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Characterization of Recombinant H9N2 Influenza Viruses Isolated from Wild Ducks in China

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Abstract

Wild birds are considered to be the natural reservoirs for avian influenza A viruses (AIV). During active influenza surveillance in Poyang Lake of southeast China, we isolated and characterized eleven H9N2 viruses from two species of wild ducks. Phylogenetic analysis showed that the eleven isolates were almost identical with 99.3% to 100% nucleotide homology in their entire genome, and they all closely related in whole eight genes (95.6-99.4% homology) to human H9N2 isolates (HK/33982/2009) and clustered in the same sublineage. The isolates belonged to triple reassortant H9N2 genotype viruses containing Ck/Bei-like NA genes, Y439-like PA genes and six other G1-like genes. We also found that the subtype of virus replicated efficiently in the lungs and tracheas of BALB/c mice and caused mortality in 20-40% of infected groups after 3-6 days, which indicates that the subtype of virus is capable of establishing lethal mammalian infections. However, whether or not the virus has features transmittable from wild ducks to humans is not known. This study showed that the subtype of virus was detected for the first time in wild birds, and also suggested that wild birds may carry the virus for a long time and spread it over long distances along migratory routes, so more attention should be paid to the continued surveillance of wild birds.

Keywords

H9N2; human infection; triple reassortant; wild ducks

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Introduction

The H9N2 avian influenza viruses have been endemic in poultry in the Eurasian continent since the mid-1990s (Guo et al., 2000). Some avian-origin H9N2 viruses can be well replicated in mice and ferrets without prior adaptation (Choi et al., 2004; Li et al., 2005; Bi et al., 2010), and occasionally are able to expand their host range to mammals, including pigs and humans (Lin et al., 2000; Cong et al., 2007). Several human cases of H9N2 infection have been reported since 1999 (Peiris et al., 1999; Butt et al., 2005). In addition, H9N2 viruses show a high genetic compatibility with other subtypes, providing their “internal” genes to other viruses. Genetic analysis shows that the H9N2 viruses might be the donors of the “internal” genes of the lethal H5N1 viruses that prevailed in Hong Kong in 1997 (Guan et al., 1999), and the human H7N9 viruses that emerged in China in March 2013 (Gao, 2013). It is also noteworthy that once H9N2 influenza virus acquired a polybasic HA cleavage site through reassortment with other subtypes of influenza viruses, the potential for pandemic spread was probably enhanced (Gohrbandt et al., 2011; Veits et al., 2012). These findings show that the H9N2 viruses pose potential hazards to human populations. Thus, longitudinal surveillance on the prevalence of H9N2 might be key factors in limiting or preventing the source of the transmission of AIV in humans.

In China, H9N2 subtype viruses have become the principal reported strains in poultry following their first report in 1992 (Liu et al., 2003). Until now, at least 74 different genotypes have been identified (Dong et al., 2011). New lineages and genotypes are being discovered throughout China. Three main lineages of H9N2 influenza viruses, Chicken/Beijing/1/94 (Ck/Bei-like), Quail/Hong Kong/G1/97 (G1-like) and Duck/Hong Kong/Y439/97 (Y439-like or Korean-like), were responsible for influenza outbreak events in China since the mid-1990s (Li et al., 2005; Xu et al., 2007a; Xu et al., 2007b; Sun et al., 2010). The G1-like viruses are primarily detected in quails and humans, and were rarely detected in domestic poultry and wild birds (Guan et al., 1999; Xu et al., 2007a). While Ck/Bei-like H9N2 viruses were mainly prevalent in chickens, and the lineage was gradually replaced by the F98-like or G9-like viruses (represented by A/Ck/Shanghai/F/98 or CK/HK/G9/97, respectively) in eastern and southeastern China (Chen et al., 2003a; Zhang et al., 2009). Y439-like (Korean-like) H9N2 viruses were isolated from domestic duck in Hong Kong and chicken in South Korea (Choi et al., 2005).

For the surveillance of H9N2 viruses in China, some researchers focused mainly on domestic poultry, such as chickens, ducks, geese and other minor poultry species (Li et al., 2003; Xu et al., 2007a; Bi et al., 2010). Although the fact that H9N2 viruses isolated from humans are likely of avian origin, they are unable to persist for a long period in avian species due to the birds' relatively short life span and public health control efforts (Suarez, 2000). Wild aquatic birds are considered the natural hosts for avian influenza A virus and they are the reservoirs of all known subtypes H1–H16 and N1–N9 (Webster et al., 1992). Avian influenza infections in wild aquatic birds are usually asymptomatic and control of AIV outbreaks in wild birds is both difficult and costly (Webster et al., 1992). Wild birds especially wild duck are very common in southern China and their populations have increased markedly. Some wild ducks (such as mallard) have been domesticated and farmed, which may alter the interspecies transmission cycle of influenza viruses in southern China. The surveillance of H9N2 influenza virus in wild aquatic birds and viral genomic information are having been limited. In order to explore the role of wild birds and the disease ecology of H9N2 virus, we characterized eleven strains of virus isolated from wild ducks in Jiangxi, China in 2011.

Material and Methods

Sampling and virus isolation

During January and February 2011, AIV surveillance was conducted in Poyang Lake (28°90'–29°25' N, 116°06'–116°69' E) located in Jiangxi Province. Poyang Lake is one of the most important habitat and overwintering sites for migratory water birds in Asia. The fertile surrounding land and rich bio-resources provided a shared habitat for wild birds and domestic fowl. 67 samples were collected from apparently healthy wild spot-billed ducks (*Anas poecilorhyncha*) and mallard ducks (*Anas platyrhynchos*). Tracheal and cloacal swabs were eluted with PBS containing 2000U/ml penicillin and 1000ug/ml streptomycin. The supernatants were inoculated into the allantoic cavity of 9- to 10-day-old embryonated specific-pathogen free (SPF) chicken eggs and were incubated at 37°C for 48–72 h as described by Li *et al.* (2003). The allantoic fluid from hemagglutination-positive infected eggs was harvested and tested for influenza A viruses by standard hemagglutination inhibition (HI) and neuraminidase inhibition (NI) tests with a panel of the World Health Organization reference antisera as previously described by Van Deusen *et al.* (1983). Virus stocks were stored at –80°C for further experiments. All manipulations of live viruses were conducted in biosafety level 2 agriculture (BSL-2AG) containment facilities.

Genetic and phylogenetic analysis

Viral RNAs were extracted from the virus-infected allantoic fluid using a QIAamp Viral RNA Mini kit (Qiagen, Valencia, CA) according to manufacturer's instructions, and reverse transcribed using the uni12 primer (5'-AGCA AAAGCAGG-3'). PCR was performed with specific primers for segment-specific primers (the primer sequences and amplification conditions used are available upon request). PCR products were purified with the Wizard® SV Gel and PCR Clean-Up System (Promega), and directly sequenced at Sangon Biotech Ltd (Shanghai, China). Nucleotide sequences were assembled, edited, and compared using the DNAMAN program (version 6.0). The appropriate nucleotide substitution models of all the eight gene segments from these viruses were estimated by using the software jModelTest 1.0.1, and the maximum likelihood trees were constructed for using the Bayesian analysis with MrBayes 3.1, two replicates of 1 million generations with six chains were run, sampling every 100 generations, and Bayesian posterior probabilities were calculated from the consensus of 15000 trees after excluding the first 5000 trees as burn-in. The maximum likelihood trees were further confirmed by using PHYLIP 3.0 with the General Time Reversible (GTR) model and 100 bootstraps.

Pathogenicity testing in mice

To assess the pathogenicity of isolates in mice, 6- to 8-week-old male SPF BALB/c mice (Xiper-Bikai Experimental Animal Co., LTD, Shanghai) were lightly anesthetized with ethylether and inoculated intranasally (i.n.) with 50 µl of the virus positive allantoic fluid diluted in phosphate-buffered saline (PBS) to yield a 10⁶egg infectious dose (EID₅₀). The control mice were inoculated i.n. with 50 µl of an equivalent dilution of noninfectious allantoic fluid.

Each group was divided into two subgroups of five mice post inoculation to assess clinical features including activity, behavior, body weight and mortality over a 14-day observation period. Fifteen mice were used to evaluate the viral replication in different organs. For this, lungs, kidneys, brains, livers, spleens, hearts and tracheas of three mice from each group were collected 3 and 6 post inoculations. Portions of the left lung lobes were fixed in buffered 10% formalin, embedded in paraffin, and five micrometers-thick sections stained with hematoxylin-eosin for histopathological evaluation. Tissues from left lung lobes and other organs were also stored at –80°C and the amount of viral RNA determined by real-

time PCR using the ABI 7300 Sequence Detection system (Applied Biosystems) for influenza virus matrix (M) gene fragments according to previously described methods (Mosleh et al., 2009) with a small change: the housekeeping gene Beta-Actin (ACTB) was used as an endogenous control. The concentration of virus in mice was analyzed by using the comparative Ct (Ct) method (Chen et al., 2003b).

All animal procedures followed the ethics guidelines of the Guide for the Care and use of Laboratory Animals (Council., 1985).

Nucleotide sequence accession numbers

The nucleotide sequences analyzed in this study are available in the GenBank database under accession numbers JQ901619-JQ901706. All of the other representative H9N2 sequences used in this study for phylogenetic comparison were obtained from GenBank (<http://www.ncbi.nlm.nih.gov>).

Results

Virus isolation and genetic analysis

A total of 67 paired tracheal and cloacal swabs were collected from spot-billed ducks and mallard ducks in Poyang Lake of Jiangxi province in China during January and February of 2011. After proliferating in SPF embryonated chicken eggs, 15 H9N2 subtype influenza viruses were isolated: 7 from the spot-billed duck and 8 from the mallard duck, with an isolation rate of 22.4 %. To better understand the evolution and ecology of H9N2 viruses in wild ducks, 11 representative viruses (abbreviated as JX/11-like viruses) were selected for further genetic characterization, and the eight gene segments were completed sequenced. Subsequently, one of the 11 isolates was used to test for lethality in mice.

The nucleotide sequences for each gene segment of the H9N2 JX/11-like viruses were aligned with other representative H9N2 viruses from GenBank. The results showed that all eight gene segments of the JX/11-like isolates shared the highest 95.6%–99.4% homology with 2009 human H9N2 isolates (HK/33982/2009 - Table 1), and furthermore shared 96.2% to 98.2% nucleotide homology with quail H9N2 isolates (representative QA/Shantou/11195/05) except for NA genes (Table 1). JX/11-like isolates were also more closely related to G1-like virus (QA/HK/G1/1997) except for the NA and PA genes, which had a higher homology with Ck/Bei-like viruses, such as CK/HK/G9/1997 virus and Y439-like viruses (DK/HK/Y439/1997) respectively (Table 1).

Phylogenetic analysis of the surface genes

To better understand the evolutionary characterization of JX/11-like viruses, 11 H9N2 viruses from wild waterfowls were phylogenetically analyzed. Phylogenetic analysis of the HA gene revealed that all 11 JX/11-like viruses belonged to the G1-like lineage and showed a very close relationship with 2009 human H9N2 isolates (HK/35820/2009 and HK/33982/2009) and 2005 H9N2 viruses isolated from quails in southern China (such as QA/Shantou/11195/05), but they were different from the 2011 isolates in southern China (Figure 1A).

Phylogenetic analysis of the neuraminidase (NA) gene revealed that JX/11-like viruses clustered into Ck/Bei sublineage. The Ck/Bei sublineage could be divided into two major subgroups: including subgroup 1 (Y280-like), represented by Dk/HK/Y280/97; and subgroup 2 (G9-like), represented by CK/HK/G9/97 as described in a previous study (Bi et al., 2011). It is noteworthy that the JX/11-like H9N2 isolates all fell into the subgroup 2 (Figure 1B), and were most closely related to the 2009 human isolates (Figure 1).

Furthermore, the NA genes of all G9-like isolates had no deletion, suggesting that they were of the same origin. In all, these results indicated that the HA and NA genes of JX/11-like isolates belong to two different sublineages of H9N2 viruses, G1-like and Ck/Bei-like sublineage respectively, revealing reassortment between the two viral sublineages.

Phylogenetic analysis of the internal genes

In the phylogenetic tree of ribonucleoprotein (RNP) complex genes (PB2, PB1, PA, and NP genes), four different evolution lineages were recognized: Ck/Bei-like, Y439-like, F98-like and G1-like (Figure 2A, 2B, 2C, and 2D). For PB2, PB1 and NP genes, JX/11-like viruses all clustered into the G1-like lineage, but the PA gene fell into the Y439-like lineage. Phylogenetic analyses of the RNP genes also indicated that the JX/11-like viruses had a close relationship with 2009 human isolates, and representative H9N2 isolates QA/Shantou/11195/05 (Figure 2). We also noted that the PB2 and NP genes of JX/11-like viruses were also closely related to the H6N1 isolates, such as QA/HK/YU421/02 and QA/HK/NT342/01, which implied that JX/11-like viruses shared a common origin with these viruses.

Phylogenetic analysis of the other two internal genes (M and NS) revealed that the topologies of these gene segments were similar to the HA, with three different lineages: Ck/Bei-like, Y439-like and G1-like (Figure 2E, 2F). JX/11-like viruses fell into the G1-like lineage. The M and NS genes of the 2009 human isolates and 2005 quail H9N2 isolates QA/Shantou/11195/05 clustered into the same sublineage with JX/11-like isolates.

Molecular Characterization

All JX/11-like isolates possessed a new motif “R-S-N-R” in spite of a typical “R-S-S-R” at the HA1-HA2 connecting peptide (Table. 2), which is associated with low pathogenicity in chickens (Steinhauer, 1999). Position Q226 and G228 of HA (H3 numbering used throughout) suggest that the JX/11-like viruses all preferentially bind to 2,3-NeuAcGal linkages of avian cell-surface receptors (Ha et al., 2001). The other amino acids in the receptor binding site (RBS) were identical to those of 2009 human isolates (Table 2). Seven N-linked potential glycosylation sites (PGS) with the N-X-T/S motif (in which X may be any amino acid except Proline) of JX/11-like viruses were present in HA protein, which were similar to QA/HK/G1/1997 virus and 2009 human isolates.

All JX/11-like viruses contained no amino acid deletion at the stalk of neuraminidase, which also were consistent with 2009 human isolates. In addition, the mutations that confer adaption to mammals at PB2 E627K and D701N were not found in the JX/11-like isolates, but one human virus (A/Hong Kong/33982/2009) contained D701N mutation (Gao et al., 2009). The JX/11-like viruses had H at position 274 of the neuraminidase protein and are predicted to be sensitive to oseltamivir (Treanor et al., 2000), and none contained point mutations at position 27, 30 and 31 in the matrix protein 2, associated with resistance to amantadine (Scholtissek et al., 1998).

Pathogenicity of the H9N2 isolates in mice

We used BALB/c mice as a model to evaluate JX/11-like viruses' ability to replicate in mammalian hosts. In our initial experiment, three representative viruses were used and the results showed that all three viruses could replicate in mouse lungs without prior adaptation after exposure to 10^6 EID₅₀ of each virus and that they exhibited the similar clinical features, such as inappetence, emaciation, weight loss, and visible labored respirations (data not shown). Subsequently, only one representative virus A/Mallard duck/Jiangxi/39/2011(H9N2) (abbreviated as MD/JX/39/11) was used to further analyze the pathogenicity of the H9N2 isolates in mice, because all three viruses gave similar clinical features and shared >99% high homology with each other.

Virus-infected mice showed severe clinical signs of infection on day 3 postinoculation, including inappetence, inactivity, ruffled fur and visual signs of respiratory distress. The body mass of the JX/39/11 virus infected mice significantly decreased on days 3 to 9 post inoculation ($P<0.01$) compared with the non-infected control groups (Figure 3A). The mice in the virus infected group began to die on day 3 post inoculation, and reached a plateau at day 6. The survival rates were significantly lower in the infected group than the control mice on days 6 to 13 post inoculation ($p<0.01$, Figure 3B), and the virus could be isolated directly from lung and tracheal tissues of infected mice on days 3 and 6 postinfection (Figure 3C). We also found that the virus titer was significantly higher in the tracheas than in lungs ($P<0.01$). No virus was recovered from other organs, such as heart, liver, kidney, spleen and brain. No deaths were observed, nor virus recovered in control mice during the experiment period.

Lungs were markedly edematous in infected mice, with areas of profuse hemorrhaging. On day 3 postinoculation, lungs of infected mice were characterized by extensive peribronchiolar lesions and bronchiolitis (Figure 4B), interstitial and alveolar edema, inflammatory cell infiltration around small blood vessels and thickening of alveolar walls (Figure 4B, black arrows). On day 6 postinoculation, lung lesions were characterized by diffuse pneumonia, with thickening of alveolar wall, shedding of bronchial epithelium, severe infiltration of neutrophils in the bronchioles (Figure 4C, black arrows), and peribronchiolar and vascular edema and hemorrhaging (grey arrows in 4C). On day 8 postinoculation, lung lesions were significantly alleviated and thickening of alveolar walls were observed in Figure 4D (black arrow). In comparison, lungs from control mice had no apparent histological changes (Figure 4A) and also not observed in infected mice on days 11 and 14 postinoculation (Figure 4E and 4F).

Discussion

As the natural reservoirs of all influenza A viruses, wild aquatic birds might be of paramount importance for the ecology of influenza virus infection, since they provide most of the gene pool for all influenza viruses of human and animal populations (Webster et al., 1992). The importance of conducting AIV surveillance in wild birds is evident. During January and February of 2011, we characterized eleven H9N2 viruses isolated from common wild ducks (mallard and spot-billed ducks, *Anseriformes*) in southeast China. The amino acid sequence of the connecting peptide of HA is considered an indicator of pathogenic potential (Steinhauer, 1999). Analysis of the HA gene showed that the cleavage site patterns of JX/11-like viruses are similar to those of two human H9N2 isolates in 2009, that are different from the other wild bird isolates. The significance of this mutation is not fully understood. All JX/11-like isolates contained Q226 and G228 at RBS (H3 numbering) of HA, suggesting that these viruses all preferentially bound to 2, 3-NeuAcGal linkages of avian cell-surface receptors. The amino acid change at RBS, cleavage sites, and PGS of HA of all our isolates are consistent with the two human H9N2 isolates in 2009, but different from representative G1-like viruses.

Previous research has indicated that mutations of E627K and D701N in PB2 protein are critical for enhanced pathogenicity in mammals (Gao et al., 2009), but molecular analysis demonstrated that all of the viruses in our study had no mutations at the two sites in PB2 proteins. However, results of pathogenicity studies in mice indicated that the JX/11-like viruses could replicate efficiently in BALB/c mice lungs and tracheas without prior adaption, resulting in 40% mortality to mice after exposure to 10^6 EID₅₀ of virus. Severe pneumonia and alveolar damage were also observed in those affected mice. Therefore, the mechanism of JX/11-like virus to replicate in mammals is likely related to other amino-acid mutations or other gene segments.

Although the eleven viruses were obtained from two different species of duck, all eight gene segments of these isolates shared high homology (99.3%–100%) with each other, and clustered into the same sublineage, suggesting that they were of the same origin. Phylogenetic results indicated that JX/11-like viruses belong to a novel triple-reassortant genotype containing the PA gene from an Y439-like sublineage, the NA gene from Ck/Bei-like sublineage, and the other six genes from G1-like sublineage. Of key significance was the observation that JX/11-like viruses shared the highest identity with 2009 human H9N2 isolates (HK/33982/2009 and HK/35820/2009) in all eight genes (95.6–99.4% homology), and they were closely clustered into the same subgroup. These findings suggest that JX/11-like isolates and the 2009 human H9N2 isolates should also originate from a common ancestor, but whether or not these viruses have a propensity to transmit to the human population is not known. The genotype virus was detected for the first time in wild birds and was different from those of other reported H9N2 viruses that have been established worldwide in terrestrial poultry and wild birds since the 1960s (Dong et al., 2011), implying that these genetic reassortments apparently occurred independently. Given the potential of this subtype influenza virus to harm people, it is vital to continue influenza virus surveillance in wild birds in order to have more information on the prevalence, variance, and whereabouts of this subtype virus as well as to make preparations against future pandemic influenza.

As a possible intermediate host, quails possess both 2,3-NeuAcGal linkages and 2,6-NeuAcGal linkages receptors (Wan et al., 2006) and harbor both G1-like and Ck/Bei-like viruses (Xu et al., 2007b) and may play an important role in the introduction of influenza viruses from aquatic birds to domestic poultry species coexisting in the same ecosystems (Perez et al., 2003). In this study, we also noted that the full genomes of JX/11-like viruses shared 96.2% to 98.2% nucleotide homology with quail H9N2 isolates in 2005 QA/Shantou/11195/05 and formed a single sublineage together except for NA genes (the homology is about 87.9% to 88.0%, and which were closely clustered with other Ck/Bei-like viruses isolated from other poultry in 2005), which suggests these viruses of quail origin might have further reassorted with Ck/Bei-like viruses endemic in quails or other animal species in this region at the same time period, and then the reassortant virus could have gradually acquired the ability to replicate in mammals and wild ducks.

Poyang Lake is one of the largest freshwater lakes in China and about 108 species of waterfowl can be found there (Zhu et al., 2012). The highly developed poultry industry for both domestic fowl and waterfowl, and the loose backyard poultry around Poyang Lake increases the contact among domestic poultry and wild birds. When these wild bird-origin viruses are transmitted to new hosts such as domestic poultry, swine, and humans they evolve rapidly and may increase in pathogenicity (Webster et al., 1997; Alexander, 2000). If the JX/11-like viruses acquired any mammalian associated mutations with further reassortment, they may have significant pandemic potential. This pandemic potential may be increased through migration of these wild birds to new localities, and because of the current lack of strategies to control avian influenza in wild birds. Due to the limited number of samples in this study, we may not know whether the JX/11-like viruses have established stable infection in wild ducks. Therefore, conducting long-term molecular epidemiological investigations of H9N2 and other influenza viruses in wild aquatic birds is critical to better define the ecology and evolution of influenza viruses on a global scale.

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Figure. 1.

Phylogenetic relationships of HA (A) and NA (B) genes of the H9N2 influenza viruses analyzed. Phylogenetic analysis was based on the following nucleotides: HA, 10-1650 and NA 58-1358. The trees were constructed with MrBayes 3.1 using the maximum likelihood method. Numbers above or below branches indicate Bayesian posterior probabilities and bootstrap values, respectively. Not all supports are shown because of space constraints. Viruses characterized in this study are highlighted in blue colors, while representative viruses from each lineage are in red colors, and the H6N1 isolates are marked with green colors. Gene sequences used in this study can be found in GenBank. Abbreviations: CK, chicken; DK, duck; GS, goose; SbD, spot-billed duck; MD, mallard duck; BbM, black-billed magpie; CHU, chukkar; GF, guinea fowl; PG, pigeon; PH, pheasant; QA, quail; FR, ferret; PT, partridge; SW, swine; WD, wild duck; HK, Hong Kong.

Figure.3.

Change in body weight (A), mortality (B) and virus replication (C) in BALB/c mice inoculated with one representative H9N2 influenza viruses (MD/JX/39/11). Body weight and survival rate of mice were observed over 14 days after infection. (A) Data are expressed as means $\pm$ SD. White bars are the uninfected control group and black bars are the infected group. * $p < 0.05$; ** $p < 0.01$ compared with the uninfected control group. (B) Data are expressed as a percentage of total survival (survival rate), uninfected control group () and virus infected group (). ** $p < 0.01$ compared with uninfected control group. (C) Lungs and tracheas were collected on 3 and 6 days p.i., the virus replication levels were measured

by real-time PCR, and normalized with ACTB. Data were expressed by 2^{-Ct} . Open bars, trachea; solid bars, lung. **: $p < 0.01$ compared with tracheas

Figure.4.

Representative histopathological changes of lungs are shown by Hematoxylin and Eosin (H&E) staining after H9N2 virus infection. The noninfected control is labeled (A). At day 3 post-inoculation there were evident interstitial edema around small blood vessels, thickening of the alveolar walls, and inflammatory cell infiltration around small blood vessels (B, black arrows). On day 6 postinoculation, there was evidence of dropout of bronchial epithelium and a great number of neutrophils infiltrating the bronchioles as well as both thickening of alveolar walls (C, black arrows) and hemorrhaging (grey arrows in C). On day 8 postinoculation, there were visible slight interstitial edema around small blood vessels and thickening of alveolar walls (D, black arrows). No apparent lung lesions were observed on day 11 (E) and 14 (F) postinoculation. Objective magnifications, $\times 10$ (A–F).

Table 1
Homology of the gene segments of JX/11-like H9N2 influenza viruses with other H9N2 representative viruses from different lineages

Gene	nucleotide sequences	% Homology with							
		CK/HK/G1/97	DK/HK/Y439/97	DK/HK/Y280/97	CK/BJ/1/94	QA/HK/G9/97	CH/SH/F/98	QA/ST/11195/05	HK/33982/09
PB2	153-2231	94.2%-94.3%	85.3%-85.4%	83.9%-84.0%	84.4%	93.7%-93.8%	84.3%-84.4%	^a 97.0%-97.1% (1037-2231)	99.1%-99.2%
PB1	48-2202	94.1%-94.3%	89.1%-89.3%	88.2%-88.4%	89.0%-89.1%	92.8%-92.9%	89.3%-89.5%	^a 96.2%-96.5%(48-1460)	95.6%-95.8%
PA	62-2104	87.9%-88.0%	94.2%-94.3%	86.1%	86.6%-86.7%	85.3%-85.4%	89.0%-89.1%	^a 98.3% (772-2104)	99.0%-99.1%
HA	24-1540	93.1%-93.7%	82.7%-83.2%	86.7%-87.4%	88.7%-89.3%	87.2%-87.9%	87.1%-87.6%	96.4%-97.0%	98.6%-99.2%
NP	19-1286	95.4%-95.5%	92.0%-92.1%	88.9%-89.0%	89.6%-89.7%	89.4%-89.5%	89.6%-89.7%	^a 97.4%-97.5% (19-969)	97.7%-97.8%
NA	61-1358	89.1%-89.2%	85.9%-86.0%	90.9%-91.0%	92.2%-92.3%	92.9%-93.0%	90.3%-90.4%	87.9%-88.0%	98.7%-98.8%
M	26-708	94.8%-95.0%	88.6%-88.7%	91.9%-92.1%	92.0%-92.1%	92.1%-92.3%	92.7%-92.9%	97.8%-97.9%	99.3%-99.4%
NS	15-693	93.9%-94.0%	88.5%-88.7%	92.9%-93.1%	92.0%-92.2%	91.7%-91.9%	91.3%-91.4%	98.0%-98.2%	98.8%-98.9%

Abbreviations: CK, chicken; DK, duck; BJ, Beijing; HK, Hong Kong; SH, Shanghai; ST, Shantou. The highest homologies are in bold face.

^a no completely compared, the nucleotide sequences are given in parenthesis.

Table 2

Comparison of amino acid residues in several key sites of HA, NA, M and PB2 proteins of H9N2 viruses tested in this study and other H9N2 representative viruses from different lineages

Virus	Amino acid sequence at the cleavage sit	Receptor binding site of HA (H3 numbering)				NA deletion	M2 residue at amantadine resistance mutation position				Residue at PB2	
		183	190	226	228		27	30	31	627	701	
QAI/HK/G1/97	RSSR	H	E	L	G	Yes	V	A	S	E	D	
DK/HK/Y280/97	----	N	T	-	-	-	-	-	-	-	-	
CK/BJ/1/94	----	N	V	Q	-	No	-	-	-	-	-	
DK/HK/Y439/97	A-N-	-	-	Q	-	No	-	-	-	-	-	
HK/33982/09	--N-	-	D	Q	-	No	-	-	-	-	N	
HK/35820/09	--N-	-	D	Q	-	No	-	-	-	/ ^b	/ ^b	
JX/2011	--N-	-	D	Q	-	No	-	-	-	-	-	

Abbreviations: CK, chicken; DK, duck; BJ, Beijing; HK, Hong Kong;

^aDashes indicate residues identical to those of QA/HK/G1/97 virus.

^bno compared