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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 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 ($TCID_{50}$) 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 $TCID_{50}$ of virus, as previously described [8]. Supernatant was collected at different time points (12, 24, 36, and 48 h) and titrated by the $TCID_{50}$ analysis. The viral titers were compared by using

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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: Neu5Acα2-3Galβ1-4GLcNAcβ1-4GlcNAcβ-PAA- biotin and Neu5Acα2-6Galβ1-4GLcNAcβ1-4GlcNAcβ-PAA-biotin (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 α2, 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 hemaglutination 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 inoculated 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) 10^4 $TCID_{50}$ 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 $TCID_{50}$ 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 previous 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.

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Table 1. Characterization of test viruses.

Virus (aberration)	Subtype	Host (year)	Phylogenetic lineage of HA gene	Amino acid at position 226 of HA[a]	Cleavage site of HA (351-341)	HA titer ($nlog_2$)	
						Untreated CRBCs	Treated CRBCs[b]
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	—[c]	—	—	—

[a] The amino acid residues at HA 226 (H3 HA numbering). L, leucine; Q, glutamine; [b] The chicken red blood cells were treated with α2, 3-sialidase; [c] Not done.

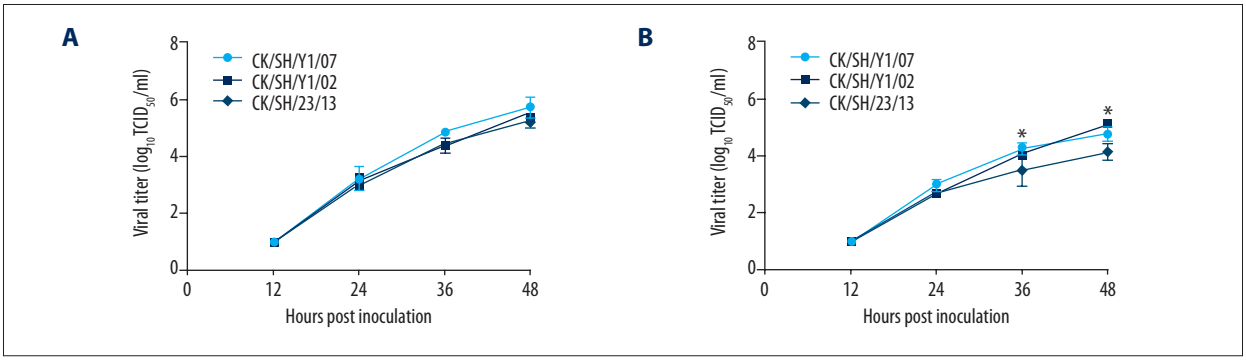

Figure 1. Replication kinetics of H9N2 AIVs in MDCK (**A**) and A549 (**B**) cells. The cells were infected with 0.1 $TCID_{50}$ of virus, and viral titers are expressed as log_{10} $TCID_{50}$/ml. Each data point on the curve indicates the means ±SD of 2 independent experiments. * $P<0.05$.

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).

Infectivity and replication of test viruses in mini-pigs

To evaluate the infectivity and replication of these H9N2 AIVs in mammals, mini-pigs (n=4 per group) were intranasally inoculated with 10^4 $TCID_{50}$ of selected H9N2 AIVs. The human seasonal H3N2 virus, which was isolated from an ILI patient in Shanghai and was confirmed to efficiently replicate in mice in another study, was used as a positive control. All 4 viruses caused a loss of body weight and higher body temperature in mini-pigs, but only SH/MH124/13-inoculated mini-pigs sneezed at 2 dpi or 3 dpi. Shedding of virus was mostly detected in the nasal swabs of mini-pigs inoculated with SH/MH124/13 and CK/SH/23/13, starting as early as 1 dpi and lasting for 1–5 days (Table 2). In contrast, 1 mini-pig inoculated with CK/SH/Y1/02 shed the virus at 7 dpi, but the other pigs inoculated with CK/SH/Y1/02 or CK/SH/Y1/07 had no detectable virus during the study period.

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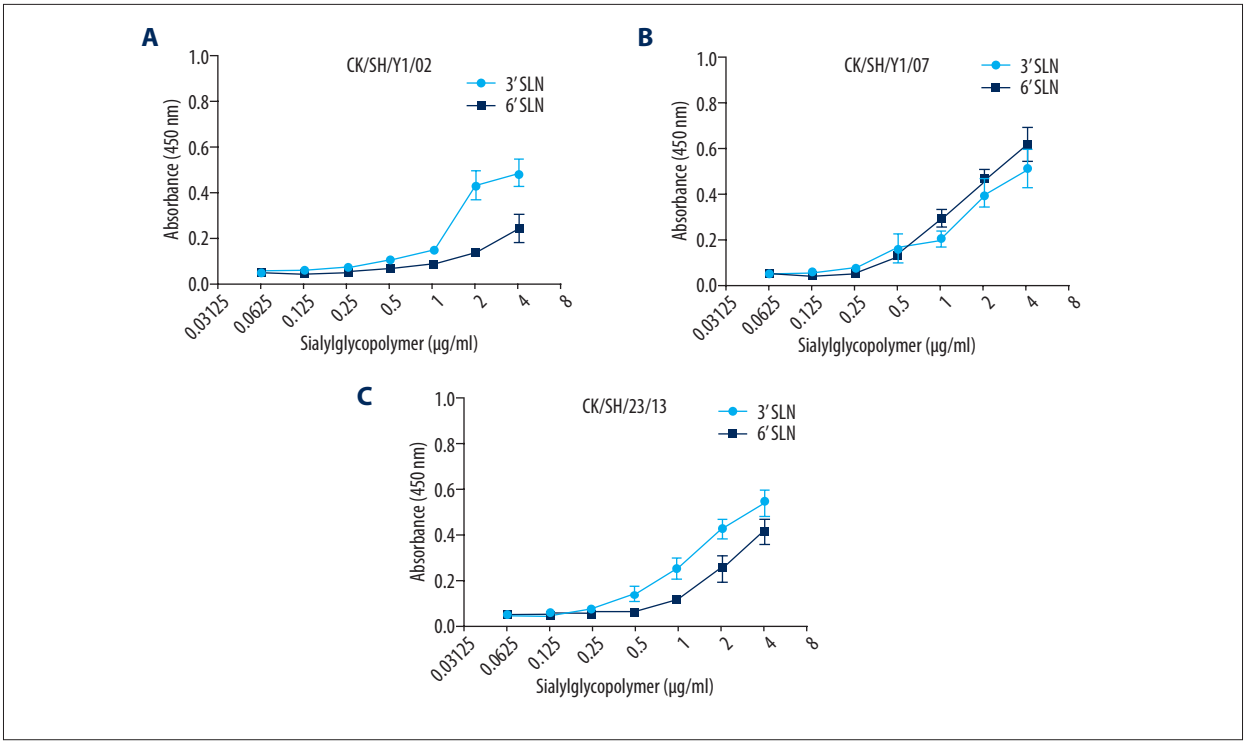

Figure 2. Solid-phase receptor-binding assay for H9N2 AIVs. The binding of viruses to 2 different sialylglycopolymers (α2, 6-linked SA; α2, 3-linked SA) were detected. The data are expressed as the means ±SD of triplicate experiments.

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

Virus	Clinical signs (day of onset)			Shedding of virus		Virus titer[a] (positive/total)		Pathological changes in lung tissues (positive/total)	
	Max body temperature increase (°C)	Max body weight loss (%)	Sneezing	Onset (dpi)	Duration (days)	Trachea	Lung	HE[b]	IHC[b]
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	—[c]
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[d]	NA[d]	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

[a] 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; [b] HE, hematoxylin-eosin staining; IHC – immunohistochemical assay; [c] Lack of anti-H3 serum, not done; [d] 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).

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Table 3. Clinical signs, virus detection, and pathological changes in direct-contact and respiratory droplet-exposed mini-pigs.

Virus	Group	Clinical signs			Shedding of virus		Virus titer		Pathological changes in lung	
		Max body temperature increase (°C)	Max body weight loss (%)	Sneezing	Onset (dpi)	Duration (days)	Trachea	Lung	HE	IHC
CK/SH/23/13	Direct-contact[a]	0.9	10.3	0/6	ND	NA	0/6 (0.00±0.00)	0/6 (0.00±0.00)	0/6	0/6
	Respiratory droplet[a]	0.5	9.2	0/6	ND	NA	0/6 (0.00±0.00)	0/6 (0.00±0.00)	0/6	0/6
CK/SH/Y1/07	Direct-contact[a]	1	14.2	0/6	ND	NA	1/6 (1.5)	1/6 (1.2)	1/6	0/6
	Respiratory droplet[a]	0.5	16.7	0/6	ND	NA	1/6 (1.2)	3/6 (1.13±0.17)	1/6	0/6
CK/SH/Y1/02	Direct-contact[b]	0.4	24.4[c]	0/4	ND	NA	2/4 (2.5±1.3)	1/4 (2.5)	1/4	0/2
	Respiratory droplet[b]	0.7	5.6	0/2	ND	NA	0/2 (0.00±0.00)	0/2 (0.00±0.00)	0/2	0/2

[a] Two pigs of each group were euthanized at 3, 5, and 7 dpi; [b] One pig was euthanized at 3 dpi and another was euthanized at 7 dpi; [c] 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.

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). In contrast, CK/SH/Y1/02 was only detected in 3 of 4 direct-contact mini-pigs. Surprisingly, 1 direct-contact pig, which died at 3 dpi with unknown disease, showed positive virus detection in both tracheal and lung tissues, and the titers were significantly higher than those in other positive mini-pigs, which were near the limit of detection (1 $\log_{10}TCID_{50}$/ml). In contrast, no viruses were detected in the tracheal and lung tissues of direct-contact or respiratory droplet-exposed mini-pigs with CK/SH/23/13 (Table 3).

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

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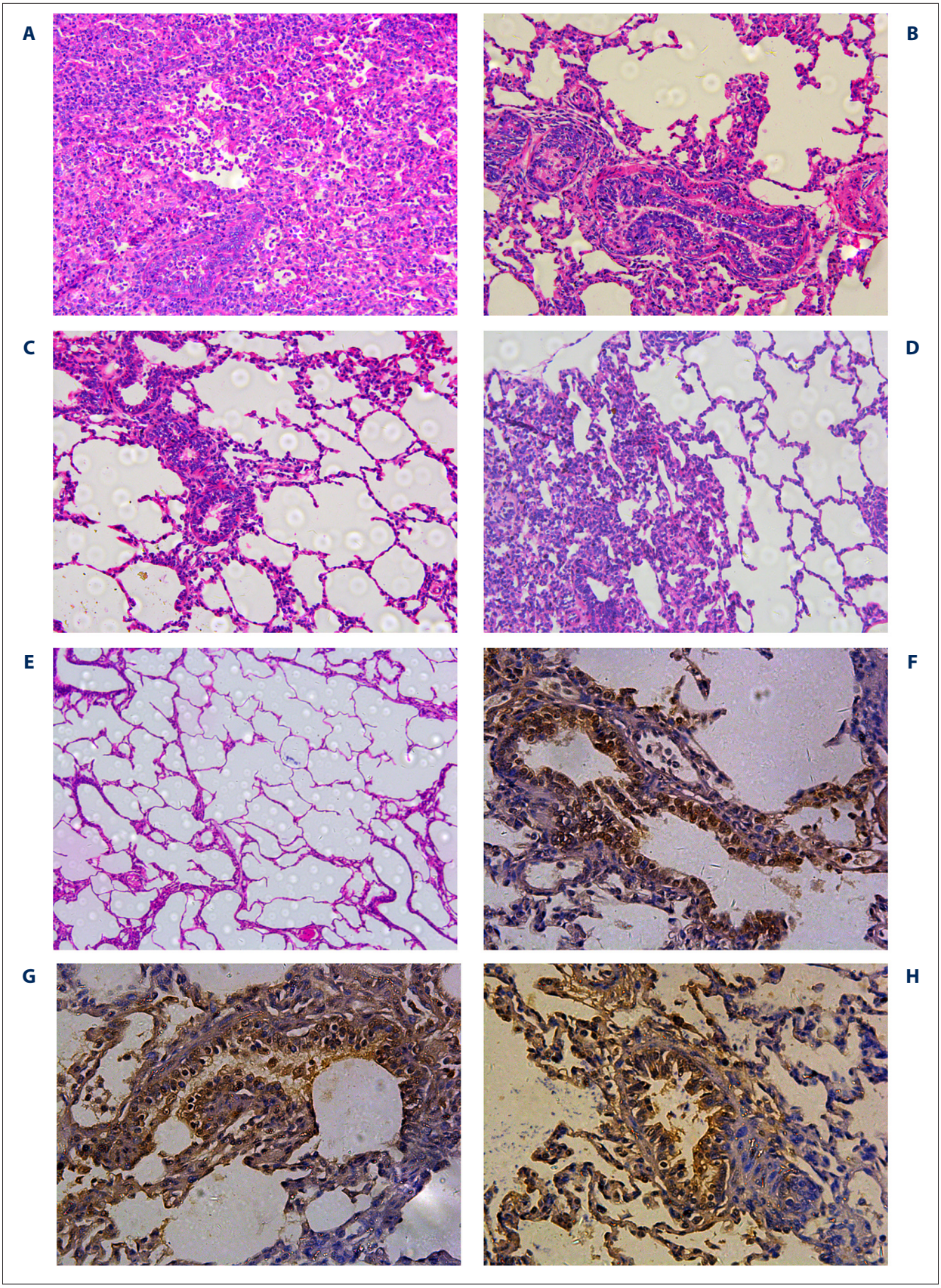

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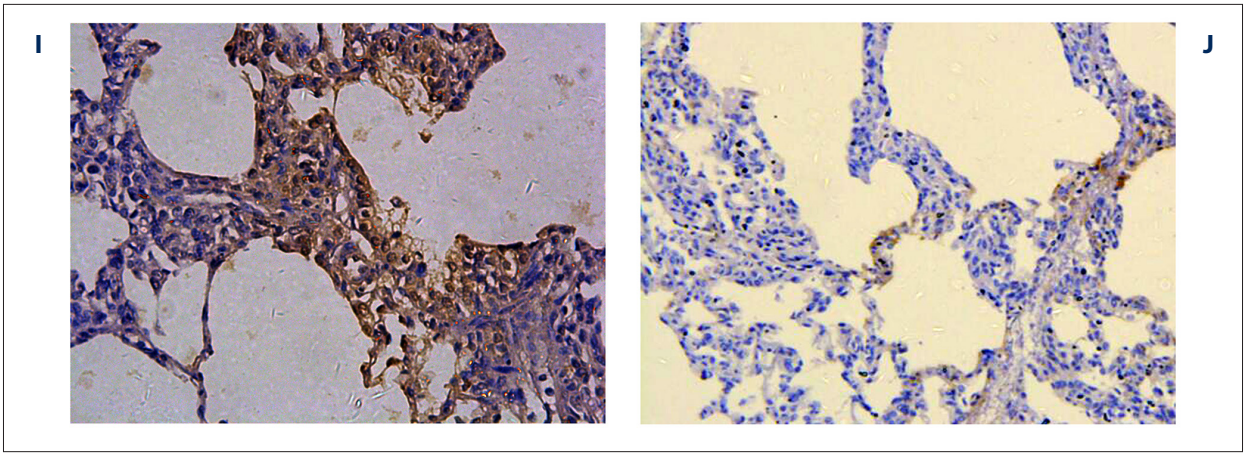

Figure 3. Pathological changes and immunohistochemical assay in the lung tissues of infected mini-pigs. Pigs were euthanized at 7 dpi and the lung tissues were collected for pathological study (**A–D** 200× magnification, **E** 100× magnification) or immunohistochemical staining (**F–I** 400× magnification, **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**).

CK/SH/23/13 (Figure 3I) were positive at autopsy. In contrast, no positive-staining cells were observed in direct-contact or respiratory droplet-exposed animals (Figure 3J).

Discussion

Recently, increasing numbers of H9N2 AIVs isolated in China possess the amino acid L226 in the HA glycoprotein, which is considered with the binding preference for human-like receptor [19,20]. In addition, the interaction between the HA protein and its cellular receptor affects virus infection, replication, and transmission [21]. In the present study, all H9N2 AIVs tested had an RSSR motif at the cleavage site of HA, and deduced amino acid sequences of HA displayed human virus-like cell tropisms [15]. Consistent with this finding, the CK/SH/Y1/02 virus with Q226 in the HA protein showed binding preference for α2 3- and 6-linked SA receptors. Interestingly, the CK/SH/Y1/07 and CK/SH/23/13 isolated from healthy chickens with L226 displayed enhanced affinity for α2, 6-sialoglycan. These results indicate that H9N2 viruses isolated naturally from poultry have gained the ability to bind to the human-type receptor.

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 asymptomatically 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.

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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 asymptomatically replicate and transmit among mammals. Therefore, long-term surveillance and investigation of H9N2 AIVs should be conducted.

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