Genetic and pathobiologic characterization of H3N2 canine influenza viruses isolated in the Jiangsu Province of China in 2009–2010

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ABSTRACT

The newly emerging canine influenza virus (CIV) causes considerable concerns for both veterinary and public health. During 2009–2010, six strains of H3N2 influenza virus were isolated from dogs in Jiangsu Province, China. Sequence and phylogenetic analysis of eight gene segments revealed that the six viruses were most similar to a recent canine-derived subtype H3N2 influenza virus isolated in cats from South Korea, which originated from avian strain. By comparing the deduced amino acid sequences of the hemagglutinin 1 (HA1) and neuraminidase (NA) genes of the six Jiangsu isolates against the most similar avian strains, we found that all isolates had several common mutations at the receptor-binding sites, potential glycosylation sites and cleavage site in HA1, and antigenic sites in both the HA1 and NA segments. Significantly, a unique two amino acid insertion in the NA stalk was found. Experimental infection of BALB/c mice revealed that viral RNA could be detected in the major rodent organs, such as brain, heart, spleen, kidney, liver and intestine, as well as the lung. All the sampled organs from infected mice showed significant lesions and viral antigen staining. This study highlights the potential of domesticated animals to become a reservoir for influenza virus and the need for surveillance programs to detect cross-species transmission.

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1. Introduction

Influenza A is a highly contagious disease that has led to severe local and pandemic disease outbreaks, seriously threatening human health and causing tremendous economic losses in the poultry industry. Influenza A viruses can be isolated from a wide variety of species, including humans, pigs, horses, sea mammals and birds. In 2004, dogs were discovered as new hosts, coming as a surprise because they were previously thought to be refractory to infection with influenza viruses. The first canine influenza virus (CIV) subtype H3N8 emerged in racing greyhounds in Florida in January 2004 (Crawford et al., 2005), and then seven months later, the virus was also isolated in shelter and pet dogs (Payungporn et al., 2008). Thousands of greyhound dogs, at tracks in nine states, were subsequently affected during multiple respiratory disease outbreaks from 2004 to 2006 (Yoon et al., 2005). In 2007, another canine influenza outbreak was confirmed in Korea (Song et al., 2008), but this time the causal agent was an H3N2 avian influenza virus. The H3N2 virus was isolated during outbreaks of severe respiratory disease in dogs at multiple facilities, and had been demonstrated to be capable of transmitting directly from dog to dog (Song et al., 2009). In 2010, the interspecies transmission of H3N2 CIV to cats was firstly reported in Korea (Song et al., 2011). Additionally, a fatal infection in dogs and cats with the highly pathogenic avian influenza virus (HPAIV) H5N1 was also reported in several countries in Asia (Desvaux et al., 2009; Songserm et al., 2006). However, transmission of the infection to in-contact dogs or cats was not observed (Butler, 2006). In China, the

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first case of H3N2 CIV was reported in Guangdong Province in 2010 (Li et al., 2010b). As all of these cases of canine influenza have caused considerable problems and concerns for both veterinary and public health, the surveillance of this virus is of great importance.

Influenza A virus (family Orthomyxoviridae) is an enveloped virus with a segmented negative stranded RNA genome encoding 12 viral proteins: hemagglutinin (HA), neuraminidase (NA), nucleoprotein (NP), M1, M2, NS1, NS2 (NEP: nuclear export protein), PA, PB2, PB1, PB1-F2 and PB1-N40, a newly identified protein that is expressed from the PB1 segment (Wise et al., 2009). Of these proteins, HA and NA are the two major surface glycoproteins on the virion used for determination of the subtype of the virus. HA and NA are targeted by the protective immune response and can vary as a result of antigenic drift and antigenic shift (Shu et al., 1994).

In this study, we provided the molecular and biological properties of six strains of H3N2 CIV, which were isolated from the dogs with severe respiratory syndrome in the Jiangsu Province of China. Genetic sequence data from six viruses were valuable for understanding the mutation frequencies associated with replication of the virus in the canine host. Experimental infections of BALB/c mice provided insights into the pathobiological behavior of these viruses in dogs.

2. Materials and methods

2.1. Samples

A total of 13 nasopharyngeal swabs of dogs with severe respiratory syndrome were collected from Animal Clinics of Nanjing Agricultural University in Jiangsu Province of China from November 2009 to July 2010. These dogs were kept as domestic pets and the diseases were sporadic. The samples were taken from individual dogs at different time but showing similar respiratory symptoms such as coughing, sneezing and copious nasal discharge. The pet dogs did not have contact with birds, but it is difficult to determine whether they have had contact with pet cats.

2.2. Virus isolation

Samples were inoculated into 9–10-day-old specific pathogen free (SPF) embryonated chicken eggs and incubated for 72 h at 37 °C before harvesting the allantoic fluid. Virus isolates were identified by RT nested PCR. Two pairs of primers were selected based on conserved sequences of the M gene of influenza A virus (Ellis and Zambon, 2001).

2.3. RNA extraction and RT-PCR

Viral RNA was extracted from infected allantoic fluid with the Virus Nucleic Acid Extraction Kit II (Geneaid, Taiwan). Reverse transcription (RT) was carried out under standard conditions using the Uni12 primer (AGGAAAAAG-CAGG), followed by PCR as described by Hoffmann et al. (2001) using primers specific for each of the eight RNA segments. PCR products were purified with the Agarose Gel DNA Purification Kit (TaKaRa, Dalian) and cloned into the pMD18-T vector (TaKaRa, Dalian). Positive clones were selected and sequenced.

2.4. Fifty-percent egg infective dose (EID_{50}) assays

EID_{50} assays were performed by injecting 200 μl of 10-fold serially diluted positive samples into 9–10-day-old SPF embryonated chicken eggs, which were harvested three days after inoculation. Allantoic fluid was tested by hemagglutination assays at room temperature by using 1% chicken red blood cells. EID_{50} titers were calculated by the Reed–Muench method (1938) with five eggs per dilution.

2.5. Sequence analysis

Reference sequences were obtained from the National Center for Biotechnology Information (http://www.ncbi.nlm.nih.gov). Comparisons of nucleotide and deduced amino acid sequences were made using DNASTAR software. The phylogenetic trees were generated with MEGA4 software by the neighbor-joining method. Bootstrap values were calculated based on 1000 replicates of the alignment.

2.6. Experimental infection of mice

For investigating virus tropism and virus shedding, we chose two of the six Jiangsu isolates to infect mice. Prior to conducting this study, we obtained approval for the experimental protocols from the Animal Ethics Committee of Nanjing Agricultural University. All animal experiments complied with the guidelines of the Animal Welfare Council of China. BALB/c mice (22–24 g, 50 days old, female) were purchased from the Animal Experiment Center, Yangzhou University. Intranasal inoculations were given to group I (n = 15) with the 01 strain, group II (n = 15) with the 06 strain and group III (n = 15) with phosphate-buffered saline (PBS). The daily weight (Narasaraju et al., 2009) and clinical symptoms were recorded for up to 16 days.

Three mice from each infected group and PBS control were euthanized humanely according to a pre-designated schedule. At each of the time points of 2, 4, 6, 8 and 16 days post-infection (d.p.i.), tissues including lung, brain, heart, spleen, liver, intestine, and kidney, as well as feces were collected. Virus shedding was detected by screening fecal samples. Tissue distribution and virus shedding were both determined by the detection of viral RNA. Tissues and feces were homogenized in PBS at a ratio of 1:1 (g/ml), respectively, centrifuged at 10,000 × g for 30 min, and the supernatants were collected for the extraction of viral RNA with the Virus Nucleic Acid Extraction Kit II (Geneaid, Taiwan). Viral RNA were also detected by the RT nested PCR assay described above.

2.7. Real-time PCR for quantitation of viral loads

Quantitative assays were carried to measure viral loads in the main organs. RNA from tissues mentioned above were reverse transcribed and run in an ABI 7300 Real Time PCR System using the SYBR® Premix Ex Taq™ (Perfect Real
Time) kit (TaKaRa). The set of primers was designed on the region of matrix gene as following: 5′ TCTATCGTCCCATT-CAGGC/GGTCTTGTCTTACATTCA. A reference standard was prepared using pMD19-T Simple Vector (50 ng/μl; TaKaRa) that contained the corresponding target virus sequences. A series of seven 10-fold dilutions equivalent to \(1 \times 10^{-3} \text{–} 1 \times 10^{-8}\) copies per reaction was prepared to generate calibration curves and was run in parallel with the test samples (To et al., 2010). The detection limit of this assay was 1120 copies of RNA per ml.

### 2.8. Histopathology and immunohistochemistry

After euthanasia, the organs from the mice inoculated with A/Canine/Jiangsu/06/2010 or PBS at 6 d.p.i. were collected and placed into 10% neutral buffered formalin. After fixation the organs were embedded in paraffin, sectioned at 4 μm and stained with hematoxylin and eosin for histological evaluation. Sequential slides were stained using an immunoperoxidase method as follows. Firstly, sections were blocked with 3% \(\text{H}_2\text{O}_2\) for 30 min, and then non-specific background staining was blocked by incubating the sections for 30 min with normal rabbit serum. The sections were then incubated for 1 h with rabbit anti-H3N2 CIV polyclonal serum (prepared in our laboratory using the purified 06 strain from this study) diluted 1/1000, followed by biotinylated goat anti-rabbit immunoglobulin (Ding-Guo, China) diluted 1/100 at 37 °C for 30 min and subsequent incubation with HRP conjugated streptavidin (DingGuo, China) at 37 °C for 30 min. Finally, the sections were developed with HRP-DAB chromogenic substrate kit (Tiangen, China) for 10 min, and then slides were counterstained with hematoxylin.

### 3. Results

#### 3.1. Virus isolation

Six strains of influenza virus were isolated out of the 13 samples and designated as A/Canine/Jiangsu/01/2009 (01), A/Canine/Jiangsu/02/2010 (02), A/Canine/Jiangsu/03/2010 (03), A/Canine/Jiangsu/04/2010 (04), A/Canine/Jiangsu/05/2010 (05) and A/Canine/Jiangsu/06/2010 (06), with virus titers of \(10^6.71 \text{EID}_{50}/200 \mu l, 10^6.16 \text{EID}_{50}/200 \mu l, 10^6 \text{EID}_{50}/200 \mu l, 10^6.71 \text{EID}_{50}/200 \mu l, 10^6.3 \text{EID}_{50}/200 \mu l,\) and \(10^7.2 \text{EID}_{50}/200 \mu l\), respectively.

#### 3.2. Homology analysis of nucleotide sequences

All eight genes of the six virus isolates were amplified, sequenced and then submitted to GenBank. GenBank accession numbers of the eight genes of the six viruses (48 segments in total) are numbered from JN247576 to JN247623. Their homologies were determined by comparison with sequences available in GenBank. There was a high degree of genetic similarity (>99%) in eight genes among the six Jiangsu canine isolates. All eight genes exhibited the highest homology (>99%) to the influenza virus in cats infected with H3N2 CIV reported in Korea, and were also highly homologous (98–99%) to H3N2 CIV circulating in Korea and Southern China. It was surprising to find that all Jiangsu isolates were most genetically similar to the CIV isolated in cats in Korea but not to the CIV isolated in Guangdong, China. All of these canine H3N2 influenza isolates originated from the avian influenza virus (AIV).

Amino acid changes for each gene segment of the six H3N2 isolates were compared to the closest AIV. Many common mutations in PB2, PB1, PA, HA, NP, NA, M and NS are shown in Table 1. Of these common mutations, some also exist in Korean feline and canine isolates while some are just unique to Jiangsu isolates (data not shown). And some of these common mutations have been identified to be functionally significant, such as E152N, E172K and V180I in N51, which lie in the region aa144–186 previously identified as the binding site for CPSF (Twu et al., 2006).

Other important mutations included R251K and I255V in PB2, which also lie in the most important region aa206–259 of PB2 for PB1 binding, and R723Q in PB1 lies in the most important region aa718–732 of PB1 for PB2 binding (Ohtsu et al., 2002). In addition, unique mutations were found in some isolates (data not shown), some of which were also significant, such as the NS1 mutations of D125G in the 02 strain and D125N and F103L in the 06 strain. The aa125 in NS1 falls within the aa123–127 region of NS1 that binds to protein kinase PKR, allowing the virus to suppress the antiviral activity of this host protein (Min et al., 2007).

<table>
<thead>
<tr>
<th>Gene segment</th>
<th>Avian strains with the highest nucleotide identity</th>
<th>Common amino acid changes of the six strains compared to the closest avian strain</th>
</tr>
</thead>
<tbody>
<tr>
<td>PA</td>
<td>A/migratoryduck/HongKong/MP2553/03(H8N4) (97.4–97.6%)</td>
<td>S65Y, L105F, T208A, E243D, S277T, A369V, N388S, M441K, K615R</td>
</tr>
<tr>
<td>NP</td>
<td>A/mallard/Jiangxi/8346/04(H6N5) (96.1–96.3%)</td>
<td>G50S, Y52H, N125G, M159L, V353I, T373K, M374I, R389K, L418I, A428T, R452K, N473K</td>
</tr>
<tr>
<td>NA</td>
<td>A/duck/Korea/J533/2004(H3N2) (96.8–97%)</td>
<td>M24L, N44S, E54K, P81S, D143N, P156S, D288N, S372L, R432G</td>
</tr>
<tr>
<td>M</td>
<td>A/duck/Hunan/5613/03(H6N5) (98–98.2%)</td>
<td>M2: G14E, K188, S22L</td>
</tr>
</tbody>
</table>
It has been reported that mutation F103L in NS1 increases virus replication and virulence (Dankar et al., 2011; Guan et al., 1999). It is worth mentioning that with the exception of the 06 strain, the amino acid sequences of the M, NP and NA genes were the same in the other five strains. Also, the substitutions of D290V and V456M in NP and E97K in M2 were found in the five strains, whereas C53K in the NA gene was found only in the 06 strain.

3.3. HA1 amino acid analysis

To investigate the genetic characteristics in detail, we compared the deduced amino acid sequences of the HA1 gene from the six canine H3N2 isolates against the canine and feline isolates from Korea, the Guangdong canine isolate and the most similar avian strain, A/aquatic bird/Korea/JN-2/2006. These four isolates were chosen for comparisons in order to determine if any of antigenic mutations had occurred among these canine influenza viruses. The proposed antigenic sites (Caton et al., 1982; Nakajima et al., 2003), receptor-binding sites, potential glycosylation sites and the cleavage site were analyzed (Fig. 1).

Some obvious common mutations were found in the nine canine or feline H3N2 isolates compared with the original avian H3N2 virus: T10A, L79F, D81N, L111I/V, A160T, D172N, G209S and W222L. Three of these eight mutations, D81N, A160T and G209S, occurred at three different antigenic sites, while the W222L mutation was at the receptor-binding site. Interestingly, the T10A mutation resulted in the loss of a potential glycosylation site, whereas the D81N mutation created an additional potential glycosylation site.

The amino acid changes of R207G in the 04 and 05 strains and Q210K in the 06 strain occurred at antigenic sites. Significantly, the mutation K326R was located at the cleavage site in all canine or feline isolates except for the Guangdong canine isolate. The R261H change was found simultaneously in the Jiangsu canine isolates and Korean feline isolates. Several other sporadic mutations were also seen in the HA1 gene of the nine viruses (Fig. 1).

3.4. NA amino acid analysis

The obvious common mutations in the deduced amino acid sequences of the NA gene from the nine canine or feline isolates against the closest avian strain, duck/Korea/JS53/2004 (Fig. 2) were M24L, N48S, E54K, P81S, D143N, P156S, I208V, D288N, S372L and R432G, of which S372L and R432G occurred at two different antigenic sites (Air et al., 1985; Colman et al., 1983; Zhirkov et al., 2009). Significantly, a two amino acid insertion unique to the Jiangsu isolates was found at the end of the NA stalk, while there were no any deletions or insertions in the stalk of related avian, canine and feline isolates. In addition, another three mutations, H36Y, R222Q and L392S, were found only in the six canine isolates from Jiangsu and the feline isolate from Korea, furthermore, L392S occurred at antigenic site. The comparison of six Jiangsu isolates showed that the first five strains shared the same amino acid sequences of the NA gene, while the 06 strain had a single difference at position 53.

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Fig. 1. Alignment of HA1 amino acid sequences of the nine canine or feline H3N2 influenza isolates and the most similar avian isolate. Boxed residues represent the antigenic sites A–E, and colored residues denote potential glycosylation sites (orange), receptor-binding sites (blue) and cleavage site (red). (For interpretation of the references to color in this figure legend, the reader is referred to the web version of the article.)
3.5. Phylogenetic analysis

Phylogenetic analysis of the eight genes showed that the influenza viruses could be segregated into three distinct lineages, avian strains, human and swine strains, and equine strains. From the eight constructed phylogenetic trees, we can see that the six Jiangsu viruses grouped together with the newly isolated canine H3N2 viruses in dogs and cats from Korea and China. Additionally, all eight genes of the six viruses were most closely related to the Korea feline isolate (Fig. 3).

The HA and NA genes of the canine H3N2 viruses were closely related and clustered in the same clade with the avian H3N2 viruses from Korea, while avian viruses of other countries were in different clades. Furthermore, the two genes of the H3N2 canine strains seemed to be both derived from the A/aquatic bird/Korea/JN-2/2006 (H3N2) strain.

In contrast, the PB2, PB1, NP and M genes of the canine viruses were most closely related to the Chinese influenza viruses of different subtypes. Interestingly, the closest BLAST hit to the NS gene, A/Quail/Nanchang/7-026/00 (H3N6), was not the nearest neighbor but rather those Korean avian strains with lower homology. In the PA gene, the canine viruses were most closely related to A/migratoryduck/Hong Kong/MP2553/03 (H8N4). Similar to the NS gene, some Korean avian strains with lower homology were in the same clades with the canine influenza viruses, while those with higher homology were not. Similar phenomena have been reported by Koski and Golding (2001).

3.6. Experimental infection of mice

Considering that there were sequence divergences between strains 01 and 06, we chose the two strains to infect mice, and compared the results of weight loss and viral loads in tissues. In order to access the pathogenicity difference, we inoculated the mice of two groups with the same dose. A total volume of 50 µl of the virus stock was inoculated per mouse with virus titers of approximately 10^4 EID50. Both groups of mice exhibited clinical signs of infection, including depression, decreased activity and huddling. The two viruses resulted in less than 4% of body weight loss. And the mice infected with 06 strain lost more body weights than 01-infected mice during challenge experiments. The body weights were reduced to a minimum in 06-infected group at 4 d.p.i., one day earlier than 01-infected group (Fig. 4).
In spite of this, we noticed that, the two viruses showed similar trend in virus shedding and tissue distribution. By 2 d.p.i., in mice of two groups, virus had begun to shed from feces (2/3) and replicate in the lung (3/3) and many other organs such as heart (2/3), liver (1/3), intestine (2/3), kidney (1/3 for 01 strain and 2/3 for 06 strain) and spleen (2/3 for 01 strain and 3/3 for 06 strain), except for brain (0/3). By 4 d.p.i., viruses were detected in all tested organs of one mouse inoculated with the 06 strain and virus shedding could also be detected, while mice inoculated with the 01 strain had fewer infected organs than at 2 d.p.i. and had no virus shedding. By 6 d.p.i., both the infected groups had a high proportion of animals (3/3) with detectable viral RNA in all tested organs. By 8 d.p.i., the detection result was similar to that at 6 d.p.i. However, at 16 d.p.i., in mice of two groups, a lower proportion of virus distribution was found than at 8 d.p.i. (Tables 2 and 3), indicating a sign of recover. No viral RNA was detected in any organs of the mice from control group.

3.7. Quantitation of viral RNA loads

Considering that mice inoculated with both strains had widest range of tissue distribution at 6 and 8 d.p.i., we chose the two time points to determine the viral RNA loads in different tissues and fecal samples (Fig. 5). At 6 d.p.i., in mice inoculated with 06 strain, the heart, spleen, lung, kidney, brain and intestine contained similar viral RNA loads, with mean titer of $10^{6.5}$ copies/g, while liver and fecal samples contained lower viral RNA loads, with mean titers of $10^{5.8}$ copies/g and $10^6$ copies/g, respectively. Similar tissue distribution was found in 01-infected mice. Viral RNA titer was a little but not significantly lower in this group than in 06-infected group, with $10^{6.3}$ copies/g in heart, spleen, kidney, brain and intestine, $10^{5.2}$ copies/g in liver, and $10^{5.8}$ copies/g in fecal samples. At 8 d.p.i., while all tissues of 06-infected mice contained higher viral RNA loads than those of 01-infected mice, viral RNA loads in all tissues from two infection groups had significantly dropped compared to 6 d.p.i., indicating that the virus might be gradually cleared from the body.
Table 2
Viral RNA detection in collected tissues and fecal samples of mice inoculated with A/Canine/Jiangsu/01/2009 strain.

<table>
<thead>
<tr>
<th>Organ</th>
<th>2 d.p.i.</th>
<th>4 d.p.i.</th>
<th>6 d.p.i.</th>
<th>8 d.p.i.</th>
<th>16 d.p.i.</th>
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</thead>
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</tr>
</tbody>
</table>

Note: Numbers 1, 2 and 3 represent the three mice euthanized from each infected group at different d.p.i.

Table 3
Viral RNA detection in collected tissues and fecal samples of mice inoculated with A/Canine/Jiangsu/06/2010 strain.

<table>
<thead>
<tr>
<th>Organ</th>
<th>2 d.p.i.</th>
<th>4 d.p.i.</th>
<th>6 d.p.i.</th>
<th>8 d.p.i.</th>
<th>16 d.p.i.</th>
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</table>

Note: Numbers 1, 2 and 3 represent the three mice euthanized from each infected group at different d.p.i.

3.8. Histopathology and immunohistochemistry

We chose the organs from 06-infected mice at 6 d.p.i. to perform histopathological and immunohistochemical analysis. All the sampled organs from infected mice showed significant lesions and viral antigen staining, while those from control group did not.

In the trachea, hyperplasia of submucosal glands with infiltration of inflammatory cells and serous effusion and infiltration of lymphocytes were found in the tracheal lumen (Fig. 6A). Abundant viral antigens were detected in the epithelial cell cytoplasm (Fig. 7A).

In the lung, large areas of serous and fibrous effusion were observed in bronchioli and extensive regions of the alveoli (Fig. 6B). Besides, interstitial pneumonia was also obvious with the alveolar septum thickened by the infiltration of a number of inflammatory cells mainly lymphocytes (Fig. 6B) and proliferation of connective tissue infiltrated with numerous lymphocytes around the bronchioli and blood vessels (Fig. 6C). Bronchiolitis with loss of bronchiolar epithelium in combination with intense lymphocytic infiltration in the bronchiolar lumen was observed (Fig. 6C). Viral antigen stainings were present in almost all bronchiolar epithelial cells and some alveolar cells (Fig. 7B and C).

In the kidney, acute glomerulonephritis was found. Glomerulus was swollen and filled up Bowman’s capsule with endothelium and mesenchymal cell in glomerulus showed proliferation. Epithelia of renal tubes also showed acute swelling. Some renal tubes and glomerulus were even necrotic (Fig. 6D). Viral antigens were present in glomerulus and renal tubes (Fig. 7D).

In the brain, glial nodules and neuronophagia were found. Cytoplasm of neuron was basophilic due to contraction (Fig. 6E). Antigen could be detected in glial nodules (Fig. 7E).

In the liver, granular and vacuolar degeneration was found in the swollen and rounded hepatocytes and the
broaders of cells were indistinct (Fig. 6F). Antigen was evident in hepatocyte cytoplasm (Fig. 7F).

In the heart, myocarditis was found with many lymphocytes infiltrated among the muscle fibers (Fig. 6G), which correlated well with heavy antigen staining (Fig. 7G).

In the spleen, splenic corpuscle was enlarged and lymphocytes in red pulp showed diffuse proliferation (Fig. 6H). Positive staining cells were detectable in red pulp of spleen by immunohistochemistry (Fig. 7H).

In the intestine, the epithelial cells underwent degeneration, necrosis, and desquamation. Necrotic tissue coagulated together with exuding fibrin (Fig. 6I). A large amount of viral antigens were detected in the necrotic epithelium (Fig. 7I).

4. Discussion

Influenza viruses are widespread in nature and have the capacity to cross interspecies barriers and adapt to new hosts by altering antigenic characteristics (Peiris et al., 2007; Webster et al., 1992). The viral HA protein is a major contributor to host range, as it is responsible for receptor-binding (Ito, 2000). Avian influenza A viruses are generally thought to preferentially bind to sialic acid α-2,3-galactose (SAα-2,3 gal) receptor, whereas human or swine influenza A viruses preferentially bind to sialic acid α-2,6-galactose (SAα-2,6 gal) receptor (Rogers and Paulson, 1983; Rogers et al., 1983). Pigs have always been considered a reservoir for these viruses (Kida et al., 1994; Kundin, 1970), because they possess both SAα-2,3 gal and SAα-2,6 gal receptors (Ito et al., 1998). Dogs may also have the potential to be infected with various influenza viruses, since they possess the two receptors on their tracheal epithelial cells as well (Chang et al., 1976; Kilbourne and Kehoe, 1976; Todd and Cohen, 1968). However, before 2004, dogs were not considered a reservoir species, because they did not appear to maintain their own influenza virus subtype, and no sustained transmission of any influenza virus between dogs had ever
been recorded (Gibbs and Anderson, 2010). Now, the frequent outbreaks of canine influenza occurring in many countries raise further speculation that, similar to the pig, infection of the dog can result in reassortant viruses and generate novel influenza strains infectious to humans.

Of the obvious common mutations in the HA1 genes of nine H3N2 isolates from canine or feline when compared against avian H3N2, five residues were of significance. Three mutations occurred at three different antigenic sites, while one mutation was found at the receptor-binding site. It was proposed that a drift variant with ≥4 amino acid changes at ≥2 out of 5 antigenic sites would be of epidemiologic importance (Wilson and Cox, 1990). It has been also observed that new antigenic variations are created either when ≥2 variations occur at antigenic sites, or when one variation occurs at an antigenic site and one at a sialic acid receptor-binding site (Li et al., 2007). The mutation W222L in receptor-binding site also occurred in the H3N8 CIV which originated from an equine strain. We looked into a lot of H3 subtype avian and equine influenza viruses, and found that 222W was quite conserved in these viruses. So the suspicion arises that this W222L mutation at receptor-binding site might be critical for the transmission of avian or equine influenza viruses to the new host species, dogs. Moreover, two observed mutations changed potential glycosylation sites, which could have a significant impact on the antigenicity and regulation of receptor-binding affinity, thereby affecting the generation of new viruses (Schulze, 1997). Previous studies have demonstrated that the glycosylation of HA together with the stalk-length of NA regulate the growth of avian influenza virus (Baigent and McCauley, 2001; Klenk et al., 2000; Mitnau et al., 2000).

Significantly, the K326R mutation was located at the cleavage site in all canine or feline isolates except the Guangdong canine isolate. An amino acid change I328T at the cleavage site also occurred in the H3N8 CIV. The amino acid sequence motif at the cleavage site was PEKQIR\textsubscript{G} for equine H3 HA, and PEKQTR\textsubscript{G} for the avian, swine and human H3 HA. Interestingly, the PEKQIR\textsubscript{G} sequence changed to PEKQTR\textsubscript{G} in the H3N8 CIV and now to PERQTR\textsubscript{G} in the H3N2 CIV. Cleavage of the HA precursor molecule HA0 is required to activate virus infectivity. The cleavage properties of HA0 and the distribution of infectivity-activating proteases in the host are major factors for virus tropism and capacity for systemic spread, and for avian viruses, this has been recognized as the most important determinant for pathogenicity (Steinhauer,
The I328T mutation in H3N8 CIV suggests that the TR1G site is suitable for HA0 cleavage by canine proteases since it is also present in H3N2 CIV. Now that most H3N2 CIV contain the amino acid change K326R may also indicate that the PERQTR1G motif is beneficial, although not necessary, for HA0 cleavage by canine proteases, since the Guangdong canine strains can also infect dogs without this mutation.

The NA of influenza viruses cleaves sialic acids from receptors, prevents self-aggregation and facilitates release of virus during budding from host cells (Palese et al., 1974). The length of NA stalk has been correlated with the ability to elute virus in binding studies (Baigent and McCauley, 2001). In our study, a two amino acid insertion in the NA stalk was unique to the Jiangsu isolates compared with the closest avian strain and related canine and feline strains. As previous reports (Di Trani et al., 2004; Li et al., 2010a, 2011) have shown that stalk deletions are commonly found in avian influenza viruses, and this insertion may suggest an evolutionary adaptation of avian influenza virus to mammals. Generally, the efficiency of virus replication in eggs is correlated closely with NA stalk length: the longer the stalk, the better the replication (Castrucci and Kawaoka, 1993). However, many studies have revealed that mutations will occur in the HA gene to compensate for reduced NA function due to stalk deletions (Baigent and McCauley, 2001; Klenk et al., 2000; Li et al., 2011; Mitalal et al., 2000). Few studies have addressed whether mutations in HA are induced when insertions occur in the NA stalk. We analyzed the HA genes of the six CIV isolates from Jiangsu, but failed to find any common mutations associated with the unique insertion.

All of the above mutations might potentially contribute to the direct transmission of H3N2 AIV to a new host species. The future use of the reverse genetics system and site directed mutagenesis could be helpful in deciphering the role of the amino acid(s) residues in interspecies transmission of viruses. Generally, mutations that allow infection of a new host may emerge from two pathways (Kuiken et al., 2006), either within pre-existing viruses transmitted from the donor species or evolved progressively in a reservoir species until multiple adaptive mutations have been acquired. The former route is a more direct pathway for successful viral emergence, while the latter necessarily requires a certain level of ongoing transmission. Our homology and phylogenetic analysis suggested that the closely related CIV in China and Korea may have originated from the same avian H3N2 virus. Since the first outbreak of H3N2 canine influenza occurred in Korea, and the HA and NA genes of the H3N2 CIV were quite prevalent in Korea poultry, we surmised that the Chinese H3N2 CIV may have been derived from Korea through frequent international travel and trade between the two countries, or by migratory wild birds. If the virus was spread from Korean birds to Chinese dogs, it is more likely that the mutations emerged through the former pathway since there were many common mutations observed in both Chinese and Korea canine influenza viruses. However, if the virus was spread from Korean dogs or cats to Chinese dogs, it is possible that mutations occurred by means of one or two of both pathways (Fig. 8). Homology analysis showed that the Jiangsu canine isolates were most similar to the feline isolate in Korea, whereas the Guangdong isolates were most similar to the canine isolates in Korea. This difference indicated that the canine influenza viruses in Guangdong and Jiangsu may have been separately derived from Korea (Fig. 8). Certainly, there is also another possibility that the Chinese dogs might be infected by being fed the affected bird meat or contacting with the affected cats. But aside from the lack of any epidemiological evidence in support of it, the H3N2 CIV isolates from cats have not been reported in China.

Mice are widely believed to be the ideal small animal model for some pathogenesis studies on influenza virus (Conenello et al., 2007; Kawaoka, 1991), including CIV (Castleman et al., 2010). In this study, the H3N2 CIV infection of mice caused slight body weight loss during challenge experiments, which is similar to what Castleman et al. (2010) found in the mice exposed to H3N8 CIV. The H3N8 CIV-inoculated mice developed tracheitis, bronchitis and bronchiolitis, but histological observations on other
organs were not recorded. Previous studies (Ilyushina et al., 2010; Narasaraju et al., 2009) showed that human influenza virus initially caused lesions just in the lung upon challenge in mice, and its adaptation to extra-pulmonary organs was established only after serial in vivo passages. However, the avian-origin H3N2 CIV isolates from Jiangsu acquired broad tissue tropism in mice without prior adaptation. Although avian and human influenza viruses preferentially bind to SAα-2,3 gal and SAα-2,6 gal receptors, respectively, Ning et al. (2009) found that the two receptors were both expressed on trachea, lung, cerebellum, spleen, liver and kidney. It suggested that there may be factors other than expression pattern of receptors distribution that contribute to tissue tropism of influenza viruses. Ibricicic et al. (2006) reported that the mouse respiratory tract has a predominance of SAα-2,3 gal versus SAα-2,6 gal receptors, and Ilyushina et al. (2010) proved that all mouse-adapted human influenza viruses showed significantly reduced binding to the “human-type” SAα-2,6 gal with enhancing binding to SAα-2,3 gal receptors. It is uncertain whether receptors density might affect the tissue tropism of influenza viruses in mice. More work is needed in this area.

The 01 and 06 viral strains showed similar trends in the infection process such as a wide range of tissue distribution and a replication pattern that reached peak levels at 6 d.p.i. But the findings that the mice inoculated with 06 strain lost more body weight and had higher viral RNA loads in tissues at 6 and 8 d.p.i. than mice inoculated with 01 strain may suggest that 06 strain had a higher virulence in mice than 01 strain. This agrees with the result of EID50 titer, in which 06 strain has a higher virus titer of EID50 than 01 strain. The sequence analysis showed that among the six CIV isolates, 06 strain was somewhat unique. Firstly, it was the only one that differed in the M, NP and NA genes from the other five strains, and it has the mutation F103L in NS1 which had been reported increasing virus replication and virulence. Moreover, in the phylogenetic analysis of all eight genes, the 06 strain was more distantly related to the other five strains.

Our study demonstrated that, the H3N2 CIV isolates from Jiangsu could replicate in intestine and be shed in feces. But in the previous studies (Lee et al., 2011; Song et al., 2009), Koren H3N2 CIV-infected beagles shed virus via nasal discharge, and no virus was found in rectal swabs. This difference may be due to viral strain. Because dogs will usually sniff where another dog has eliminated, viral particles shed by infected dogs in feces become a source of transmission.

The six viruses in our study were isolated from 13 nasal swabs of dogs which exhibited clinical signs of respiratory disease from the same one animal clinic over the course of nine months, indicating that the CIV may now be a common pathogen for domesticated dogs in China. Several new mutations had accumulated in the Jiangsu isolates, and these variations may have facilitated adaptation of the avian influenza virus to mammals. It is unclear whether the newly emerging influenza virus will cause a pandemic among canine or feline populations in China. Influenza viruses of the H3 subtype have proven to be highly adaptable and are able to infect avian and mammalian hosts including humans. Since dogs are in regular contact with their owners and other people, it is a concern that the illness could potentially spread from dogs to humans in the future. All these findings demand that we develop better surveillance and control strategies for emerging influenza diseases in companion animals.

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References


