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Identification and Genetic Evolution Analysis of One Strain of H3N2 Canine Influenza Virus Isolated from Nanjing, China

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Authors' Contribution
DHK, SL, MSK, FP and YL conceived and designed the experiments. GHK and SL performed the experiments. DHK, SL, NR, MN and YJL analyzed the data. DHK, SL, SAP and YL wrote the paper.

Key words
Canine influenza viruses, Genetic evolution, H3N2 subtype, Isolation and identification.

ABSTRACT

Nasopharyngeal swabs were collected from 6 dogs with severe respiratory syndrome at the Animal Clinics of Nanjing Agricultural University in 2014. One viral strain was isolated from SPF embryonated chicken eggs. Sequencing analysis of hemagglutinin (HA) and neuraminidase (NA) genes showed 93 to 99% similarity between each other. The CIV isolates shared high similarity (above 98%) to the H3N2 viruses from dogs in China. On the comparison of gene sequence four unique mutations were found in the amino acid of HA (A144T, R158K, D291N, L383F) and NA (T19A, V33L, V82A, S336N). The HA and NA genetic evolution analysis revealed that one isolate was most similar to the newly isolated H3N2 viruses from dogs in China and had the same evolutionary branching. The result provided a foundation for further studies on biological characteristics of CIV.

INTRODUCTION

Influenza A virus, which belongs to family *Orthomyxovirus*, is an eight segmented, enveloped, negative stranded RNA genome encoding 12 viral proteins: HA, NA, NP, M1, M2, NS1, NS2, PA, PB2, PB1, PB1-F2 and PB1-N40 (Wise *et al*., 2009). HA and NA glycoproteins on the virion determines the subtype of the virus; they can vary due to antigenic drift and antigenic shift (Shu *et al*., 1994). Influenza viruses are capable of crossing interspecies barriers, altering antigenic characteristics for adaptation to new hosts (Webster *et al*., 1992). In January 2004 canine influenza virus (CIV) subtype H3N8 of equine origin was first isolated from racing greyhounds in USA (Crawford *et al*., 2005) and then in the same year the virus was isolated from shelter and pet dogs (Payungporn *et al*., 2008). Avian origin H3N2 CIV epidemics were observed in South Korea and China (An *et al*., 2010; Lee *et al*., 2009). H3N2 CIV was isolated from Korea in 2007 (Song *et al*., 2008), then in 2009, H3N2 CIV was demonstrated to be capable of transmitting directly from dog to dog (Song *et al*., 2009). Interspecies transmission of H3N2 CIV to cats was firstly reported in Korea in 2010 (Song *et al*., 2011). The first case of H3N2 CIV in China was reported in Guangdong Province, and most of outbreaks of CIV in pet dogs were reported from Animal Clinics, so avian-origin H3N2 CIV might be circulating in the pet dogs in southern China (Lin *et al*., 2012; Qi *et al*., 2011). Avian-origin H3N2 CIV [A/canine/ Guangdong/2/2011 (H3N2)] was firstly isolated from a farm dog with outbreak of severe respiratory diseases in Guangdong Province, southern China in 2011 (Su *et al*., 2012). CIV H3N2 in dogs caused severe respiratory syndrome including high fever, coughing and severe lesions in lung.

Influenza A virus infection in domestic animals has been studied extensively, but very little is known in companion animals. Companion animals such as dogs and cats are in close contact with humans and domestic animals, so research on influenza A virus infection in companion animals is important for veterinary and public health (Said *et al*., 2011). During the last decade influenza A virus infections from dogs has been reported, which, is an alarming situation for veterinary practitioners, virologists, and epidemiologists. Many new strains of H3N2 CIV of avian origin have emerged in China due to ecological changes over the last 20 years. Number of dogs has been increased and kept as companions and for food purpose, particularly in densely populated areas (Li *et al*., 2010). In the present study, we isolated H3N2 influenza of avian origin virus from dogs in Nanjing, China and

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isolates were genetically characterized and compared with previous H3N2 CIVs.

MATERIALS AND METHODS

Collection of samples

During February to March 2014, a total of six nasopharyngeal swabs of dogs suffering from respiratory diseases were collected from Animal clinics of Nanjing Agricultural University in Jiangsu province of China in a transport medium containing streptomycin.

Specific pathogen free eggs (SPF)

SPF chicken embryos were purchased from Nanjing, China Animal Husbandry Industry Co., Ltd. Equipment plant. Approval for conducting the experiments was obtained from the Animal Ethics Committee of Nanjing Agricultural University.

Inoculation of samples

The collected samples were inoculated in allantoic cavity of ten days old embryo. Alcohol 70% with a cotton wool was used to wash ends of the eggs and allowed for few minutes to evaporate. A hole was made by 1ml syringe at the marked inoculation site of the egg. Needle was penetrated approximately 16mm into the egg to reach the allantoic cavity. Inoculum 0.2 ml of was injected into the egg and the needle was withdrawn from the egg. After inoculum hole in the shell was sealed with melted wax, inoculated eggs were placed in the incubator. Those embryos which died within 24 h post inoculation were discarded. Mortality between 2 and 4 days post inoculation was considered to be virus specific

Harvesting of allantoic fluids

The eggs were chilled at 4°C for at least two hours to kill the embryo and to reduce the contamination of the allantoic fluid with blood during harvesting. Egg shells were cleaned with 70% alcohol cotton and wax was removed. The eggshell above the air space was removed with sterilized forceps and scissors. A sample of allantoic fluid was removed from each egg with the help of micropipette and sterile tip. Haemagglutination test was done to for the presence of CIV. HA negative embryos for CIV were discarded. Allontoic fluid was harvested by using sterile glass Pasteur pipettes, collected into sterile containers and stored at -80°C for further experiments. This step was repeated 2 times.

Viral RNA extraction

Viral RNA was extracted from allantoic fluids by Virus Nucleic Acid Extraction Kit II (Geneaid, Taiwan) according to the manufacturer's instructions.

Hemagglutination assay

Hemagglutination (HA) test is used to harvest allantoic fluid from embryonating chicken eggs for hemagglutinating agents, such as type A influenza (Killian, 2008).

Reverse transcription PCR

Reverse transcription was done by using influenza virus oligonucleotide universal primer (AGCAAAAGCAGG) (Lin *et al*., 2012). Primers for the detection of viral genes haemagglutination (HA), neuraminidase (NA) and matrix protein (M) were designed by using the Primer 3 program (Table I). PCR products were analyzed by electrophoresis in 1% agarose gel containing ethidium bromide. PCR products were purified by Agarose Gel DNA Purification Kit (TaKaRa, Dalian) and cloned into the pMD18-T vector (TaKaRa, Dalian). Positive clones were selected and sequenced.

Table I.- The sequence of the primers.

S. No	Gene	Primer
1	M	FP: 5-AGCCAGCTCTTCAGCCAGCAAAAGCAGGTAGATAT-3 RP: 5-GACCCGCTCTTCGATTAGTAGAAACAAGGTAG-3
2	HA	FP: 5-AGTTCGCTCTTCAGCCAGCAAAAGCAGGGGATA-3 RP: 5-GACCCGCTCTTCGATTAGTAGAAACAAGGGTG-3
3	NA	FP: 5 -AGTTCGCTCTTCAGCCAGCAAAAGCAGGAG-3 RP: 5-GACCCGCTCTTCGATTAGTAGAAACAAGGAGT-3

PCR for M gene was performed by reaction initiation at 94 °C for 5 min; amplification for 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 60 s and final extension at 72 °C for 10 min. PCR for HA gene was performed by reaction initiation at 94 °C for 5 min; amplification for 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 90 s and final extension at 72 °C for 10 min. PCR for NA gene was performed by reaction initiation at 94 °C for 5 min; amplification for 30 cycles at 94 °C for 30 s, 56 °C for 30 s, 72 °C for 100 s and final extension at 72 °C for 10 min.

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 MEGA5 software by the neighbor-joining method. Bootstrap values were calculated based on 1,000 replicates of the alignment.

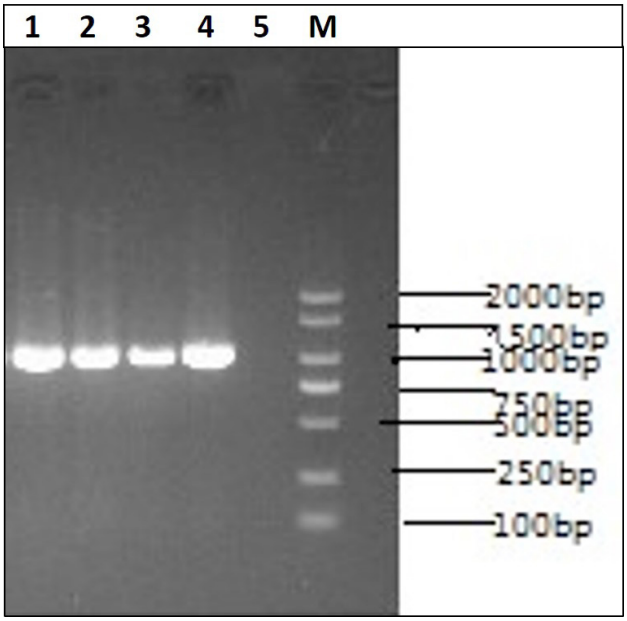

Fig. 1. Reverse transcription PCR for the amplification of M gene. Lane M, DNA marker; Lanes 1 to 4, M gene; Lane 5, negative control.

RESULTS

Isolation and identification of viruses

All the samples have hemagglutination activity with titer of 64 on 1% chicken red blood cells. Reverse transcription PCR was performed to detect M gene (Fig. 1), HA and NA gene of influenza virus (Fig. 2).

Molecular sequence homology of HA gene

HA gene sequence of Nanjing isolates [A / canine / Nanjing / 01/2014 (H3N2)] was compared with other strains and have similarity of 95%-99%. Other strains include one strain of Nanjing isolate [A / canine / Nanjing / 11 / 2012 (H3N2)], five strains of Guangdong isolates [A / canine / Guangdong / 01/2011 (H3N2), A / canine / Guangdong / 05 / 2011 (H3N2), A / canine/ Guangdong / 12/2012 (H3N2), A / canine / Guangdong / 23/2012 (H3N2) and A / canine /Guangdong / 1/2007 (H3N2)], four strains of Jiangsu Province [A / canine / Jiangsu / 03/2010 (H3N2), A / canine / Jiangsu / 04/2010 (H3N2), A / canine / Jiangsu / 05 / 2010 (H3N2) and A / canine /Jiangsu / 06/2010 nucleotide sequence of the highest (H3N2)] and one strain of Korean isolates [A / feline / Korea / 01/2010 (H3N2)], having 98%-99% similarity with the Nanjing isolate [A / canine / Nanjing / 01 / 2014 (H3N2)]. Besides, HA gene homology was 97% similarity with the H3N2 subtype avian influenza virus [A / duck / Korea / JS53 / 2004 (H3N2)], 95% similarity with the avian H3N8 and H3N6 subtypes [A / duck / Korea / U8-1 / 2007 (H3N8), A / duck / Korea / JJ72 / 2007 (H3N6)].

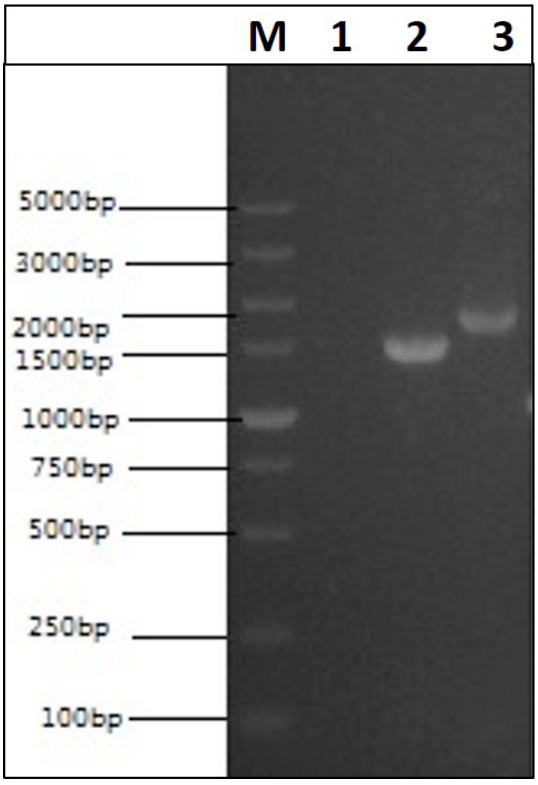

Fig. 2. Reverse transcription PCR for the amplification of HA and NA gene of influenza virus. Lane M, DNA marker; Lane 1, negative control; Lane 2, HA gene; Lane 3: NA gene.

Phylogenetic analysis

CIV HA gene from our study and the ones isolated from Guangdong, Jiangsu and Nanjing in 2006 to 2012 are from same cluster and most closely related to Guangdong strain. Avian influenza virus subtype H3N2 also has close genetic relationship to our isolate. While avian H3N8, H3N6 subtype strains and H3N2 of feline origin are not closely related to our isolate (Fig. 3). Therefore, virus isolated from dogs belongs to CIV H3 subtype.

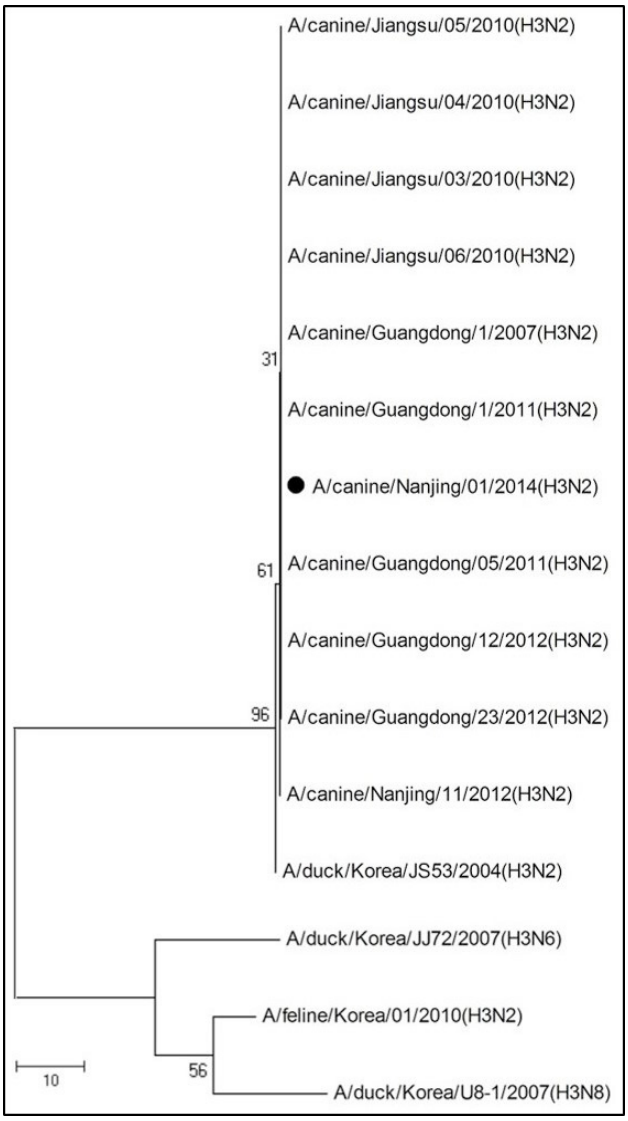

Fig. 3. Phylogenetic trees for the A/canine/Nanjing/01/2014 (H3N2) HA gene.

Molecular sequence homology of NA gene

NA gene sequence of Nanjing isolate [A / canine / Nanjing / 01/2014 (H3N2)] was compared with other strains and have similarity of 93%-99%. Strains include one strain of Nanjing isolate [A / canine / Nanjing / 11 / 2012 (H3N2)], three strains of Guangdong isolates [A / canine / Guangdong / 05/2011 (H3N2), A / canine / Guangdong / 12 / 2012 (H3N2), A / canine/ Guangdong / 23/2012 (H3N2)], four strains of Jiangsu Province [A / canine / Jiangsu / 01/2010 (H3N2), A / canine / Jiangsu / 02/2010 (H3N2), A / canine / Jiangsu / 04 / 2010 (H3N2), A / canine / Jiangsu / 06/2010 nucleotides (H3N2)] and one strain of Korean isolates [A / feline / Korea / 01/2010 (H3N2)], having 98%-99% similarity with the Nanjing isolate [A / canine / Nanjing / 01/2014 (H3N2)]. Besides, NA gene homology was 96% similarity with the H3N2 subtype avian influenza virus [A / duck / Korea / JS53 / 2004 (H3N2)], 93%-94% similarity with the H9N2 and H6N2 avian subtypes CIV [A / duck / Hong Kong / Y439 / 1997 (H9N2) and A / duck / Hokkaido / 120/2001 (H6N2)].

Phylogenetic analysis

CIV NA genes from our study and the ones isolated from Guangdong, Jiangsu, and Nanjing in 2010 to 2012 are from the same cluster while Jiangsu strain was most closely genetic relationship strains. Avian H9N2, H6N2, H3N2 subtypes and H3N2 of feline origin are not closely related to our isolate (Fig. 4). Therefore, virus isolated from dogs belongs to CIV N2 subtype.

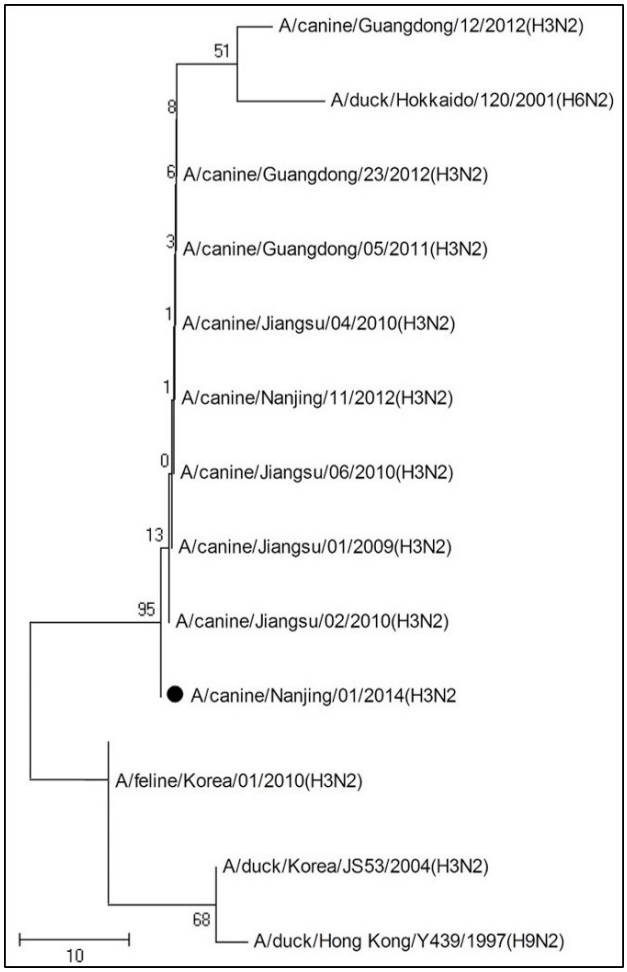

Fig. 4. Phylogenetic tree for the A/canine/Nanjing/01/2014 (H3N2) NA gene and those of other influenza A viruses. The trees were generated with the MEGA program (version 5.0) by using neighbor-joining analysis.

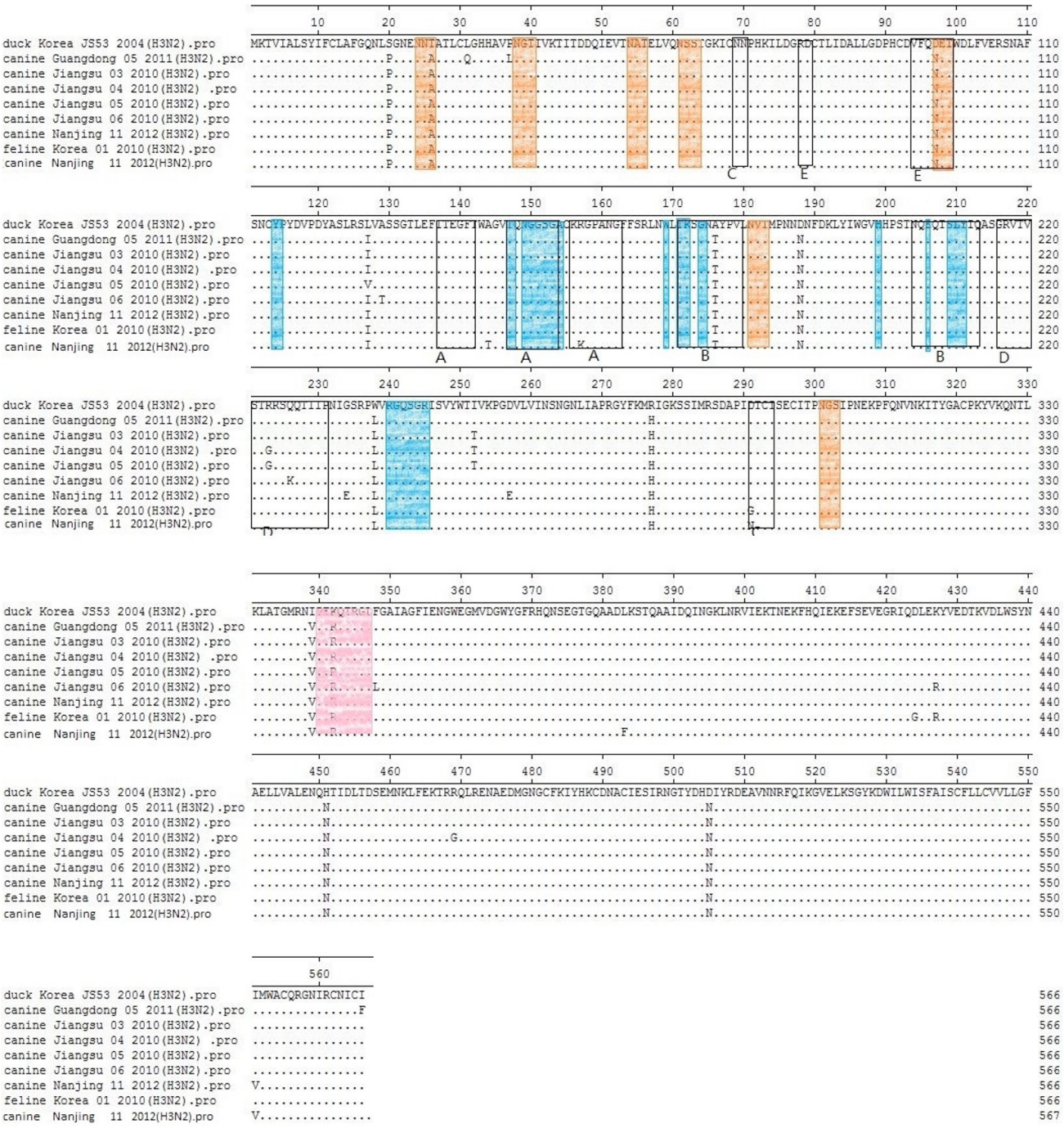

Fig. 5. Alignment of HA1 amino acid sequences of H3N2 influenza isolates and the most similar avian isolate. Boxed residues represent the antigenic sites A–E, and orange colored residues denote potential glycosylation sites, blue indicates receptor-binding sites and pink represent cleavage site.

Variability analysis of gene coding regions

HA gene coding region

HA gene of the Nanjing isolates, strains isolated from cats in Korea and strains isolated from dogs in Guangdong, Jiangsu, and Nanjing were compared with the viral strain A/duck/Korea/JS53/2004 (H3N2) having highest similarity with them. Common mutation sites in all were S20P, T26A, D97N, L127I, A176T, D188N, W238L,

R277H, I339V, F342R, H451N, D505N while T26A and D97N resulted in a change of potential glycosylation sites, F342R occured in restriction sites. D97N and A176T occurred in two distinct antigenic sites. It is worth mentioning that Nanjing isolates have unique mutations: A144T, R158K, D291N, L383F and R158K and D291N occurred in the antigenic site area. The proposed antigenic sites, receptor-binding sites, potential glycosylation sites and the cleavage sites were analyzed (Fig. 5).

NA gene coding region

NA gene of the Nanjing isolates, strains isolated from cats in Korea and strains isolated from dogs in Guangdong, Jiangsu, and Nanjing were compared with the viral strain A/duck/Korea/JS53/2004 (H3N2) having highest similarity with them. Common mutation sites were M24L, H36Y, E54K, P81S, D143N, P156S, I208V, R222Q, D288N, S372L, L392S, R432G while S372L, L392S, R432G occured in three different antigenic sites. In addition our isolate and previous NA Jiangsu isolates have two amino acid insertions at the end .Unique mutations were T19A, V33L, V82A, and S336N. S336N occurred at antigenic site. The proposed antigenic sites, receptor-binding sites, potential glycosylation sites and the cleavage sites were analyzed (Fig. 6).

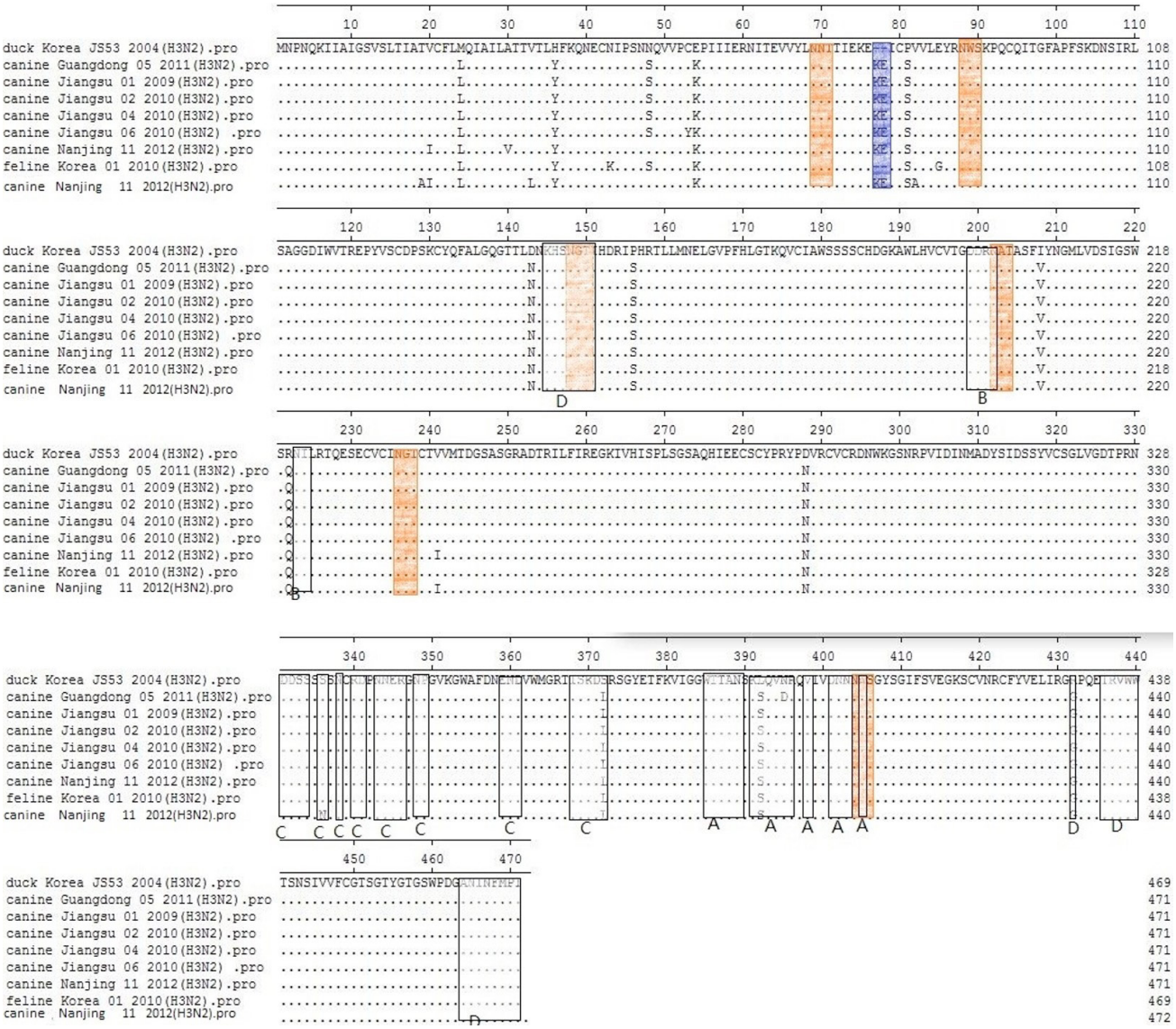

Fig. 6. Alignment of NA amino acid sequences of H3N2 influenza isolates and the most similar avian isolate. Black boxed residues represent antigenic sites A-E, and orange colored residues denote potential glycosylation sites, blue indicates receptor-binding sites.

DISCUSSION

Influenza virus A interacts with their hosts mostly through two critical glycoproteins, hemagglutinin (HA-16) and neuraminidase (NA-9) subtypes. HA is responsible for receptor binding and NA is responsible for cleaving sialic acids from receptors and work as facilitator to release virus during budding from host cells (Ito, 2000). Dogs possess two receptors on their tracheal epithelial cells so they can be infected by various viruses glycosylation of HA and stalk-length of NA regulate the growth of avian influenza virus (Baigent and McCauley, 2001; Mitnaul *et al*., 2000), mutations in the HA gene mostly occurs to compensate for reduced NA function due to stalk deletions (Baigent and McCauley, 2001; Li *et al*., 2011). Antigenic variations occured either when one variation occurs at a sialic acid receptor-binding site and another at an antigenic site or ≥2 variations occur at antigenic sites (Li *et al*., 2011; Shih *et al*., 2007). During present study phylogenetic analysis of HA and NA gene were done to compare the sequences of other influenza viruses obtained from the GenBank database. In the present study HA and NA genes of CIV H3N2 were phylogenetically close to the avian H3N2 viruses from Guangdong, Jiangsu and Nanjing China and Korean isolate from felines. Our results are in agreement with other authors who observed that the A/canine/Guangdong/3/2011 (H3N2) was most closely related to newly isolated H3N2 CIVs in dogs and cats from Korea and China (Jeoung *et al*., 2013; Song *et al*., 2011; Su *et al*., 2013). Sequence analysis revealed two unique amino acid insertion in the NA stalk in the Jiangsu isolate (H3N2) isolates when compared with the avian strain ,canine and feline strains (Lin *et al*., 2012). CIV H3N2 was isolated from a cat and showed 2% divergence in its nucleotide and amino acid sequences (Song *et al*., 2011). The neuraminidase of influenza viruses is responsible for the cleaving sialic acids from receptors, thus preventing self-aggregation and enabling the release of virus during budding from host cells (Palese *et al*., 1974). NA stalk length has been correlated with the ability to elute virus in binding studies (Baigent and McCauley, 2001). Several mutations were identified during present study. Gene sequence analysis revealed four amino acid mutations were observed in HA (A144T, R158K, D291N, L383F) and NA (T19A, V33L, V82A, S336N). Seven amino acid changes differentiated the avian and canine H3 consensus amino acid sequences: T10A, D81N, L111I, A160T, D172N, H435N and D489N while D81N and A160T were found in the antigenic sites however, none were located in the receptor binding sites (Sun *et al*., 2013). Six mutations in HA1 T10A, D81N, L111I/V, A160T, D172N and W222L were found in HA protein from four isolates in southern china (Li *et al*., 2010).

CONCLUSION

In conclusion one strain of canine influenza virus H3N2 was isolated from dogs and phylogenetic analysis showed the CIV isolate was most similar to the newly isolated H3N2 viruses from dogs in China.

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Statement of conflict of interest

Authors have declared no conflict of interest.

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