild birds, but the other internal genes shared the highest identities with H9N2 AIVs [1,2]. However, H9N2 AIVs have been endemic and of low pathogenicity in domestic poultry since the early 1990s, and investigations revealed that H9N2 AIVs can occasionally be transmitted from poultry to humans [3–5], suggesting that H9N2 AIVs poses a potential public health threat.

In addition to circulating in poultry, H9N2 AIVs were also reported to heavily contaminate the sewage and poultry drinking water in live poultry markets [6]. Another survey in Shanghai revealed that almost all positive samples of H9 AIVs tested from chickens were obtained from local poultry farms or live poultry markets [7]. These investigations strongly suggest that H9N2 AIVs are prevalent in live poultry markets and that the occurrence of human infection is not rare. We [8] and other researchers using serosurveys [9–11] confirmed that retail poultry workers and poultry-exposed workers have higher rates of positive H9N2 antibodies than in the general population. However, no H9N2 viruses were isolated from swabs collected from poultry-exposed workers or patients with influenza-like illnesses (ILI) in our previous virological surveillance. No evidence so far suggests that H9N2 AIVs have acquired the capacity for human-to-human transmission; however, it remains a concern whether they can replicate and transmit among other mammals.

It has been demonstrated that the adaptation of H9N2 AIVs in mammals through serial passage or reassortant H9N2 viruses containing genes from H3N2 or 2009/H1N1 virus are transmissible in mammals [12–14]. In addition, the amino acid residue leucine at position 226 (L226, H3 numbering) in the receptor-binding site (RBS) of HA protein is a critical motif for enhanced viral binding affinity, and L226-containing H9N2 viruses display human-like cell tropisms (α2, 6-linked sialic acid [SA] receptors). However, most H9N2 AIVs isolated from China have a leucine at HA 226 (http://blast.ncbi.nlm.nih.gov/Blast.cgi), and limited reports of human H9N2 infections suggest that this motif may not be sufficient for a human-to-human transmission.

During previous surveillance for AIVs in eastern China, several H9N2 viruses were isolated from healthy chickens and some of them had amino acid residue L at position 226 [15,16]. Whether these viruses can replicate and transmit in mammals needs further investigation. In this study, we selected a panel of 3 H9N2 AIVs, isolated from healthy chicken in Shanghai between 2002 and 2013, to evaluate their biological characteristics, including their receptor-binding ability, their infectivity in different cell lines, and their replication and transmission in mini-pigs.

Material and Methods

Facility

Studies with H9N2 AIVs were conducted in a negative-pressure biohazard suite (biosafety level 2) with high-efficiency particulate air-filtered exhaust. The room conditions for these experiments were set at about 25°C and 30–40% relative humidity. All animal experiments were authorized by the Animal Ethics and Welfare Committee of Fudan University.

Cell and viruses

Human-type II alveolar epithelial (A549) cells and Madin-Darby canine kidney (MDCK) cells were maintained and inoculated with viruses in minimal essential medium (Gibco, USA) as previously described [8].

The viruses used in this study, including 3 low-pathogenic H9N2 AIVs and a seasonal human H3N2 virus, are described in Table 1. The H9N2 AIVs CK/SH/Y1/02, CK/SH/Y1/07, and CK/SH/23/13 were isolated from healthy chickens in Shanghai and were kindly provided by Dr. J. P. Zhou (the Shanghai Animal Disease Control Center). The human seasonal H3N2 virus SH/MH124/13 was isolated from an ILI patient by our laboratory in Shanghai, as previously described [8]. All virus strains were propagated in MDCK cells, and they were plaque-purified 3 times. The viral titers were determined by calculating the 50% tissue culture infectious dose (TCID50) based on the method of Reed and Muench [17].

Growth kinetics of test viruses

To evaluate the growth kinetics of H9N2 viruses, monolayers of MDCK or A549 cells were infected with 0.1 TCID50 of virus, as previously described [8]. Supernatant was collected at different time points (12, 24, 36, and 48 h) and titrated by the TCID50 analysis. The viral titers were compared by using
analysis of variance (ANOVA) and differences were considered to be significant at \( P < 0.05 \).

**Receptor-binding assay**

Hemagglutination assays using sialidase-treated chicken red blood cells (CRBCs) were performed as described previously [18] with a modification of using 2 different glycopolymers: Neu5Ac\(\alpha\)2-3Gal\(\beta\)1-4GlcNAc\(\beta\)-PAABiotin and Neu5Ac\(\alpha\)2-6Gal\(\beta\)1-4GlcNAc\(\beta\)-PAABiotin (GlycoTech Corporation, USA). Briefly, CRBCs were washed and diluted to 10% (vol/vol) in phosphate-buffered saline (PBS). We inoculated 0.1 ml of CRBCs with 1.25 units of \( \alpha \text{2, 3-sialidase} \) (Takara, Japan) for 1 h at 37°C. Viruses in a volume of 50 μl were serially diluted in 50 μl of PBS and were mixed with 0.5% (vol/vol) of CRBCs in a 96-well plate at room temperature. The hemagglutination assay titers were read after 15-min incubation.

The treated CRBCs were also adjusted to 0.5% final working concentration with PBS, and different concentrations of 2 biotinylated glycopolymers were added to a 96-well plate coated with streptavidin (Pierce, USA). The plates were blocked with 2% skim milk and inoculated at 4°C overnight. We added 64 HA units of influenza virus in PBS after washing and inoculation at 4°C overnight. The chicken antiserum against H9 was added and incubated for 4 h at 4°C. After washing 5 times with PBS, the plates were inoculated with HRP-linked goat anti-chicken antibody (Sigma-Aldrich, USA) for 1 h at 37°C. TMB substrate (Sigma-Aldrich, USA) was used and the optical density at 450 nm was recorded.

**Animal studies**

A total of 50 mini-pigs (weighing 2.5–4 kg), aged from 4 to 6 weeks, were purchased from the Shanghai Jiao Tong University Agricultural College. They were confirmed to be sero-negative for influenza antibodies by HI assay before infection, and they were fed a commercial pelleted diet. Lidocaine (2 mg/kg) and xylazine (3 mg/kg) were used to anesthetize mini-pigs by intramuscular injection.

The mini-pigs (n=4) were anesthetized and we instilled into the nostrils (1 ml on each side) 10⁴ TCID₅₀ of test viruses at 0 days post-inoculation (dpi). In each of the virus-inoculated groups, 4 mini-pigs were housed separately into 2 large cages (n=2 each) placed inside the room. To study the transmissibility of H9N2 viruses, 4 or 6 naïve mini-pigs were respectively transferred into the cage holding inoculated pigs, and another 2 or 6 mini-pigs were placed in an adjacent cage (50 cm away) in the same room at 1 dpi. The use of separate cages prevented the occurrence of direct-contact, allowing only respiratory droplets to be transmitted. Four mini-pigs were inoculated with 2 ml of PBS and served as negative controls.

Body weight, body temperatures, and clinical signs of infection were monitored, and nasal swabs were collected during the experiments. At 7 dpi, all mini-pigs from each inoculated group were euthanized. One or 2 mini-pigs from direct-contact or respiratory droplet-exposed groups were euthanized at 3, 5, and 7 dpi. Tracheal and lung tissues were collected for the virus detection and pathology study.

**Virus detection in swab samples and tissue samples**

The trachea and lung tissues (no more than 1 g) were frozen in liquid nitrogen, homogenized, and then resuspended in 3 ml of PBS. Supernatant of swab and tissue samples were passed through 0.22-μm filters and cultured in MDCK cells, and viral titers of tissues were then determined by TCID₅₀ analysis in MDCK cells, as above.

In addition, we also conducted real-time PCR to confirm the virus infection in tracheal and lung tissues. Extraction of viral RNAs from tissue supernatants was performed as previously described [8], and TaqMan-based real-time PCR was performed by using 7500 Fast Real-Time PCR equipment (Applied Biosystems, USA). The Subtype H3 of Influenza Virus A Real-Time RT-PCR kit and Avian Influenza Virus H9 Real-Time RT-PCR kit (LifeRiver Bio-Tech, China) were used according to the manufacturers’ instructions.

**Histological analysis and immunohistochemistry**

Lung samples were preserved in 10% PBS-buffered formalin for 24–30 h, embedded in paraffin, and cut into 3-μm sections. Subsequently, the sections were stained with hematoxylin-eosin (H&E) for histopathological examination or used in immunohistochemical (IHC) assay. The paraffin-embedded serial sections were deparaffinized, rehydrated in distilled water, and immersed in 3% hydrogen peroxide for 30 min at room temperature to eliminate endogenous peroxidase activity. A solution of 5% bovine serum albumin was used as blocking agent for 1 h. Sections were then incubated with a poultry-derived serum (Harbin Veterinary Institute, China) specific for H9 AIVs as the primary antibody (1: 10 dilution) for 1 h. The labeled tissue sections were then stained with biotin-labeled goat anti-chicken antibody. Finally, IHC staining was performed with 3, 3′-diaminobenzidine (DAB).

**Results**

**Characterization of H9N2 AIVs**

To characterize the H9N2 viruses, we selected 3 viruses – CK/SH/Y1/02, CK/SH/Y1/07, and CK/SH/23/13 – isolated from healthy chickens in Shanghai between 2002 and 2013.
Nucleotide sequence analysis of H9 HA gene revealed that CK/SH/Y1/02 and CK/SH/Y1/07 were derived from CK/Bei/1/94 and their proteolytic cleavage site was RSSR¯GLF [15], which is a characteristic of low pathogenicity in chickens. Analysis of the deduced amino acid sequence showed that CK/SH/Y1/02 had the amino acid residue glutamine (Q) at position 226, while CK/SH/Y1/07 and CK/SH/23/13 had L226 in the HA gene (Table 1).

The replication kinetics of the 3 viruses was evaluated in MDCK and A549 cells. All 3 H9N2 AIVs replicated to similar titers in MDCK cells (Figure 1A), but CK/SH23/13 grew to significantly lower titers than the other 2 viruses in A549 cells throughout the infection course at 36 h and 48 h (Figure 1B).

To determine the receptor-binding properties of these viruses, all test viruses were determined by evaluating the ability to agglutinate untreated and α2, 3-sialidase-treated CRBCs. The result showed that all viruses could agglutinate well with both the untreated and α2, 3-sialidase-treated CRBCs, indicating that these viruses have affinity for α2, 6-linked SA moieties. Further study of solid-phase binding assay showed that all the 3 H9N2 AIVs were able to bind to both α2, 3-linked SA and α2, 6-linked SA glycopolymers (Figure 2A–2C).

### Table 1. Characterization of test viruses.

| Virus (aberration)                     | Subtype | Host (year) | Phylogenetic lineage of HA gene | Amino acid at position 226 of HA* | Cleavage site of HA (351-341) | HA titer (nlog.) Untreated CRBCs | HA titer (nlog.) Treated CRBCs |
|---------------------------------------|---------|-------------|-------------------------------|----------------------------------|-------------------------------|----------------------------------|------------------------------|
| A/Chicken/Shanghai/23/13 (CK/SH/23/13) | H9N2    | Chicken (2013) | CK/Bei | L | RSSR¯GLF | 5 | 4 |
| A/Chicken/Shanghai/Y1/07 (CK/SH/Y1/07) | H9N2    | Chicken (2007) | CK/Bei | L | RSSR¯GLF | 5 | 4 |
| A/Chicken/Shanghai/Y1/02 (CK/SH/Y1/02) | H9N2    | Chicken (2002) | CK/Bei | Q | RSSR¯GLF | 6 | 5 |
| A/Shanghai/Minhang/124/13 (SH/MH124/13) | H3N2    | Human (2013) | Human seasonal | — | — | — | — |

* The amino acid residues at HA 226 (H3 HA numbering). L, leucine; Q, glutamine; † The chicken red blood cells were treated with α2, 3-sialidase; ‡ Not done.

Figure 1. Replication kinetics of H9N2 AIVs in MDCK (A) and A549 (B) cells. The cells were infected with 0.1 TCID<sub>50</sub> of virus, and viral titers are expressed as log<sub>10</sub> TCID<sub>50</sub>/ml. Each data point on the curve indicates the means ±SD of 2 independent experiments. * P<0.05.
Trachea and lung tissues were collected from the euthanized animals at 7 dpi for virus detection. All test viruses except CK/SH/23/13 were detected in the lungs of inoculated pigs (1.4 to 4.8 log_{10} TCID_{50}/ml), but they were not all detectable in the tracheal tissues of mini-pigs (1.1 to 3.2 log_{10} TCID_{50}/ml). However, the titers of tracheal and lung tissues from SH/MH124/13-infected mini-pigs were significantly higher than those from the other 3 viruses (Table 2).

![Graph](image)

**Figure 2.** Solid-phase receptor-binding assay for H9N2 AIVs. The binding of viruses to 2 different sialylglycopolymers (α2, 6-linked SA; α2, 3-linked SA) were detected. The data are expressed as the means ±SD of triplicate experiments.

**Table 2.** Clinical signs, virus detection, and pathological changes in inoculated mini-pigs.

| Virus         | Clinical signs (day of onset) | Shedding of virus | Virus titer* (positive/total) | Pathological changes in lung tissues (positive/total) |
|---------------|-------------------------------|-------------------|-------------------------------|-----------------------------------------------------|
|               | Max body temperature increase (°C) | Max body weight loss (%) | Sneezing (dpi) | Duration (days) | Trachea (positive/total) | Lung (positive/total) | HE* | IHC* |
| SH/ MH124/13  | 1.6 (5)                       | 18.9 (3)          | 2/4 (2,3)       | 1,1,1,1       | 3,4,4,5                 | 2/4 (3.85±0.95)           | 3/4 (2.57±0.45)           | 4/4 | —   |
| CK/SH/23/13   | 2.3 (5)                       | 15.3 (3)          | 0/4             | 1,1,1,1       | 3,3,3,1                 | 1/4 (3.0)                  | 2/4 (2.25±0.25)           | 2/4 | 1/4 |
| CK/SH/Y1/07   | 0.9 (2)                       | 16.6 (5)          | 0/4             | ND*           | NA*                     | 3/4 (2.67±0.67)           | 4/4 (1.95±0.86)           | 3/4 | 4/4 |
| CK/SH/Y1/02   | 0.8 (2)                       | 20 (2)            | 0/4             | 7             | 1                       | 2/4 (2.1±0.7)             | 3/4 (1.33±0.17)           | 3/4 | 2/4 |
| Control       | 0.3 (3)                       | 3 (1)             | 0/4             | ND            | NA                      | 0/4 (0.00±0.00)           | 0/4 (0.00±0.00)           | 0/4 | 0/4 |

*The virus titers are presented by log_{10} TCID_{50}/ml, means ±SD. No SD is shown where only 1 pig shed virus. The limit of virus detection was 1 log_{10} TCID_{50}/ml; *HE, hematoxylin-eosin staining; IHC – immunohistochemical assay; †Lack of anti-H3 serum, not done; ND – not detectable; NA – not applicable.

Trachea and lung tissues were collected from the euthanized animals at 7 dpi for virus detection. All test viruses except CK/SH/23/13 were detected in the lungs of inoculated pigs (1.4 to 4.8 log_{10} TCID_{50}/ml), but they were not all detectable in the tracheal tissues of mini-pigs (1.1 to 3.2 log_{10} TCID_{50}/ml). However, the titers of tracheal and lung tissues from SH/MH124/13-infected mini-pigs were significantly higher than those from the other 3 viruses (Table 2).
Transmissibility of H9N2 viruses in mini-pigs

To compare the transmission of H9N2 AIVs from infected to uninfected mini-pigs, we introduced 6 or 4 naïve pigs into the cage of each infected animal at 1 dpi. We also placed 6 or 2 naïve animals in adjacent cages, as described above. The infection status of each animal was determined by observation of clinical signs, as well as virus detection in their tracheal and lung tissues. The results showed that none of the direct-contact or respiratory droplet-exposed mini-pigs of the CK/SH/Y1/07, CK/SH/Y1/02, and CK/SH/13/13 groups had any clinical signs of sneezing or shed virus. However, they had a low-grade fever or weight loss during the study, and 2 direct-contact mini-pigs exposed to CK/SH/Y1/02 became ill at 2 dpi and subsequently died at 3 dpi (Table 3). Whether this was due to influenza virus infection could not be determined.

In the CK/SH/Y1/07 group, transmission was observed to both the direct-contact and respiratory droplet-exposed mini-pigs, as determined by the virus detection in their tracheal or lung tissues. One direct-contact mini-pig had virus detected both in the trachea and lung tissues at 5 dpi, while 3 respiratory droplet-exposed mini-pigs had positive lung tissues (3, 5, and 7 dpi, respectively). Two pigs of each group were euthanized at 3, 5, and 7 dpi; one pig was euthanized at 3 dpi and another was euthanized at 7 dpi; two direct-contact pigs had poor health and max weight loss at 2 dpi and subsequently died at 3 dpi. One of the dead pigs had positive virus detection in tracheal and lung tissues.

Pathological changes in the lung tissues of mini-pigs

The lung tissues harvested from mini-pigs inoculated with either H3N2 or H9N2 viruses were stained with H&E to determine pathological damages. The extent and character of lesions were variable among mini-pigs in the transmission groups and in the virus-inoculated groups. The lung tissues from the mini-pigs inoculated with the SH/MH124/13 virus showed moderate to severe lesions, with acute neutrophil-predominant inflammation (Figure 3A). Whether this was due to a bacterial infection was not tested. In CK/SH/Y1/07-, CK/SH/Y1/02-, and CK/SH/23/13-inoculated mini-pigs, slight to mild interstitial pneumonia with a few lymphocytes was observed (Figure 3B–3D). However, pathological studies showed that the lung samples taken at 5 dpi from 1 CK/SH/Y1/07 respiratory droplet-exposed mini-pig and 1 CK/SH/Y1/02 direct-contact animal had slight damages (data not shown). In contrast, the lung tissues from uninfected mini-pigs did not show obvious pathological changes (Figure 3E).

Immunohistochemically, viral antigens were only detected in the lungs of mini-pigs inoculated with CK/SH/Y1/02, CK/SH/Y1/07, and CK/SH/23/13 viruses, which were also positive on virus detection. The lung tissues taken at 7 dpi from all mini-pigs infected with the CK/SH/Y1/07 virus had positive staining in several cells (Figures 3F, 3G), but very few cells in tissues of the lungs from pigs infected with CK/SH/Y1/02 (Figure 3H) or tissues of direct-contact or respiratory droplet-exposed mini-pigs with CK/SH/23/13 (Table 3).
Shi Q. et al.: H9N2 AIVs isolated from chickens in Shanghai

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and there were no significant differences in growth kinetics as expected, all H9N2 viruses replicated well in MDCK cells activity and replication of influenza viruses [16,22]. In our study, A549 cells are an appropriate cell line for examining the infec

As MDCK cells possess both avian- and human-like receptors, they are commonly used to evaluate the replication of influenza viruses. Previous studies have also demonstrated that A549 cells are an appropriate cell line for examining the infectivity and replication of influenza viruses [16,22]. In our study, as expected, all H9N2 viruses replicated well in MDCK cells and there were no significant differences in growth kinetics assay. However, CK/SH/23/13 had lower titers in A549 cells at 36 h and 48 h when compared with CK/SH/Y1/07 and CK/SH/ Y1/02, suggesting that growth kinetics and virulence are not always consistent with the viral titer. This phenomenon may be contributed to by the HA-NA balance or other factors [23].

Furthermore, in vitro results sometimes are not consistent with in vivo viral infectivity, and an ideal mammal model should be introduced to evaluate the infectivity and replication. Pigs are considered as an intermediate model for the adaptation and transmission of AIVs because their tracheal epithelium can express the receptors for both avian influenza viruses and human influenza viruses [24–26]. The Chinese Bama miniature pigs (mini-pigs) used in this study are genetically highly inbred and their small size makes them easier to handle than normal domestic swine [27,28]. They have been developed as an experimental animal for hepatitis E virus infection [29] and human rotavirus infection [30]. In our study, we clearly demonstrated that mini-pigs are susceptible to both H3N2 seasonal influenza virus and H9N2 AIVs. Although none of the H9N2 viruses caused obvious clinical signs or severe lung lesions in inoculated mini-pigs, virus detection in tissues and positive immunohistochemical staining indicated that H9N2 AIVs were able to asymptptomatically infect mini-pigs. Interestingly, the CK/ SH/23/13 and SH/MH124/13 viruses were shed in mini-pigs, whereas CK/SH/Y1/07 and CK/SH/Y1/02 showed no shedding in this host, suggesting that infectivity and replication of H9N2 viruses in pigs are not enhanced by HA 226.

Figure 3. Pathological changes and immunohistochemical assay in the lung tissues of infected mini-pigs. Pigs were euthanized at 7 dpi and the lung tissues were collected for pathological study (A-D 200× magnification, E 100× magnification) or immunohistochemical staining (F-I 400× magnification, J 200× magnification). The lungs from the SH/MH124/13 virus-inoculated pig showed severe lesions (A), whereas those from the pigs inoculated with CK/SH/Y1/07 showed slight to mild interstitial pneumonia (B). CK/SH/Y1/02- (C) and the CK/SH/23/13- (D) inoculated pigs showed mild lesions and the naïve pigs showed no histopathological changes (E). Viral antigen was detected in the lung cells from the CK/SH/Y1/07-inoculated pigs (F, G), CK/SH/Y1/02-inoculated pigs (H), and CK/SH/23/13- inoculated pigs (I). No positive cells were found in the direct-contact or respiratory droplet-exposed animals (J).
In the transmission study, although none of the viruses replicated in tissues, greater weight loss was observed in the CK/SH/Y1/07 group than in the CK/SH/23/13 group and the CK/SH/Y1/02 group. We believe that the season during which experiments were carried out during might have contributed to this phenomenon. The transmission study of CK/SH/Y1/07 virus was conducted in July, while others were carried at different seasons, and their increasing fecal output or loss of appetite might decrease their body weight. However, the CK/SH/23/13 virus was not transmissible among mini-pigs, whereas both the CK/SH/Y1/02 and CK/SH/Y1/07 viruses showed transmissibility in this host. As both CK/SH/23/13 and CK/SH/Y1/07 viruses have L226 in HA, these results indicate that this motif is not necessary for transmission among mammals. Sang et al. found that after 9 serial passages of H9N2 virus through guinea pigs, 3 amino acid substitutions – HA1-Q227P, HA2-D46E, and NP-E434K – were important for transmission in guinea pigs [14]. Li et al. demonstrated that the 627K and 701N mutations of H9 basic polymerase 2 (PB2) enhance virulence and transmissibility in mammals [31]. In addition, other studies have reported that reassortant H9N2 virus bearing genes from 2009 pandemic H1N1 has enhanced transmissibility in ferrets [13]. As these viruses were isolated from healthy chickens and they have been shown to replicate and transmit efficiently among pigs without prior adaptation, indicating that the H9N2 viruses isolated from eastern China are likely to acquire enhanced interspecies transmissibility.

The environment and lifestyle of people in China, especially in southern China, include constant close proximity to birds, poultry, pigs, and other humans [32], which increases the opportunity for generation of new reassortant influenza viruses. Therefore, reasonable protection measures and better working environments are important to reduce this risk.

**Conclusions**

The H9N2 AIVs isolated from healthy chickens displayed both avian-like and human-like receptors, and they could asymptptomatically replicate and transmit among mammals. Therefore, long-term surveillance and investigation of H9N2 AIVs should be conducted.

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**References:**

1. Chen H, Yuan H, Gao R et al: Clinical and epidemiological characteristics of a fatal case of avian influenza A H10N8 virus infection: A descriptive study. Lancet, 2014; 383: 714–21
2. Liu D, Shi W, Shi Y et al: Origin and diversity of novel avian influenza A H7N9 viruses causing human infection: Phylogenetic, structural, and coalescent analyses. Lancet, 2013; 381: 1926–32
3. Butt KM, Smith GJ, Chen H et al: Human infection with an avian H9N2 influenza A virus in Hong Kong In 2003. J Clin Microbiol, 2005; 43: 5760–67
4. Guo Y, Li J, Cheng X: Discovery of men infected by avian influenza A (H9N2) virus. Zonghua Shi Yan He Lin Chuang Bing Du Xue Za Zhi, 1999; 13(2): 105–8 [in Chinese]
5. Peiris M, Yuen KY, Leung CW et al: Human infection with influenza H9N2. Lancet, 1999; 354: 916–17
6. Huang Y, Zhang H, Li X et al: Detection and genetic characteristics of H9N2 avian influenza viruses from live poultry markets in Hunan Province, China. PLoS One, 2015; 10: e0142564
7. Ge FF, Li HB, Yang DQ et al: Epidemiological situation and genetic analysis of H7N9 influenza viruses in Shanghai in 2013. Arch Virol, 2014; 159: 3029–41
8. Wang Q, Lu J, Liu P et al: Serological and virological surveillance of avian influenza A virus H9N2 subtype in humans and poultry in Shanghai, China, between 2008 and 2010. Zoonoses Public Health, 2015; 62: 131–40
9. Pawar SD, Tandale BV, Raut CG et al: Avian influenza H9N2 seroprevalence among poultry workers in Pune, India, 2010. PLoS One, 2012; 7: e36374
10. Huang R, Wang AR, Liu ZH et al: Seroprevalence of avian influenza H9N2 among poultry workers in Shandong Province, China. Eur J Clin Microbiol Infect Dis, 2013; 32: 1347–51
11. Zhou P, Zhu W, Gu H et al: Avian Influenza H9N2 seroprevalence among swine farm residents in China. J Med Virol, 2014; 86: 597–600
12. Pawell EM, Wu J, Araya Y et al: Minimal molecular constraints for respiratory droplet transmission of an avian-human H9N2 influenza A virus. Proc Natl Acad Sci USA, 2009; 106(18): 7565–70
13. Kimble JB, Sorrell E, Shao H et al: Compatibility of H9N2 avian influenza surface genes and 2009 pandemic H1N1 internal genes for transmission in the ferret model. Natl Acad Sci USA, 2011; 108: 12084–88
14. Sang X, Wang A, Ding J et al: Adaptation of H9N2 AI in guinea pigs enables efficient transmission by direct contact and inefficient transmission by respiratory droplets. Sci Rep, 2015; 5: 15928
15. Ge FF, Zhou JP, Liu J et al: Genetic evolution of H9 subtype influenza viruses from live poultry markets in Shanghai, China. J Clin Microbiol, 2009; 47: 3296–3300
16. Zhu Y, Yang Y, Liu W et al: Comparison of biological characteristics of H9N2 avian influenza viruses isolated from different hosts. Arch Virol, 2015; 160: 917–27
17. Reed LJ ML. A simple method of estimating fifty percent endpoints. The American Journal of Hygiene, 1938; 27: 493–97
18. Imai M, Watanabe T, Hatta M et al: Experimental adaptation of an influenza H5 HA confers respiratory droplet transmission to a reassortant H5N1 virus in ferrets. Nature, 2012; 486: 420–28
19. Wan H, Perez DR: Amino acid 226 in the hemagglutinin of H9N2 influenza viruses determines cell tropism and replication in human airway epithelial cells. J Virol, 2007; 81: 5181–91
20. Wan H, Sorrell EM, Song H et al: Replication and transmission of H9N2 influenza viruses in ferrets: Evaluation of pandemic potential. PLoS One, 2008; 3: e2923
21. Imai M, Kawaoka Y: The role of receptor binding specificity in interspecies transmission of influenza viruses.Curr Opin Virol, 2012; 2: 160–67
22. Qiao C, Liu Q, Bawa B et al: Pathogenicity and transmissibility of reassortant H9 influenza viruses with genes from pandemic H1N1 virus. J Gen Virol, 2012; 93: 2337–45
23. Wagner R, Matosovitch M, Klenk HD: Functional balance between hemagglutinin and neuraminidase in influenza virus infections. Rev Med Virol, 2002; 12: 159–66
24. Ito T, Couceiro IN, Kelm S et al: Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. J Virol, 1998; 72: 7367–73
25. Landolt GA, Karasin AI, Phillips L, Olsen CW: Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. J Clin Microbiol, 2003; 41: 1936–41

26. Scholtissek C, Ludwig S, Fitch WM: Analysis of influenza A virus nucleoproteins for the assessment of molecular genetic mechanisms leading to new phylogenetic virus lineages. Arch Virol, 1993; 131: 237–50

27. Liu HB, Lv PR, He RG et al: Cloned Guangxi Bama minipig (Sus scrofa) and its offspring have normal reproductive performance. Cell Reprogram, 2010; 12: 543–50

28. Wu FC, Wei H, Gan SX, Wang AD: Analysis of genetic diversity of Bama miniature pigs and Guizhou miniature pigs by RAPD. Shi Yan Sheng Wu Xue Bao, 2001; 34: 115–19

29. Li JT, Wei J, Guo HX et al: Development of a human rotavirus induced diarrhea model in Chinese mini-pigs. World J Gastroenterol, 2016; 22: 7135–45

30. Tang ZM, Wang SL, Ying D et al: The Bama miniature swine is susceptible to experimental HEV infection. Sci Rep, 2016; 6: 31813

31. Li X, Shi J, Guo J et al: Genetics, receptor binding property, and transmissibility in mammals of naturally isolated H9N2 Avian Influenza viruses. PLoS Pathogens, 2014; 10: e1004508

32. Su S, Zhou P, Fu X et al: Virological and epidemiological evidence of avian influenza virus infections among feral dogs in live poultry markets, China: A threat to human health? Clin Infect Dis, 2014; 58: 1644–46