Interspecies transmission of the canine influenza H3N2 virus to domestic cats in South Korea, 2010

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In the past 4 years, incidences of endemic or epidemic respiratory diseases associated with canine influenza H3N2 virus in Asian dogs have been reported in countries such as South Korea and China. Canine species were considered to be the new natural hosts for this virus. However, at the beginning of 2010, influenza-like respiratory signs, such as dyspnoea, were also observed among cats as well as in dogs in an animal shelter located in Seoul, South Korea. The affected cats showed 100 % morbidity and 40 % mortality. We were able to isolate a virus from a lung specimen of a dead cat, which had suffered from the respiratory disease, in embryonated-chicken eggs. The eight viral genes isolated were almost identical to those of the canine influenza H3N2 virus, suggesting interspecies transmission of canine influenza H3N2 virus to the cat. Moreover, three domestic cats infected with intranasal canine/Korea/GCVP01/07 (H3N2) all showed elevated rectal temperatures, nasal virus shedding and severe pulmonary lesions, such as suppurative bronchopneumonia. Our study shows, for the first time, that cats are susceptible to canine influenza H3N2 infection, suggesting that cats may play an intermediate host role in transmitting the H3N2 virus among feline and canine species, which could lead to the endemic establishment of the virus in companion animals. Such a scenario raises a public health concern, as the possibility of the emergence of new recombinant feline or canine influenza viruses in companion animals with the potential to act as a zoonotic infection cannot be excluded.

INTRODUCTION

Canine influenza is caused by two subtypes of influenza A virus: H3N8 and H3N2. The H3N8 canine influenza virus (CIV) was first identified in dogs in the USA in 2005, and is known to originate from equine H3N8 influenza viruses. The H3N2 CIVs are of avian origin and were detected in Asian countries, including South Korea and China (Crawford et al., 2005; Lee et al., 2010; Li et al., 2010; Payungporn et al., 2008; Song et al., 2008). The aetiologies of the disease caused by these two CIVs differ geographically, and do not share any genetic or antigenic features (Crawford et al., 2005; Song et al., 2008). However, they commonly infect and replicate in the upper and lower respiratory tract and sustain successful transmission among dogs (Harder & Vahlenkamp, 2010; Lee et al., 2010). The

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diseases caused by the two CIVs have a similar epidemic pattern, although the CIV-related epidemic respiratory disease may have changed into an endemic form depending on the herd immunity (Hayward et al., 2010). Interestingly, recent reports have shown that the pandemic A/H1N1/09 human influenza virus can also infect dogs (Dubovi, 2010).

In recent times, feline species, especially cats, have become a noticeable host for influenza infections. They are susceptible to infections of avian influenza H5N1 virus and pandemic H1N1 influenza (Kuiken et al., 2004; Sponseller et al., 2010). Also, there has been serological evidence for H3N2 influenza in cats (McCullers et al., 2011; Said et al., 2011; Seiler et al., 2010). These observations indicate that cats may play an intermediate host role in transmitting the virus to humans and other mammals and have stimulated surveillance of cats for influenza virus infections. At the beginning of 2010, we isolated a canine-origin feline H3N2 influenza virus from a lung specimen of a dead cat, which had suffered from a severe respiratory disease. Experimentally, we infected domestic cats with CIV H3N2 (A/canine/Korea/GCVP01/07) to investigate if the virus would induce respiratory infection and disease in these hosts. The infected animals all showed fever, nasal virus shedding and severe pulmonary lesions.

RESULTS

History of outbreak

From December 2009 to January 2010, an explosive flu-like severe respiratory disease was identified in cats and dogs in an animal shelter located in the capital of South Korea, Seoul. There were a total of 200 dogs and 50 cats afflicted with this disease. Dogs showed 25% mortality after the onset of the respiratory signs, whereas cats showed 100% morbidity and approximately 40% mortality after the onset of the respiratory signs, such as tachypnea, dyspnoea and lethargy. Upon necropsy of the afflicted cats, severe bronchopneumonia was observed, characterized by dark-reddish consolidation affecting 30–70% of the whole lung, frequently accompanied by pulmonary oedema in five cats. In March 2010, lung specimens from five dead cats with severe respiratory disease were submitted to the National Veterinary Research and Quarantine Service (NVRQS) for diagnosis, which was a surveillance programme of NVRQS to monitor CIV infections in animal shelters. Because dogs and cats in the shelter were parcelled out to the pet owners continuously and only frozen cadavers of cats were submitted to the NVRQS, there were some difficulties in obtaining fresh specimens from cats with an acute outbreak. The use of frozen specimens may explain the low virus detection (3/5; 60% by antigen detection rapid kit) and isolation rates [1/5; 20% in embryonated-chicken eggs (ECEs)] observed in this study.

Virus isolation from field samples

An influenza virus was isolated from ECE inoculated with the lung suspension of a dead cat. The isolate was confirmed by RT-PCR as subtype H3N2 influenza virus (Fig. 1). By PCR methods and using a commercially available antigen detection kit (Bionote), the lung specimen was found to be negative for other viral agents, including feline calicivirus, herpesvirus, feline infectious peritonitis virus and feline immunodeficiency virus. However, co-infection with Bordetella bronchiseptica was identified with PCR using purified colony from cultured specimen blood agar.

Serological surveillance

Twenty-four of 69 dog samples (34.8%) were seropositive for the influenza virus, whereas all the 18 cat sera tested were seronegative.

Nucleotide sequencing and phylogenetic analysis of the A/feline/Korea/01/2010 (H3N2) virus

From the feline influenza isolate, eight gene segments (H3, N2, PB1, PB2, PA, NP, M and NS) were sequenced, and homologous sequences for them were sought in GenBank. Overall, nucleotide sequences of the feline isolate displayed 98.0–99.8% similarities with all eight gene segments of H3N2 CIVs (data not shown). The influenza isolate was designated A/feline/Korea/01/2010 (H3N2). Phylogenetic analysis revealed that the HA and NA gene segments of A/feline/Korea/01/2010 (H3N2) were clustered very closely with those of CIV H3N2 circulating in Korea from 2007 to 2010 (Fig. 1). Similarly, the internal genes (PB2, PB1, PA, NP, M and NS) were also closely related to those of a prototype virus of the isolate A/canine/Korea/GCVP01/2007 (H3N2).

Pathogenicity of the CIV in domestic cats

Clinical signs, such as sneezing, coughing, abdominal breathing and nasal discharges, were observed on day 3 post-infection (p.i.) in the inoculated group of cats (Felis catus domestica) with the H3N2 CIV. Cats in the inoculated group developed a fever in 2–7 days p.i. (rectal temperature ~39.5–39.9 °C). The peak mean rectal temperature (39.7 ± 0.2 °C) was observed at 4 days p.i. (Fig. 2). Fever and clinical signs were not observed in the two cats in the non-infected group.

Virus shedding was assessed on days 2, 4 and 7 p.i. by realtime RT-PCR. In infected cats, mean nasal shedding viral titres were 10^{5.8}, 10^{5.7} and 10^{5.8} egg infectious dose (EID_{50}) ml^{-1} on days 2, 4 and 7 p.i., respectively. Shedding viral titres were highest on day 2 p.i., whereas no nasal shedding was identified in the non-infected cats. Nucleoprotein-specific ELISA showed that the cats in both the infected and the non-infected groups remained seronegative until 7 days p.i.

All three cats with intranasal inoculation of a high-dose (10^{6.5} EID_{50}) of canine H3N2 influenza developed severe macroscopic and microscopic pulmonary lesions, limited
only to the respiratory tract at day 7 p.i. Macroscopic lung lesions were commonly observed as dark reddish-tan consolidation of the lung, especially in the intermediate lobes (Fig. 3a). Microscopic lung lesions at 7 days p.i. were so diffuse that most of the bronchi, bronchioles and alveoli in the tissues were affected by viral infection. Three major characteristics of histological lesions were: (i) ‘necrotic’ – mild to moderate necrosis of bronchial, bronchiolar and alveolar epithelial cells, and occasionally, neutrophils within the lesions (Fig. 3b); (ii) ‘inflammatory’ – moderate to severe infiltration of macrophages, neutrophils, and to a lesser extent, monocytes in bronchiolar and alveolar lumens; and (iii) ‘proliferative’ – mild to moderate thickness of alveolar septa following type 2 pneumocyte hyperplasia and infiltration of inflammatory cells, such as macrophages. Collectively, cats with CIV infection at 7 days p.i. developed a typical influenza pneumonia in a mid-stage of infection, i.e. severe diffuse chronic-active necrotizing suppurrative bronchointerstitial pneumonia. In addition, pulmonary (alveolar) oedema frequently accompanied such pneumonic lesions, and frequent observation of atypical, enlarged type 2 pneumocytes was also notable in the lung of CIV-infected cats (Fig. 3c). Large amounts of influenza A viral (NP) antigens were detected by immunohistochemistry (IHC) in the bronchial epithelium of the infected lung (Fig. 3d).

**Fig. 1.** Phylogenetic analysis of canine origin feline influenza virus H3N2 based on partial HA gene. Phylogenetic trees were generated by the neighbour-joining method with the maximum composite likelihood model and bootstrapping values were calculated with 1000 replications. A/canine/Korea/02/07 (H3N2) and A/canine/Korea/03/07 (H3N2) are not registered in GenBank. The phylogenetic trees based on the other genes are presented in Supplementary Fig. S1.
DISCUSSION

Interspecies transmission of the influenza virus has been a public health concern because of the possibility that through reassortment, a novel strain with zoonotic potential could emerge (Sponseller et al., 2010). Not only dogs but also cats are susceptible to natural influenza virus infections mostly considered as spillover transmission of whole viruses. Equine H3N8, avian H3N2, avian H5N1 and pandemic human H1N1 influenza viruses have been identified in dogs, while cats had been shown to be susceptible to avian H5N1 and pandemic human H1N1 influenza viruses (Harder & Vahlenkamp, 2010; Songserm et al., 2006). The epidemiology of CIV H3N2 infection is most probably similar to that of CIV H3N8 of equine origin (Hayward et al., 2010). A recent study investigating CIV H3N8 indicated that viral infection and transmission were mainly established and maintained in those animal facilities, such as animal shelters and kennels, with large numbers of incoming susceptible dogs (Hayward et al., 2010). It has also been found that CIV H3N8 has not spread widely among the household dog population (Hayward et al., 2010). We speculated that some environmental factors present in the animal shelter, such

**Fig. 2.** Nasal virus shedding and rectal temperature of domestic cats with CIV infection. Grey and white bars indicate the rectal temperatures of the CIV-infected (three cats) and non-infected cats (two cats), respectively. The closed and open circles indicate the nasal virus shedding of the CIV-infected and non-infected cats, respectively.

**Fig. 3.** (a) Macroscopic lung lesion (ventral view) of a cat with CIV H3N2 infection at 7 days p.i., showing dark reddish-tan consolidation, especially in the intermediate lobes (arrow). (b) Microscopic lung lesion of a cat with CIV H3N2 infection at 7 days p.i., showing severe suppurative necrotizing bronchopneumonia. Bronchiolar airways (B) are plugged with a number of necrotic and inflammatory cells, such as neutrophils, accompanied by oedema in alveolar spaces. H&E stain. (c) Microscopic lung lesion of a cat with CIV H3N2 infection at 7 days p.i., showing suppurative bronchopneumonia. Alveolar lumens are accumulated with large numbers of macrophage-like cells and neutrophils, and homogeneous pale eosinophilic fluid (alveolar oedema). Note atypical, enlarged type 2 pneumocytes with prominent eosinophilic nucleoli (arrows). H&E stain. (d) Bronchial epithelium of a cat with CIV H3N2 infection at 7 days p.i., having large amounts of influenza A viral antigens (brown stain; arrows). Original magnification ×200. IHC.
as swift movement of animals, might affect the direct transmission of CIV H3N2 to cats. The CIV H3N2-infected dogs had nasal virus shedding at high titres, which was infectious and also transmissible to contact naive dogs (Song et al., 2008, 2009a). In the shelter, water, food and air sources might be contaminated with the virus shedding from the infected dogs and by direct contact with inside workers. Therefore, cats might be infected with CIV H3N2 through horizontal transmission, similar to sporadic infections of avian H5N1 influenza in cats during human outbreaks (Desvaux et al., 2009; Kuiken et al., 2004). In our study, it was not verified if the CIV H3N2 infection was sustained within the cat population; continuous surveillance is required to address this issue. Pepin and others have previously categorized the mechanisms relating to viral transmission and adaptation to new hosts, i.e. viral-host jumping (Pepin et al., 2010). On the basis of the authors’ classification, transmission of H3N2 CIV to cats can be considered to be spillover cases of fortuitously adapted genotypes, which do not require significant genetic mutations for host jumping. The spillover effect is defined as a situation in which specific viral genotypes capable of sustained spread in new host species are selected over other genotypes that are doomed to fail (Pepin et al., 2010).

From full-sequence analysis of the eight gene segments of the feline H3N2 virus isolate, no genetic reassortment was identified between the isolate and other influenza viruses. The HA and NA genes of the virus showed ~99.7–99.8 % similarity with Korean H3N2 CIVs, with high genetic similarity of six internal protein genes (~98.0–99.0 %), indicating horizontal transmission of whole CIV H3N2 to cats. After an inoculation of CIV H3N2 into domestic cats, we evaluated the kinetics of virus shedding in the infected animals. Nasal shedding is critical for mammalian intraspecies or interspecies transmission of influenza viruses. The cats with the experimental CIV H3N2 infection had nasal shedding of median viral titres (ranging from 10^{2.8} to 10^{3.8} EID_{50} ml^{-1}), which is lower than that described in an earlier study of infection in dogs (ranging from 10^{4.8} to 10^{7.3} EID_{50} ml^{-1}) (Song et al., 2008). Upon gross lesion, histopathology including IHC in the lung and serological response, inoculated feline influenza virus was infected in the experimental animal. The viral shedding originated from these infections, not by the inoculum. It was not certain if the low shedding viral titres affect intraspecies or interspecies transmission of the virus, because we did not assign any naïve cats as contact animals with the infected cats. Therefore, further studies are needed to verify both whether low infectivity of the CIV H3N2 in cats depends on species-specific interaction and whether it affects the transmission of the CIV H3N2 to cats.

The CIVs H3N2 and H3N8 have successfully sustained infectivity and transmissibility in dog populations (Lee et al., 2010; Payungporn et al., 2008). Moreover, dogs were recently reported to be infected by pandemic H1N1 (Dubovi, 2010). Cats are susceptible to the pandemic human H1N1, avian H5N1 and CIV H3N2, as verified in our current study. Due to co-infection by different genotype or subtype influenza viruses, the emergence of recombinant CIVs or feline influenza viruses with potential zoonotic infection cannot be excluded in companion animals, which have more opportunity to transmit zoonotic viruses to humans. Therefore, intense monitoring for influenza infections in pet dogs and cats is necessary for public health (Harder & Vahlenkamp, 2010).

Our study shows infection and transmission of whole CIV H3N2 to domestic cats housed in an animal shelter in South Korea. During the outbreak, cats showed 100 % morbidity and high mortality (40 %). Domestic cats with experimental CIV H3N2 infection reproduced the symptoms fever, nasal virus shedding and severe pneumonia as observed in natural cases. In this study, cats were susceptible to CIV H3N2 infection. Thus, cats may act as a potential intermediate host for the transmission of CIV H3N2 among feline and canine species, possibly resulting in endemic establishment of the virus in companion animals. This raises a public health concern.

**METHODS**

**Serological surveillance.** In March 2010, that is 2 months after the outbreak, sera samples were collected from 69 dogs and 18 cats for serological surveillance. The animals had been raised in the animal shelter where the influenza outbreak occurred. Serum antibodies against the nucleoprotein of influenza A viruses were assessed by a commercially available competitive ELISA kit targeting nucleocapsid protein of influenza A viruses (Bionote). The competitive ELISA kit was validated for mammals and avian species (Song et al., 2009b).

**Detection and characterization of the isolate.** Before submission of specimens to NVRQS, rapid detection of influenza viral antigens in lung specimens or nasal swabs was performed with a commercially available kit (Bionote), which detected the nucleocapsid protein (NP) of influenza A virus, according to the manufacturer’s instructions. For the virus isolation, each lung sample was processed into 10 % (w/v) tissue suspension in PBS (pH 7.4). The suspensions were clarified by centrifugation at 1500 g for 20 min, and the supernatants were ultracentrifuged through a 0.2-μm syringe filter. Virus isolation was conducted with 11-day-old ECEs and subtyping was carried out by RT-PCR (Song et al., 2008). Full genome nucleotide sequencing of the eight gene segments from the isolate was performed as described previously, with some modifications (Hoffmann et al., 2001). The sequences of the isolated viral genes were edited using the BioEdit software (www.mbio.ncsu.edu/BioEdit/bioedit.html). Partial HA gene (540 bp) sequences of the isolate and reference strains were analysed by multiple pairwise alignments with the CLUSTAL_X algorithm (www.megasoftware.net). Phylogenetic trees were generated by the neighbour-joining method with the maximum composite likelihood model and supported by bootstrapping values calculated with 1000 replications using the MEGA version 4.0 software (Tamura et al., 2007).

**Experimental infection of the A/canine/Korea/GCVP01/07 (H3N2) virus in domestic cats.** All animal experiments complied with the current laws of South Korea. Animal care and treatment were conducted in accordance with guidelines established by the Green Cross Veterinary Products Institutional Animal Care and Use Committee. Five 4-month-old conventional Korean shorthair cats were assigned to two treatment groups [infected (n=3) and non-infected (n=2)]. Each
group of cats was placed in a separate, negatively pressurized isolator in the
isolation facility at the Green Cross Veterinary Products (Yongin, Korea). At the start, all cats were determined to be seronegative for
influenza virus. The A/canine/Korea/GCVP01/07 (H3N2) virus isolated
from a dog with severe respiratory disease was used as an inoculum
(Song et al., 2008). The virus could be transmitted dog-to-dog (nose-to-
) via contact infection (Song et al., 2009a). Cats in the infected
host were inoculated intranasally with a 1 ml 10^6.5 EID_{50}, while those
in the non-infected group were given 1 ml PBS. After inoculation, rectal
temperatures and clinical signs were examined. Nasal swab samples
were collected at 0, 2, 4 and 7 days p.i. for the purpose of assessing
shedding viral titres using real-time RT-PCR, as described previously
(Song et al., 2008, 2009a). On day 7 p.i., all cats were euthanized for
pathological examination and viral antigen detection by IHC, as
described previously (Song et al., 2008). Serum antibody titres against
influenza A nucleoprotein were tested using a commercially available
competitive ELISA kit (Bionote) as mentioned earlier (Song et al.,
2009b).

**Nucleotides sequence accession numbers.** Sequences of the eight
 viral gene segments of influenza virus isolated from domestic cat
were submitted to GenBank. Accession numbers assigned to
sequences determined in this study are HQ316191 (HA),
HQ316192 (NA), HQ454980 (M), HQ454981 (NS), HQ454982
(NP), HQ454983 (PA), HQ454984 (PB1) and HQ454985 (PB2).

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