A Novel Canine Influenza H3N2 Virus Isolated from Cats in an Animal Shelter

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Running title: Interspecies Transmission of Influenza Virus from Dogs to Cats

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Abstract

The interspecies transmission of avian-origin H3N2 canine influenza virus (CIV) to dogs was first reported in 2007. The present study characterized a novel CIV H3N2 isolate from cats in an animal shelter. A comparative analysis of the deduced amino acid sequences of the A/Canine/Korea/CY009/2010(H3N2) (CY009) and A/Feline/Korea/FY028/2010 (H3N2) (FY028) strains isolated from dogs and cats, respectively, in the animal shelter identified point mutations in 18 amino acid positions within eight viral genes. Interestingly, CY009 and FY028 replicated well in specific pathogen-free embryonated chicken eggs and in mice, respectively. Mice infected with the FY028 strain exhibited significant overexpression of IL-10, TNF-α, and IFN-γ \( (p < 0.001) \) at 3 days postinfection. Thus, an emergency monitoring system should be developed to identify influenza mutations that occur during interspecies transmission in companion animals and for continuous public health surveillance.

Keywords: CIV • Feline • H3N2
1. Introduction

Influenza viruses (H2N2, H7N7, and H7N3) isolated from birds, seals, and humans can all replicate in experimentally-inoculated cats (Hinshaw et al., 1981; Paniker and Nair, 1970, 1972; Romva’ry et al., 1975), while human influenza viruses, H3N2 and H2N2, and influenza B virus can also be transmitted to uninfected cage mates (Hinshaw et al., 1981; Harder and Vahlenkamp, 2010). These observations highlight the need to determine the epidemiological role played by infected cats. However, none of these viruses has been isolated naturally from asymptomatic/symptomatic cats, with the exception of H5N1, which was isolated from cases of avian-to-cat interspecies transmission in Thailand (Songserm et al., 2006), Iraq (Yingst et al., 2006), and Germany (Klopfleisch et al., 2007a,b). Recently, cats have emerged as potential hosts for influenza infections. Several studies have reported the susceptibility of felids to infection by the avian H5N1 and the pandemic H1N1 influenza viruses (Kuiken et al., 2004; Sponseller et al., 2010). Furthermore, there is serological evidence of H3N2 influenza infections in cats (Jeoung et al., 2012; McCullers et al., 2011; Said et al., 2011; Seiler et al., 2010).

The interspecies transmission of avian influenza virus H3N2 to dogs was first reported in South Korea during 2007 (Song et al., 2008a) and the results of an investigation into the systemic transmission of canine influenza virus (CIV) H3N2 between dogs was published in the following year (Song et al., 2009). The pathogenicity of CIV H3N2 was demonstrated in dogs, which had a severe respiratory syndrome (associated with high fever and coughing) and pathogenic lung lesions (Jung...
et al., 2010). Similarly, sporadic cases of respiratory diseases associated with CIV H3N2 infections were detected in the dog population in southern China (Li et al., 2010) and these results corroborated those of a 2007 study that found evidence of CIV H3N2 infections in Korean dogs. Recently, the interspecies transmission of CIV H3N2 to domestic cats was reported in South Korea (Song et al., 2011). The eight viral genes (HA, NA, PB1, PB2, PA, NP, M, and NS) isolated from infected cat were almost identical to those of the canine influenza H3N2 virus, thereby suggesting interspecies transmission from dogs to cats (Song et al., 2011). Thus, there is a public health concern related to the possible emergence of new recombinant feline or canine influenza viruses in companion animals and a threat of zoonotic infections (Song et al., 2011).

The present study aimed to determine whether CIV H3N2 is transmitted naturally from dogs to cats, as well as performing a comparative analysis of CIV H3N2 strains isolated from dogs and cats.

2. Material and Methods

2.1. The animal shelter and the disease outbreak history

The interspecies spread of CIV H3N2 infection was investigated in an animal shelter in Gyunggido, which had an average population of 400 dogs and 60 cats. The shelter had four buildings for housing animals (three for dogs and one for cats). Each room in the animal houses could accommodate approximately 20–40 individuals. Most animals were kept in individual cages, although dogs were kept together in a floor-type
animal room in one building. During October 2010, mild to severe symptoms of respiratory disease, including dyspnea, coughing, and high fever, were observed in 25 street dogs at the shelter. Three weeks later, over 300 dogs were infected with avian-origin influenza virus H3N2, while four cats exhibited similar clinical signs (dyspnea and coughing) at the same time. Two weeks later, 28 cats began to present significant signs of respiratory disease. The affected dogs experienced 77% morbidity and 23% mortality within the first 3 weeks of CIV infection, while the affected cats experienced 46.6% morbidity and 21.7% mortality 2 weeks after the disease onset in cats.

2.2. Antigen detection

The influenza viral antigens in lung specimens from dead animals and nasal swabs from infected animals were detected rapidly using a commercially available Antigen Rapid CIV Ag Test Kit (Bionote Cat No. RG 11-07, South Korea). This test kit was used to detect influenza A virus nucleocapsid protein (NP) in the tissue and/or swab samples, according to the manufacturer’s instructions.

2.3. Genome sequencing

Virus isolation and propagation were performed in 10-day-old specific pathogen-free (SPF) embryonated chicken eggs (ECEs) and the virus subtypes were determined by RT-PCR (Song et al., 2008a). The nucleotide sequencing of the eight gene segments from the virus isolates was performed as described previously, with some modifications (Song et al., 2008a). The sequences of the isolated viral genes were analyzed and aligned using the Bioedit program.
(www.mbio.ncsu.edu/BioEdit/bioedit.html). The HA gene nucleotide sequence similarities of the influenza viruses isolated from five species (pig, chicken, duck, dog, and cat) were compared using SimPlot version 3.5.1 (http://sray.med.som.jhmi.edu/SCRoftware/simplot). The sequences of the complete genes for CIV H3N2 strains was deposited at the GenBank under accession numbers KC755899-KC755926.

2.4. Passages in embryonated chicken eggs and mice

The swab and homogenized tissue samples (10% w/v) were prepared in 1 ml of PBS containing 1% gentamycin. The debris was pelleted by centrifugation (2000 × g, 10 min) and the supernatant was collected. The supernatant samples were serially diluted 10-fold and inoculated into 10-day-old ECEs. After 3 days’ incubation, the allantoic fluid was collected and the HA activity was tested. The virus titer of each sample was calculated using the Reed-Muench method (Reed and Muench, 1938).

Four female BALB/c mice (8 weeks old) were inoculated intranasally with 30 µl of the supernatant sample. Five days postinoculation (p.i.), blood and lung tissue samples were collected from all of the mice by necropsy and analyzed to detect CIV antigen using the rapid test kit.

2.5. Cytokine profiling

Mice were injected intranasally with 30 µl inocula of the A/Feline/Korea/FY028/2010 (H3N2) (FY028) and A/Canine/Korea/CY009/2010(H3N2) (CY009) strains and PBS (control), and blood
samples were taken at 3 and 9 days p.i. The blood samples were collected using sodium citrate tubes and were centrifuged at 1,000 × g at 4°C for 10 min. The plasma samples were harvested and filtered through a sterile 0.22 µm filter system and stored immediately at −20°C until use. Next, cytokine assays were performed using a Bio-Plex™ Mouse Cytokine 8-plex panel (BioRad, USA) according to the manufacturer’s instructions.

2.6. Statistical methods

One-way ANOVA was used to analyze the data. Data from at least three independent experiments were expressed as the mean ± S.E.M. The main effects were compared using a Newman-Keuls post-test. p < 0.001 was considered significant.

3. Results

3.1. Analysis of the HA, NA, and M genes in CIV H3N2

The divergence and percentage identity between the HA protein from CY009 and FY028 strains were 0.4% and 99.6%, respectively. For the NA protein and the M protein, the results were 0.4% and 99.1%, and 0.3% and 99.7%, respectively (data not shown). Compared with the A/canine/Korea/GCVP01/2007 strain (the first strain isolated in 2007), the deduced amino acid sequences of the FY028 and CY009 strains shared 98.8% and 98.8% identity with the HA gene, 97.7% and 98.1% with the NA gene, and 98.5% and 98.8% with the M gene, respectively. The HA gene nucleotide
sequences of influenza viruses isolated from five different species shared high similarity with the FY028 and CY009 strains (Fig. 1).

3.2. Amino acid sequence analysis of CIV H3N2 isolates from dogs and cats

The two strains (CY009 and FY028) from different species harbored mutations in two amino acid (aa) positions (P-123-S and G-132-S) in HA, four aa positions (L-23-P, I-215-T, A-398-V, and T-455-A) in NA, one (E-382-G) in PA, six (L-10-Q, S-79-N, L-130-F, H-134-R, R-190-K, and M-726-I) in PB2, two (G-449-E and V-451-A) in NP, one (F-64-S) in M, and two (A-124-V and I-180-V) in NS1 (Table 1).

3.3. Phylogenetic tree

A phylogenetic tree containing the complete HA gene sequences from 53 influenza virus (H3N2, H3N8, and H3N6) strains (avian, swine, and equine) was constructed using MEGA 4.1 and the CLUSTALX alignment algorithm, based on the neighbor-joining (NJ) method. The phylogenetic tree was divided into three major clusters, i.e., avian, swine, and equine. Six CIV H3N2 isolates from three dogs and three cats in this study were included in the avian lineage cluster (Fig. 2).

3.4. Comparison of the culture passages

To determine any differences in the characteristics of the CY009 and FY028 strains, we attempted to passage the two strains in SPF ECEs and in mice. Interestingly, the virus titers of the CY009 strain were $10^{3.4}$ EID$_{50}$ to $10^{5.6}$ EID$_{50}$ when passaged in ECEs, whereas this strain could not replicate after the second passage in mice (Table 2). By
contrast, the FY028 strain was detected in the lungs of all mice during all passages using the Antigen Rapid CIV Ag Test Kit, whereas this strain was not detected after the third passage in ECEs (Table 2).

3.5. Mice cytokines

Suitable standard curves for the cytokine assays were obtained using a Bio-Plex™ Mouse Cytokine 8-plex panel (BioRad, USA) for IL-2, IL-4, IL-5, IL-10, IL-12p70, GM-CSF, IFN-γ, and TNF-α using a standard photomultiplier tube (PMT) setting and 10-fold serial dilutions from 10^{-1} to 10^{-8} (data not shown). The data for FY028, CY009, and PBS (control) were analyzed using a one-way ANOVA. The results of the cytokine assays showed that the FY028 strain increased the level of IL-10 (p < 0.001), TNF-α (p < 0.001), and IFN-γ (p < 0.001) at 3 days p.i. (Fig. 3).

4. Discussion

The interspecies transmission of CIV H3N2 virus was reported in cats and dogs at an animal shelter in Seoul, South Korea during 2010 and the affected cats experienced 100% morbidity and 40% mortality (Song et al., 2011). By contrast, the affected cats in the present study experienced 46.6% morbidity and 21.7% mortality. The transmission of CIV H3N2, which has similar characteristics to influenza-associated endemic or epidemic respiratory diseases, was rapid from cat to cat. It has been suggested that cats are susceptible to CIV H3N2 infection and that dogs may play a role as intermediate hosts during the transmission of H3N2 virus among cats and dogs (Song et al., 2011).
The comparative analysis of CIV H3N2 strain isolates from dogs and cats, and the first CIV H3N2 strain (GCVP01) isolated in 2007, showed that there was no more than 2% divergence among HA genes of all the strains. In a previous study, a CIV H3N2 isolate from a cat had several differences in its amino sequence from that of CIV H3N2 (Song et al., 2011), but it also had no more than 2% divergence in its nucleotide and amino acid sequences. These results may suggest that antigen drift conserved these sequences, despite intraspecies transmission between two species. The possibility of antigen drift and antigen shift may be very high if CIV H3N2 infections occur continuously in dogs and cats, or if CIV H3N2 is co-infected with other virus serotypes such as pandemic influenza H1N1. Indeed, a novel CIV H3N1 has emerged, which is a putative reassortant between pandemic H1N1 and CIV H3N2 (Song et al., 2012). Similarly, the 18 amino acid mutations detected in FY028 in this study based on CY009 suggest the possible emergence of a novel and highly pathogenic strain. Thus, further study is needed to test whether these mutations are necessary for the transmission of the H3N2 virus from dogs to cats.

The NJ tree showed that six CIV H3N2 strains isolated from three dogs and three cats belonged to the same group as the Feline/Korea01/2010 (HQ316191) strain, which was isolated recently from cat in a study by Song et al. (2011). In the CIV H3N2 sub-cluster, the isolated strains were further sub-divided into two groups according to their country of origin. The first group contained the virus strain isolated in South Korea in 2007 while the second group contained the four avian-origin canine influenza H3N2 strains isolated from Guandong province in China during 2006 and 2007 (Li et al., 2010).
A previous study (based on virus replication in mice) demonstrated that avian Korean H3 viruses have the potential to expand their host range to include mammalian species (Song et al., 2008b). Interestingly, the detection of antigens from the CY009 strain showed that it replicated well when the virus was passaged in ECEs. By contrast, FY028 was found to replicate well when the virus was passaged in mice. This may indicate that the FY028 strain isolated from cats harbored mutations in its amino acid sequence, which differed from those in the CY009 strain isolated from dogs.

Neutrophils and their related chemoattractant cytokines (e.g., IFN-γ, IL-1, and IL-8) normally promote the defense against secondary bacterial infections, but they may also play a role in pathogenesis in H3N2 CIV-infected dogs (Jung et al., 2010). Dogs infected with CIV also exhibited the strong induction of chemokines, including MCP-1 (a blood mononuclear cell chemoattractant) and IL-8 (a neutrophil chemoattractant), which suggests that the dysregulation of chemokines during H3N2 CIV infection might contribute to viral pneumonia characterized by extensive immune cell infiltration (Lee et al., 2011). Cytokines such as IL-1, IL-8, and MCP-1, which form part of the immune response in infected dogs, were not examined in CIV-infected mice in the present study, but three cytokines (IL-10, TNF-α, and IFN-γ) were induced at high levels by strain FY028 at 3 days p.i. These levels returned to normal by 9 days p.i. IL-10 is produced by Th2 cells and inhibits cytokine production, particularly IFN-γ and TNF-α, which are produced by pro-inflammatory Th1 cells in response to antigen. Thus, these three cytokines may have been involved with the early viral pneumonia observed in mice infected with the CIV H3N2 isolate from cats.

In conclusion, this study investigated a novel influenza virus H3N2 isolate from
cats and conducted a comparative analysis of its genomic sequences, cytokine production, and replication. The emergence of a new recombinant CIV strain in companion animals cannot be excluded at any time. Therefore, systematic surveillance is required to monitor this disease and the evolutionary behavior of the virus in the cat population of South Korea.

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Experimental infection of dogs with avian-origin canine influenza A virus (H3N2).


Figure Legends

Fig. 1. Similarity of the HA gene nucleotide sequences of influenza viruses isolated from five species. The HA gene analysis was based on the A/Canine/Korea/GCVP01/2007(H3N2) strain. The viruses isolated from the five species were A/Chicken/Korea/LPM88/2006 (pink), A/Duck/Korea/LPM91/2006 (green), A/Swine/Korea/CY05/2007 (black), A/Canine/Korea/CY009/2010 (blue), and A/Feline/Korea/FY028/2010 (red).

Fig. 2. Phylogenetic tree based on the complete HA gene sequences from 53 avian, swine, and equine strains of influenza virus (H3N2, H3N8, and H3N6), which was constructed using Mega 4 and the neighbor-joining method. The bootstrap percentages are shown above nodes that were supported in > 70% of 1,000 replicates. The scale bar indicates the number of nucleotide substitutions per site. The box with the dotted line indicates the CIV H3N2 strains isolated from dogs and cats in Korea.

Fig. 3. Murine cytokines induced by the FY028 and CY009 strains. One-way ANOVA was used to analyze the data, which are expressed as the mean ± S.E.M. p < 0.001 was considered significant on days 3 (D3) and 9 (D9) postinoculation. The gray, black, and white bars represent CY009, FY028, and the control, respectively. MFI = median fluorescence intensity.
Table 1. Mutations in the deduced amino acid sequences of proteins from strains A/Canine/Korea/CY009/2010 (H3N2) and A/Feline/Korea/FY028/2010 (H3N2)

<table>
<thead>
<tr>
<th>Strain</th>
<th>HA</th>
<th>NA</th>
<th>PA</th>
<th>PB2</th>
<th>NP</th>
<th>M1</th>
<th>NS1</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>123*</td>
<td>132</td>
<td>23</td>
<td>215</td>
<td>398</td>
<td>455</td>
<td></td>
</tr>
<tr>
<td>A/Canine/Korea/CY009/2010</td>
<td>P</td>
<td>G</td>
<td>L</td>
<td>I</td>
<td>A</td>
<td>T</td>
<td></td>
</tr>
<tr>
<td>A/Feline/Korea/FY028/2010</td>
<td>S</td>
<td>S</td>
<td>P</td>
<td>T</td>
<td>V</td>
<td>A</td>
<td></td>
</tr>
</tbody>
</table>

* Amino acid sequence position.
Table 2. Comparison of the antigen detection results using two passaging methods

<table>
<thead>
<tr>
<th>Strain</th>
<th>Mice Passages</th>
<th>Embryonated Chicken Egg Passages</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1  2  3  4  5  6</td>
<td>1  2  3  4  5  6</td>
</tr>
<tr>
<td>CY009</td>
<td>4/4a</td>
<td>0/4  0/4  0/4  0/4  0/4</td>
</tr>
</tbody>
</table>

a The number of antigen-positive mice using the rapid test kit.
b Virus titers are expressed as log_{10} EID_{50}/ml.1
Fig 1

Figure

A/Chicken/Korea/LPM88/2006 (H3N2)
A/Duck/Korea/LPM91/2006 (H3N2)
A/Swine/Korea/CY05/2007 (H3N2)
A/Canine/Korea/CY009/2010 (H3N2)
A/Feline/Korea/FY028/2010 (H3N2)
Figure
Fig 3.