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Brief Communication

Rapid adaptation of avian H7N9 virus in pigs

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ABSTRACT

How the H7N9 avian influenza virus gained the distinct ability to infect humans is unclear. Pigs are an important host in influenza virus ecology because they are susceptible to infection with both avian and human influenza viruses and are often involved in interspecies transmission. Here, we passaged one avian isolate and one human isolate in pigs to examine the mammalian host adaptation of the H7N9 virus. The avian virus replicated to a high titer after one passage, whereas the human isolate replicated poorly after three passages in pig lungs. Sequence analysis found nine substitutions in the HA, NA, M and NS segments of the avian isolate, which enhanced the binding affinity for human-type receptors. These results indicate that avian H7N9 influenza viruses can be easily adapted to pigs and that pigs may act as an important intermediate host for the reassortment and transmission of such novel viruses.

Introduction

In March 2013, a novel avian-origin H7N9 subtype influenza virus was recognized as the causative agent of an influenza-like illness in humans in eastern China (Gao et al., 2013). Since then, it has caused more than 130 human infections in China, resulting in 45 deaths (World Health Organization, 2013). More recently, on October 15, 2013, two new confirmed cases appeared in Zhejiang Province, China. Most patients presented with respiratory infections that progressed to severe pneumonia and dyspnea (Gao et al., 2013). The identification of the potential virus source and possible routes of virus reassortment is currently ongoing. The majority of patients were reported to have a history of contact with live poultry (Chen et al., 2013). However, whether H7N9 can be transmitted from other sources, such as pigs, is unclear.

Pigs are susceptible to infection with avian and human influenza viruses and have been suggested to serve as intermediate hosts for the generation of pandemic human strains of influenza virus (Kida et al., 1988; Webster et al., 1971, 1973; Yasuda et al., 1991). The potential to generate novel influenza viruses has resulted in pigs being labeled as "mixing vessels." The mixing vessel theory provides one mechanism by which unique viruses can be transmitted from an avian reservoir to humans.

To date, no H7N9 infections from the recent outbreak have been reported in pigs. Surveillance of pig farms in the affected regions has yielded more than 4000 animal and environmental samples, all of which have been negative (Centers for Disease Control and Prevention (CDC), 2013). However, a recent study by Zhu et al. (2013) demonstrated that a human H7N9 isolate was able to infect pigs experimentally. Watanabe et al. (2013) also reported that a mild infection of H7N9 virus with no clinical symptoms was observed in pigs. The potential of these viruses to infect pigs and generate additional mammalian adaptations is a significant public health concern. In this study, we passaged one avian isolate and one human isolate in pigs to further our understanding of mammalian host adaptation of the H7N9 virus. Amino acid sequence alignment shows that there are seven variations in HA (including Q226L), three variations in NA, and one each in M and NS between these two original viruses. We believe that our results will facilitate a better understanding of the role of pigs in the outbreak and transmission of novel H7N9 viruses.

Results

Consecutive passages of H7N9 viruses in pigs

Successive virus passages were initiated in six naïve 4-week-old, outbred, specific pathogen-free Bama minipigs (three pigs for

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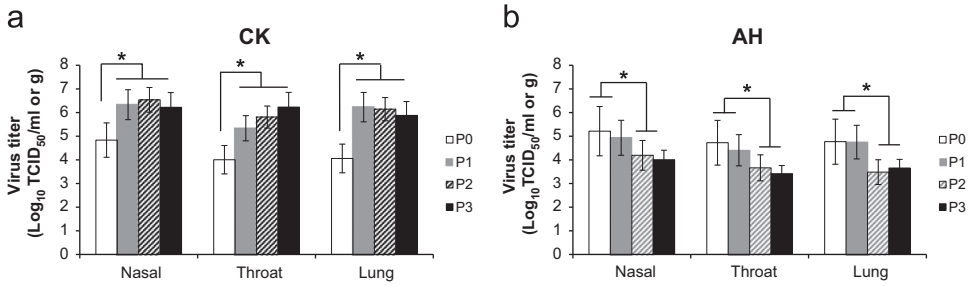

Fig. 1. Viral titers from nasal and throat swabs and from lung tissue. Successive viral passages were initiated in six naïve pigs (three pigs for each virus) by intranasal inoculation with 2 ml of allantoic fluid containing 10^6 $TCID_{50}$ of A/Anhui/1/2013 (AH) or A/chicken/Zhejiang/DTID-ZJU01/2013 (CK). Nasal and throat swabs were collected daily. The pigs were euthanized at 4 d.p.i., and the lung tissues were collected. Virus replication in the nasal and throat swabs taken at 4 d.p.i. was compared. The geometric mean titers are displayed, and the error bars indicate standard deviation. *$P < 0.05$.

each virus) by intranasal inoculation with 10^6 $TCID_{50}$ of A/chicken/Zhejiang/DTID-ZJU01/2013 (CK) or A/Anhui/1/2013 (AH). The pigs were euthanized 4 days post-inoculation (d.p.i.), and the nasal swabs that contained viruses with mutations were used for the next passage inoculation. The results showed that none of the inoculated pigs developed any clinical signs of infection during the 4-day observation period or had virus-induced lesions upon necropsy. Virus shedding from the upper respiratory tract started at 1 d.p.i. for all inoculated pigs, reaching peak titers at 2 or 3 d.p.i. The virus titers in the nasal and throat swabs were generally comparable for each individual pig inoculated with the same virus ($P > 0.05$, Supplementary Fig. S1). However, we found that the nasal and throat swabs and the lung tissues collected from pigs inoculated with the CK-P1-to-P3 virus had higher titers than those from animals inoculated with the CK-P0 virus ($P < 0.05$) (Fig. 1(a)). In contrast, for the human AH virus isolate, the titers from the nasal and throat swabs and the lung tissues of pigs inoculated with the AH-P2 or P3 virus were generally lower than those from animals inoculated with the AH-P0 or P1 virus ($P < 0.05$) (Fig. 1 (b)). Furthermore, by using one-way ANOVA and DUNCAN and LSD methods analysis, we convinced that throughout the all 4 day sampling periods of nasal and throat swabs, there were still significant differences between CK-P1-to-P3 virus with CK-P0 virus, and AH-P2 or P3 virus with AH-P0 or P1 virus ($P < 0.05$). Additionally, histopathological analyses revealed that on 4 d.p.i., lung tissues from the pigs inoculated with the CK-P0 or AH-P2-to-P3 virus had focal mild interstitial pneumonia, with alveolar septa that were slightly broadened (Fig. 2(b, n, and o)). For pigs inoculated with the CK-P1-to-P3 or AH-P0-to-P1 virus, the lung tissue lesions were larger, and interstitial inflammatory hyperemia and exudative pathological changes could be observed in parts of the lungs (Fig. 2(c–e, l, and m)). Immunohistochemistry was also performed to assess the presence of viral antigens in lung tissues. In pigs inoculated with the CK-P1-to-P3 or AH-P0-to-P1 virus, greater numbers of infected cells were detected in the lung tissues (Fig. 2(h–j, q, and r)) compared with those from pigs inoculated with the CK-P0 or AH-P2-to-P3 virus (Fig. 2(g, s, and t)).

All the above data indicated that the pathogenicity of the CK virus increased while the AH virus pathogenicity decreased after adaptation over four passages in the pig host.

Receptor binding specificity of the viruses passaged in pigs

We next tested the receptor specificity of the CK-P0, CK-P3, AH-P0 and AH-P3 viruses using a solid-phase binding assay. The results showed that all four viruses could bind to all glycans. However, the binding affinity of the CK-P3 virus to α2,6 glycans was significantly increased compared with that of CK-P0 (Fig. 3 (a and b)). Notably, compared with AH-P0, which showed a mixed α2,3 and α2,6 receptor preference, AH-P3 largely bound to α2,3 glycans (Fig. 3(c and d)). We therefore speculated that after three passages in the pig host, the avian-isolated CK virus and human-isolated AH virus exhibited increased human-type receptor binding activity and avian-type receptor binding properties, respectively.

Amino acid substitutions in the viral genomes during consecutive passages in pigs

The nasal swabs collected from each inoculated pig at 4 d.p.i. were grown in MDCK cells, and five plaque-picked viral clones were obtained and sequenced individually to obtain the H7N9 viral genome sequence. Compared with CK-P0, there were nine mutations in CK-P1, which were substituted by the amino acids present in the human-origin virus; no additional mutations appeared until CK-P3 (Table 1). The nine mutations were identified in eight plaque clones from three pig nasal swabs. Six mutations (Gly63Cys, Pro65Leu, Arg66Leu, and Met67Ile in HA [H3 numbering] and Ala436Thr and Gly437Ser in NA) existed simultaneously in five plaque clones from one pig nasal swab. The mutations Gly33Val in HA and Val423Leu in NA existed in two plaque clones from one pig nasal swab, and the mutation Phe3Ser in NS existed in one plaque clone from a separate pig nasal swab. All of these changes were in amino acids identical to those in human-isolated H7N9 strains. Meanwhile, compared with AH-P0, one mutation (Leu267Pro in M) appeared in the AH-P1 virus and existed in all five plaque clones from one pig nasal swab. In the AH-P2 viruses, two more mutations appeared: Arg56Lys in HA, which existed in four plaque clones from one pig nasal swab, and Leu226Gln in HA, which existed in all five plaque clones from another pig nasal swab (Tables 1 and 2). All three changes were in amino acids identical to those in avian-isolated H7N9 strains. The sequences of all above-mentioned mutated viral genes were deposited in GenBank under accession numbers KF768178-KF768182.

Discussion

Avian H7N9 influenza virus may be introduced into humans by direct transmission or through an intermediate host susceptible to both human and avian viruses. Pigs are susceptible to infection with both avian and human influenza viruses and are thought to play an important role in human influenza ecology. Pigs are the only domesticated mammalian species that is reared in abundance and is susceptible to and allows the productive replication of avian and human influenza viruses. This susceptibility is due to the presence of both α2,3 and α2,6 galactose–sialic acid linkages in cells lining the pig trachea, which can result in the modification of the receptor-binding specificities of avian influenza viruses from α2,3 linkages to α2,6 linkages (Ito et al., 1998). This latter linkage is the native linkage in humans, thereby providing a potential link from birds to humans. Although Chan et al. (2013) found that there

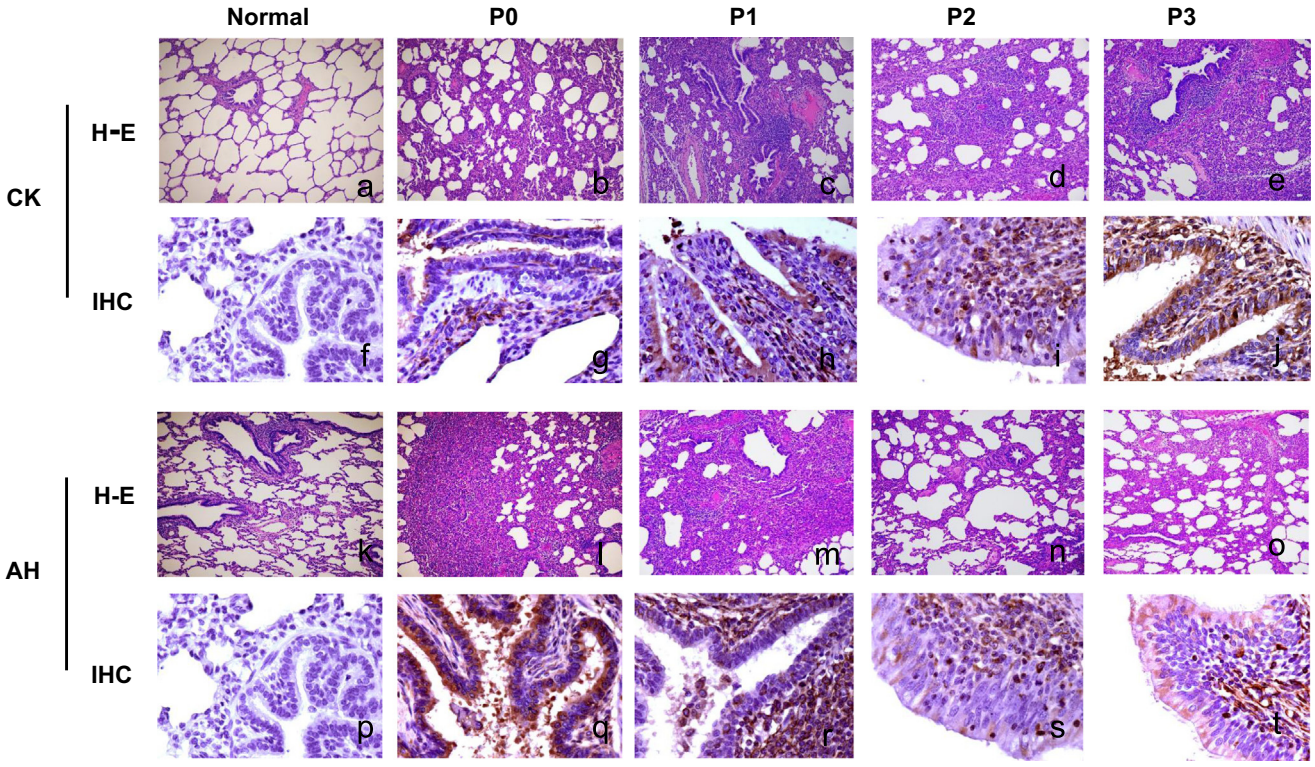

Fig. 2. Histopathological and immunohistochemical (IHC) analyses of the lung tissues of pigs inoculated with wild-type or passaged H7N9 influenza viruses. (b–e): H–E staining results of the lung tissues of pigs inoculated with the P0–P3 CK viruses; ×100. (i–o): H–E staining results of the lung tissues of pigs inoculated with the P0–P3 AH viruses; ×100. (g–j); immunohistochemical results of the lung tissues of pigs inoculated with the P0–P3 AH viruses; ×400. (q–t): immunohistochemical results of the lung tissues of pigs inoculated with the P0–P3 AH viruses; ×400.

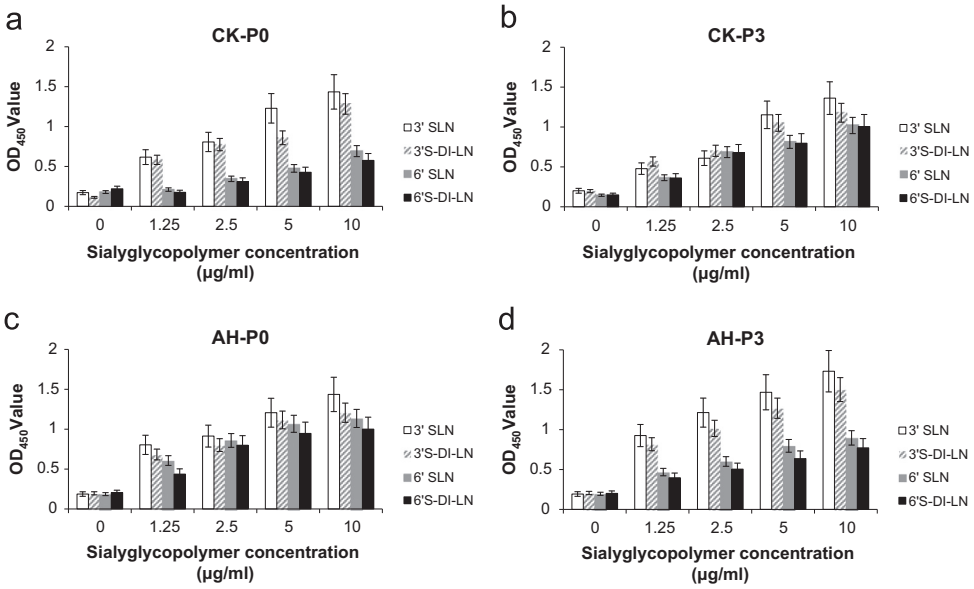

Fig. 3. Direct binding assay of wild-type and passaged H7N9 influenza viruses with synthetic sialylglycopolymers. (a) Affinity for synthetic 3′SLN. (b) Affinity for synthetic 3′S-DI-LN. (c) Affinity for synthetic 6′SLN. (d) Affinity for synthetic 6′S-DI-LN.

was not as much expression of α2,3 glycans on the surface of the trachea of pigs. Infection of α2,3 binding avian viruses was restricted to the lower respiratory tract bronchioles, which may diminish the ability of the swine to act as an intermediary in the transmission of avian viruses to humans. However, Jones et al. (2013) assessed the replication ability of three human H7N9 viruses (A/Anhui/1/2013, A/Shanghai/1/2013, A/Shanghai/2/2013) in swine tissue explants, and viruses tested replicated efficiently in tracheas and bronchi of the explants, with limited replication in alveolar cells.

This study demonstrated that H7N9 viruses isolated from an avian species and a human were capable of infecting pigs. However, the avian virus was able to replicate to a high titer after one passage, whereas the human isolate replicated poorly after three passages in pig lungs. In the avian isolate, sequence analysis found nine substitutions that led to enhanced binding affinity for

Table 1
Amino acid mutations in passaged CK and AH viral genomes.

Virus	Passage number	Amino acid position											
		HA							NA			M	NS
		33	56	63	65	66	67	226	423	436	437	267	3
CK	P0	Gly	Lys	Gly	Pro	Arg	Met	Gln	Val	Ala	Gly	Pro	Phe
	P1	**Val**	Lys	**Cys**	**Leu**	**Leu**	**Ile**	Gln	**Leu**	**Thr**	**Ser**	Pro	**Ser**
	P2	**Val**	Lys	**Cys**	**Leu**	**Leu**	**Ile**	Gln	**Leu**	**Thr**	**Ser**	Pro	**Ser**
	P3	**Val**	Lys	**Cys**	**Leu**	**Leu**	**Ile**	Gln	**Leu**	**Thr**	**Ser**	Pro	**Ser**
AH	P0	Val	Arg	Cys	Leu	Leu	Ile	Leu	Leu	Thr	Ser	Leu	Ser
	P1	Val	Arg	Cys	Leu	Leu	Ile	Leu	Leu	Thr	Ser	**Pro**	Ser
	P2	Val	**Lys**	Cys	Leu	Leu	Ile	**Gln**	Leu	Thr	Ser	**Pro**	Ser
	P3	Val	**Lys**	Cys	Leu	Leu	Ile	**Gln**	Leu	Thr	Ser	**Pro**	Ser

Note: Abbreviation: CK, A/chicken/Zhejiang/DTID-ZJU01/2013 (H7N9) virus; AH, A/Anhui/1/2013 (H7N9) virus; HA, hemagglutinin protein; NA, neuraminidase protein; M, matrix protein; NS, non-structural protein.
The mutations during the adaption process are underlined in bold.
GenBank accession numbers: CK-P0-HA: KC899669.1; CK-P0-NA: KC899671.1; CK-P0-NS: KC899673.1; CK-P1-to-P3-HA: KF768178; CK-P1-to-P3-NA: KF768179; CK-P1-to-P3-NS: KF768180; AH-P2-to-P3-HA: KF768181; AH-P2-to-P3-M: KF768182.
GISAID accession number: AH-P0-HA: EPI439507; AH-P0-M: EPI439506.

Table 2
Distribution of mutations in each passage and each individual animal.

Viruses	Passage	Pig no.	Total plaque clones from each pig	Plaque clone with mutations	Mutation amounts	Mutations
CK	P1	1	5	5 (100%)	6	Gly63Cys, Pro65Leu, Arg66Leu, Met67Ile in HA; Ala436Thr, Gly437Ser in NA
		2	5	2 (40%)	2	Gly33Val in HA; Val423Leu in NA
		3	5	1 (20%)	1	Phe3Ser in NS
AH	P1	1	5	5 (100%)	1	Leu267Pro in M
		2	5	0 (0%)	0	/
		3	5	0 (0%)	0	/
	P2	1	5	4 (80%)	1	Arg56Lys in HA
		2	5	5 (100%)	1	Leu226Gln in HA
		3	5	0 (0%)	0	/

Note: Abbreviation: CK, A/chicken/Zhejiang/DTID-ZJU01/2013 (H7N9) virus; AH, A/Anhui/1/2013 (H7N9) virus; HA, hemagglutinin protein; NA, neuraminidase protein; M, matrix protein; NS, non-structural protein.

human-type receptors. Except for Met67Ile in HA, which has not been reported previously, the observed mutations were found to exist in a number of H7N9 viruses in GenBank, with most identified in human isolates. A series of recombinant viruses should be constructed by reverse genetics to determine whether any of these substitutions are critical to receptor binding specificity.

It is unknown why the human H7N9 isolated virus, after successive passages in pigs, a mammalian host, developed enhanced affinity for avian-type receptors, but the H7N9 virus appears to not be well adapted to its new mammalian host species. We observed three substitutions in the human H7N9 isolate during three adaption passages in pigs. All three mutations were found to exist in a number of H7N9 viruses in GenBank, in isolates either from human patients or avian species. Notably, the Leu226Gln substitution in the HA protein was identified after two passages of the human-origin virus in pigs and was accompanied by a decrease in virus titer in the pig lungs and enhanced binding affinity for avian-type receptors. The Leu226Gln substitution has long been recognized to modulate receptor binding specificity from α2,6 to α2,3 galactose-sialic acid receptors (Herfst et al., 2012; Imai et al., 2012). Previous serosurveillance studies have indicated that the prevailing human viruses of both H1N1 and H3N2 subtypes are transmitted to pigs, but fail to persist (Brown, 2000). It is not clear why these viruses fail to persist in pigs, but since immune selection is not considered important in pigs, strains with different antigenic characteristics may be disadvantaged compared to the 'highly-adapted' established viruses which continually circulate within a large susceptible population (Brown, 2000).

We also observed that there were genome polymorphisms of plaque-picked virus clones from each passage in each individual animal. Some mutations existed in all five plaque clones of one inoculated pig while others appeared only in one virus clone. This may reflex the virus evolution preference when adapted to a new mammalian host.

As avian H7N9 influenza viruses can be easily adapted to pigs, the infection of pigs with avian-isolated H7N9 strains should be recognized to result in viruses gaining adaptations necessary to either maintain infection in pigs, thereby providing a reservoir for human infection, or allow transmission to humans, leading to the establishment of this virus in the human population (Londt et al., 2012). Therefore, H7N9 influenza virus genes could potentially be introduced to humans via pigs. To date, there is little evidence of the human-to-human transmission of H7N9. However, the potential circulation of this virus in pigs may result in critical changes that alter its pathogenicity or transmission potential.

Materials and methods

Viruses

The A/Anhui/1/2013 (H7N9) virus (AH-P0) was isolated from the third case of laboratory-confirmed human A (H7N9) virus infection. The patient was a 35-year-old woman who had visited a chicken market 1 week before the onset of symptoms. A throat swab was collected from the patient, propagated in the allantoic

sacs and amniotic cavities of 9 to 11 day old embryonated chicken eggs and then passaged once in Madin–Darby canine kidney (MDCK) cells. Substitution Q226L (H3 numbering) in the 210-loop of the HA gene was found in this virus. This site has been previously shown to change the receptor binding of avian-origin viruses to human-type receptors and may increase the airborne transmission ability of the virus. The virus also encoded PB2-627K, an amino acid essential for the efficient replication of avian influenza viruses in mammals. The accession numbers of genome of A/Anhui/1/2013 (H7N9) virus (AH-P0) virus in GISAID database are EPI439503 to EPI439503.

A/chicken/Zhejiang/DTID-ZJU01/2013 (H7N9) virus (CK-P0) was isolated from fecal samples taken from a chicken in a poultry market in Zhejiang Province in April 2013. This virus was also propagated in the allantoic sacs and amniotic cavities of 9 to 11 day old embryonated chicken eggs and then passaged once in MDCK cells. No molecular markers for human-type receptor binding properties were identified in the viral genome. The accession numbers of genome of A/chicken/Zhejiang/DTID-ZJU01/2013 (H7N9) virus (CK-P0) virus in Genbank are KC899666 to KC899673.

Cells

MDCK cells were obtained from the ATCC and were tested for mycoplasma contamination. The cells were maintained in Eagle's minimal essential medium (MEM, Invitrogen, USA) supplemented with 10% fetal bovine serum (FBS), 100 IU/ml penicillin and 100 μg/ml of streptomycin and were cultured at 37 °C with 5% CO_2.

Successive passages of H7N9 viruses in pigs

Four-week-old outbred specific pathogen-free Bama minipigs were kept in self-contained isolator units in an ABSL-3 facility. Prior to virus inoculation, the serum samples were evaluated for antibodies against currently circulating human and swine influenza viruses using hemagglutination-inhibition (HI) tests. Successive viral passages were initiated in six naïve pigs (three pigs per virus) by intranasal inoculation with 1 ml of allantoic fluid containing 10^6 $TCID_{50}$ of AH-P0 or ZJ-P0. Nasal and throat swabs were collected daily and transferred to 1 ml of phosphate-buffered saline (PBS). Virus titers were determined by end-point titration in MDCK cells. The pigs were euthanized with a mixture of anesthetics (25 mg/kg ketamine, 2 mg/kg xylazine and 0.05 mg/kg atropine) 4 d.p.i., and the lung tissues were collected for pathological and virological studies. The nasal swabs collected from each inoculated pig at 4 d.p.i. were grown in MDCK cells, and five plaque-picked viral clones were obtained and sequenced individually to obtain the H7N9 viral genome sequence, and specimens with mutations were used for the next passage. The inoculation titer used was 10^6 $TCID_{50}$ for both viruses. If mutations existed in more than one sample, each nasal swab was mixed equally and intranasally re-inoculated into the three naïve pigs. Virus passaging was repeated three times. The experimental protocol was evaluated and approved by the Institutional Animal Use and Care Committee of the Institute of Laboratory Animal Science, Peking Union Medical College (ILAS-PC-2013-009). All pigs were allocated randomly to experimental groups and processed. All experiments were performed under ABSL-3 conditions.

Virus titrations

Virus titrations were performed by end-point titration in MDCK cells. The MDCK cells were inoculated with tenfold serial dilutions of homogenized tissue and nasal and throat swabs. One hour after inoculation, the cells were washed once with PBS and grown in 200 μl of infection media, which consisted of MEM supplemented with 100 U/ml penicillin, 100 μg/ml streptomycin and 1 μg/ml TPCK-trypsin. At 3 d.p.i., the supernatants of infected cell cultures were tested for agglutinating activity using turkey erythrocytes as an indicator of cellular infection. Infectious titers were calculated from five replicates by the Reed and Muench method (Reed and Muench, 1938).

HI assays

Standard HI assays were performed on pig sera using 0.5% turkey erythrocytes in accordance with the WHO guidelines for established procedures (World Health Organization—Global influenza surveillance network, 2011). Sera were collected from pigs and tested for H7N9 virus-specific antibodies.

Histopathology and immunohistochemistry

Animal necropsies were performed according to a standard protocol. Samples for histological examination were stored in 10% neutral-buffered formalin (lungs after inflation with formalin), embedded in paraffin and sectioned at 4 μm. The sections were stained with hematoxylin and eosin (H–E) for examination by light microscopy or using an immunohistochemical method with a monoclonal antibody against the nucleoprotein of influenza A virus (1:200 dilution, IRR Ltd, Catalog no.: FR-51) at 4 °C overnight. The sections were washed three times with PBS and then incubated with HRP-conjugated goat anti-mouse secondary antibody (1:5000 dilution, Sigma, Catalog no.: PV-9002). The sections were developed with 3-3′ diaminobenzidine (DAB) and examined with a light microscope.

Genome sequencing

For sequencing, a total of 198 primer sets were used to amplify the full genome with the Qiagen OneStep RT-PCR Kit. The PCR products were purified from an agarose gel using the QIAquick Gel Extraction Kit (Qiagen, Germany). We performed sequencing using an ABI 3730xl automatic DNA analyzer (Life Technologies, USA) and the ABI BigDye Terminator v3.1 cycle sequencing kit (Life Technologies, USA) according to the manufacturer's recommendations.

Solid-phase binding assay

Synthetic Neu5Acα2-3Galβ1-4GlcNAcβ-Sp (3′SLN), Neu5Acα-2-3[Galβ1-4GlcNAcβ1-3]$_2$β-Sp (3′S-Di-LN), Neu5Acα2-6Galβ1-4GlcNAcβ-Sp (6′SLN) and Neu5Acα2-6[Galβ1-4GlcNAcβ1-3]$_2$β-Sp (6′S-Di-LN) were obtained through the resource request program of the Consortium of Functional Glycomics. Flat-bottom, polystyrene, 96-well plates were incubated with sialylglycopolymers in PBS at 4 °C overnight. The glycopolymer solution was then removed, and the plates were blocked with 0.15 ml of PBS containing 4% BSA at room temperature for 1 h. After four successive washes with ice-cold PBS, the plates were incubated with a solution containing the virus (32 HAU) at 4 °C overnight. The plates were then washed as described above and incubated for 2 h at 4 °C with a monoclonal antibody against the influenza A virus nucleoprotein (1:1000 dilution, IRR Ltd., Catalog no. FR-51). The plates were washed as before and incubated with horseradish peroxidase (HRP)-conjugated goat-anti-mouse IgG antisera for 2 h at 4 °C. After another wash step, the plates were incubated with tetramethylbenzidine substrate solution, and the absorbance at 450 nm was measured.

Statistical analysis

Statistical analysis of the differences in the virus titers were performed by SPSS 11.5 software, and the DUNCAN and LSD

methods were used for detection in one-way ANOVA means. A probability value < 0.05 was considered statistically significant.

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Appendix A. Supplementary information

Supplementary data associated with this article can be found in the online version at http://dx.doi.org/10.1016/j.virol.2014.01.016.

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