Genetic characterization of a novel group of H3N2 canine influenza viruses isolated from Guangdong Province in southern China in 2018

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Abstract

Canine influenza virus (CIV) is an emerging pathogen that can infect canines, causing a series of respiratory symptoms. H3N2 CIV emerged in dogs in China and Korea in approximately 2005 but was first reported in 2007. In 2015, H3N2 CIV was detected in the USA, where it caused a large outbreak. For continuous monitoring of H3N2 CIV in China, a total of 180 dog nasal swabs were collected from veterinary hospitals in southern China between 2017 and 2018 and tested for CIV RNA. Three H3N2 CIV strains were isolated. Following genome sequencing, sequences of the isolates were found to be divergent from the sequences of reported Chinese H3N2 CIV strains. Phylogenetic analyses indicated that these viruses are clustered in a novel group and genetically close to strains from the USA. Several unique aa substitutions in HA and NA were observed in the H3N2 CIV strains isolated in this study. These findings reveal unique evolutionary characteristics of recently identified H3N2 CIV strains in China.

Keywords: H3N2 CIV; Genetic characterization;

Introduction

Although a few epidemiological and artificial infection studies in the 1970s indicated that dogs can be infected with influenza A virus (IAV), the IAV strain was not isolated from dogs until 2004(Payungporn*et al.* , 2008;Song *et al.* , 2008). This virus, now known as canine influenza virus (CIV), is responsible for canine influenza (CI), a respiratory disease in canines with the clinical features of cough, sneeze, fever, and nasal discharge. CIV was classified as the H3N8 and H3N2 subtypes, which were derived from interspecies transmission of equine and avian influenza virus, respectively(Sun*et al.* , 2017;Crawford *et al.* , 2005). H3N8 CIV was first documented in the USA in 2004 and later determined in the UK and Australia. H3N2 CIV was first reported in Korea in 2007. Subsequently, we also reported H3N2 CIV in China, indicating that this virus was circulating in Chinese canine population in 2006(Payungporn *et al.* , 2008;Lee *et al.* , 2010). In 2015, H3N2 CIV emerged in the USA, causing a large outbreak. Sequencing results and further analysis indicated that the virus might have been introduced from Korea(Voorhees*et al.* , 2018;Payungporn *et al.* , 2008).

In China, H3N2 CIV was detected in different canine populations, including pet dogs, farmed dogs, stray dogs, and even Tibetan mastiffs, which have a broad geographical distribution from southern to northern China(Chen *et al.* , 2018;Li *et al.* , 2018). The RNA polymerase of IAV lacks proofreading ability, and IAV continually mutates and evolves, which was also observed in CIV. However, since 2014, no studies on the isolation and genetic sequencing of H3N2 CIV in China have been published, and no related genetic

information is available. In this study, we isolated emerging H3N2 CIV strains from sick dogs in China in 2018 and provide their genetic information.

Materials and methods

From January to May 2018, a total of 80 nasal swabs from sick dogs with respiratory disease were collected in veterinary hospitals in Guangdong Province in southern China. The presence of CIV RNA/antigen was detected using a commercial RT-qPCR kit (Anheal, China) or an immunochromatographic strip kit (RapiGEN, Korea). The positive nasal swab samples were processed for virus isolation in SPF eggs as reported in a previous study. A hemagglutination (HA) test was performed to determine the viral titer.

Total RNA was extracted from the viral stocks using TRIzol (Takara, Japan) following the manufacturer's instructions and then reverse transcribed into cDNA using the Uni12 primer (AGCAAAGCAGG). The viral genome was obtained by PCR using universal primers targeting IAV. DNA fragments with the expected size by 1% agarose gel electrophoresis were purified, cloned into pCloneEZ-blunt (CloneSmarter, USA) and sequenced (BGI, China).

The raw sequencing data were assembled, processed using SeqMan 7.1.0 and then aligned with other H3N2 CIV strains from China, the USA, and Korea using BioEdit 5.0.7.0. Their genomic sequences in eight segments were compared at the nucleotide (nt) and amino acid (aa) level. A phylogenetic tree was established with MEGA 6.0 using the neighbor-joining method based on the bootstrap values of 1,000 replicates.

Results and discussion

To sequence the genomes of CIV strains from southern China, 180 nasal swab samples were collected from veterinary hospitals. Among the tested nasal swabs collected from sick dogs with respiratory disease, 3 samples (3.75%) were positive for CIV RNA/antigen. After 3 passages in SPF eggs, allantoic fluids collected from eggs incubated with the positive samples tested positive in the HA test, with a titer of 2^7. After sequencing, three H3N2 subtype CIV strains (A/canine/China/Guangdong/1/2018, A/canine/China/Guangdong/2/2018, and A/canine/China/Guangdong/3/2018) were finally determined (Table 1). Their genomic sequences in eight segments have been deposited in the GenBank database (Nos. MK119981 - MK120004).

To further characterize the genome of H3N2 CIV in China, the nt and aa similarities of the genome sequences of H3N2 CIV strains in eight segments were calculated (Tables 2). The HA and NA genes of the H3N2 CIV isolates in this study showed genetic similarities of 99.4%-99.8% and 99.6%-99.9% among each other, respectively, and 96.1%-99.5% and 95.2%-99.8% with previously reported H3N2 CIV strains, respectively. HA and NA of the H3N2 CIV isolates in this study showed aa similarities of 96.1%-99.5% and 95.2%-99.8% among each other, respectively, and 95.7%-99.5% and 94.3%-99.8% with previously reported H3N2 CIV strains, respectively. In addition, all eight segments of these three field strains were more genetically similar to the recently determined strains from the USA than to Asian strains. All internal genes of the isolates in this study were most genetically similar to the H3N2 CIV strain A/canine/Georgia/89750.1/2017 from the USA.

The HA and NA aa sequences of the Chinese and USA isolates were aligned and compared (Table 3). The results indicated that the HA sequences of A/canine/China/Guangdong/1/2018, A/canine/China/Guangdong/2/2018, and A/canine/China/Guangdong/3/2018 contained five (V128I, N187S, A289S, K328S, N481S), four (V128I, N187S, A289S, K328S), and two (V128I, K328S) aa substitutions, respectively. In the analysis of the antigenic epitope sites, we found two (G162S, N204D) aa substitutions in the HA1 of H3N2 CIV isolates in this study which are same as USA isolates and different from other Chinese isolates(Nakajima *et al.* , 2003;Caton*et al.* , 1982).

When the NA aa sequences were aligned and compared, A/canine/China/Guangdong/1/2018, A/canine/China/Guangdong/2/2018, and A/canine/China/Guangdong/3/2018 contained two (V/I20A, N43K), two (V/I20A, N43K), and three (V/I20A, A/R30T, N43K) aa substitutions, respectively. In the analysis of the NA stalk region, we found that two (V50I, Y60H) aa substitutions in the NA of H3N2 CIV isolates in this study which are same as USA and Korea isolates and different from other Chinese isolates.

And A/canine/China/Guangdong/1/2018 and A/canine/China/Guangdong/2/2018 have an(N43K) aa substitutions in NA stalk region. Furthermore, GD isolates in this study have three(G147S, R338K, E357D) aa substitutions in the NA antigenic epitope sites which are same as USA isolates and different from other Chinese isolates(Lin*et al.* , 2012;Bunpapong *et al.* , 2014;Sun *et al.* , 2013).

To understand the genetic relationship between the three field isolates in this study and other CIV strains, we conducted phylogenetic analyses based on the HA and NA genes (Figure 1). The HA and NA genes of H3N2 CIV were clustered into three groups (groups A-C). The three groups included H3N2 CIV from China, Korea, and the USA. The strains in the present study were clustered in a new group (group D) and had were most closely related with the strains from the USA (group C) but were distant from the previously reported H3N2 CIV strain in China (group A).

Notably, all the H3N2 CIV strains in China before 2018 were located in group A. The CIV strains in group C from the USA were determined from 2015-2017. However, the molecular characteristics of the Chinese CIV strains circulating during the same period are unclear (the genomic sequences of only two strains were available online.). Accordingly, the origin of the CIV strains in group D remains unclear. Group D may be the result of continuous evolution of the field CIV strains in China. Alternatively, group D may be from the transboundary transmission of group C strains from the USA to China. A retrospective epidemiological investigation may help determine the true origin of group D.

In conclusion, in this study, we isolated and sequenced three emerging H3N2 CIV strains from China (A/canine/China/Guangdong/1/2018, A/canine/China/Guangdong/2/2018 and A/canine/China/Guangdong/3/2018), which are divergent from the reported sequences of Chinese H3N2 CIV strains but closely related with strains from the USA. This study will strengthen the understanding of the epidemiology and genetic diversity of H3N2 CIV.

The results of this study indicate that H3N2 CIV may have been transmitted from the USA to China, where it caused an epidemic in Chinese canines. These results suggest that we should strengthen monitoring of this disease at borders during the transportation of canines and continue to monitor CIV in China for a long period to observe the recombination of domestic and foreign-like strains. It is also important to monitor whether the coexistence presence of the two origin viruses of different origin has a greater impact on canines, which requires further research.

Compliance with Ethical Standards

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Conflict of Interest Statement: The authors declare no conflicts of interest.

Ethical Approval: No studies involving human participants or animals performed by any of the authors are described in this article.

Data Availability Statement: The data that support the findings of this study are available from the corresponding author upon reasonable request.

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Figure legends

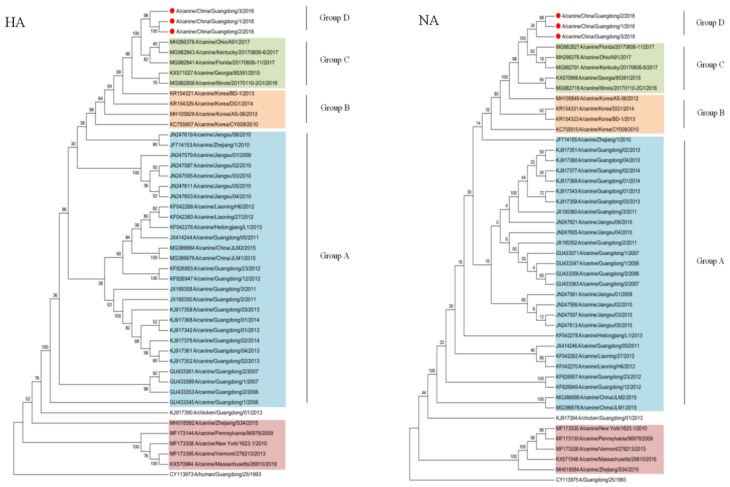

Figure 1 Phylogenetic analysis of H3N2 CIV in China, Korea, and the USA based on the HA and NA protein. Colors indicate H3N8 CIV (red) and isolates from different geographical regions of H3N2 CIV originating from the USA (green), Korea (yellow), and China (blue). The three Chinese strains sequenced in this study are indicated by red circles.

Table 1. Information on the samples collected from dogs in this study

Sample ID	Isolate	Isolation source	Collection date	Host	Detection method
F1	A/canine/China/Guangdong/1/2018	nasal swabs	2018.5.14	Corgi	RT-qPCR
W2	A/canine/China/Guangdong/2/2018	nasal swabs	2018.5.16	Pomeranian	RT-qPCR
J3	A/canine/China/Guangdong/3/2018	nasal swabs	2018.5.17	Corgi	Immunochromatogra

Abbreviation: RT-qPCR, RealTime QuantitativePCR.

Table 2. Nucleotide and amino acid similarity with the H3N2 CIV strains in this study

Gene	Nucleotide similarity with the H3N2 CIV strains in this study	Amino acid similarity with the H3N2 CIV strains i
PB2	99.7%-99.9%	99.7%-100%
PB1	99.6%-99.9%	99.5%-99.9%
PA	99.8%-100%	99.9%-100%
HA	99.4%-99.8%	99.1%-99.8%
NP	99.4%-99.9%	99.2%-99.6%
NA	99.6%-99.9%	99.4%-100%
M1	99.3%-99.9%	100%
M2	100%	100%
NS1	100%	100%
NS2	99.4%-99.7%	100%

Table 3. HA and NA amino acid differences between the H3N2 CIV strains analyzed in this study and previously analyzed H3N2 CIV strains

Isolate	HA 128	HA 187	HA 289	HA 328	HA 481	NA 20	NA 30	NA 43
A/canine/China/Guangdong/1/2018	V I	N S	A S	K S	N S	V/I A	-	N K
A/canine/China/Guangdong/2/2018	V I	N S	A S	K S	-	V/I A	-	N K
A/canine/China/Guangdong/3/2018	V I	-	-	K S	-	-	A/R T	-