A novel reassortant canine H3N1 influenza virus between pandemic H1N1 and canine H3N2 influenza viruses in Korea

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During recent canine influenza surveillance in South Korea, a novel H3N1 canine influenza virus (CIV) that is a putative reassortant between pandemic H1N1 2009 and H3N2 CIVs was isolated. Genetic analysis of eight genes of the influenza virus revealed that the novel H3N1 isolate presented high similarities (99.1–99.9 %) to pandemic influenza H1N1, except for in the haemagglutinin (HA) gene. The HA gene nucleotide sequence of the novel CIV H3N1 was similar (99.6 %) to that of CIV H3N2 isolated in Korea and China. Dogs infected with the novel H3N1 CIV did not show any notable symptoms, in contrast to dogs infected with H3N2 CIV. Despite no visible clinical signs of disease, nasal shedding of virus was detected and the infected dogs presented mild histopathological changes.

In 2009, a quadruple-reassortant H1N1 strain of influenza virus (pandemic H1N1 virus) emerged in Mexico and spread to the USA. Its strong infectivity and the absence of pre-existing immunity in humans subsequently caused the first influenza pandemic of this century (Garten et al., 2009). Although infection and transmission of the virus had occurred primarily among humans (Sponseller et al., 2010), it has been reported that human-to-animal (e.g. swine, ferret, dog, cat and turkey) transmission occurs occasionally (Dundon et al., 2010; Lin et al., 2011; Sponseller et al., 2010), implying a broad range of host species and reverse zoonotic transmission. Such transmission of the pandemic H1N1 virus beyond its primary host barrier raises concern about the emergence of non-human reservoirs and reassortant influenza viruses.

Influenza viruses of subtypes H1N1 (Lin et al., 2011), H3N8 (Crawford et al., 2005), H3N2 (Song et al., 2008), H5N1 (Songserm et al., 2006) and H5N2 (Zhan et al., 2011) in dogs have been reported. We recently isolated an H3N1 virus, which had not been reported before, from dogs through the surveillance programme of the Korean National Veterinary Research and Quarantine Service (NVRQS) to monitor canine influenza virus (CIV) infections in animal shelters and kennels. Nucleotide sequencing analysis revealed that our H3N1 isolate was a reassortant between an avian-origin H3N2 CIV (Song et al., 2008) and
the pandemic H1N1 virus. Here, we report the genetic, virological and pathological features of the novel CIV H3N1 isolate.

From May 2007 to December 2010 in South Korea, 252 nasal swabs were collected from sick dogs showing clinical respiratory signs, such as cough, nasal discharge and fever, through a surveillance programme of the NVRQS along with Green Cross Veterinary Products (GCVP) and Bionote. To confirm the presence of influenza A virus, we performed a CIV-specific RT-PCR as described previously (Song et al., 2008). After the 2009 pandemic H1N1 influenza outbreak, we also performed a commercial real-time RT-PCR (Bionote) specific for pandemic H1N1 influenza virus and targeting its nucleoprotein.

Fifty samples (50/252) were found to be positive for influenza A viruses (data not shown). In order to identify the subtype of isolates, RT-PCR and sequencing analysis of the haemagglutinin (HA)- and neuraminidase (NA)-encoding genes was performed as described by Hoffmann et al. (2001) with slight modifications. Of the 50 isolates, 49 were identified as subtype H3N2, but one isolate was identified as subtype H3N1. The H3N1 CIV isolate was purified by plaque assay and its genetic characteristics were examined further. The full-length nucleotide sequences of each gene segment were edited and analysed using the BioEdit program v. 7.0.5.3 (Hall, 1999) and compared with previously reported influenza virus sequences listed in GenBank. Nucleotide sequence similarity analysis revealed that the HA gene of the H3N1 CIV isolate was most similar (99 %) to that of A/canine/Korea/GCVP01/2007 (H3N2), a CIV currently circulating in South Korea (Song et al., 2008), whilst the other seven gene segments were related closely to those of the pandemic H1N1 virus. Here, we report the genetic, virological and pathological features of the novel CIV H3N1 isolate revealed that its HA and NA genes clustered closely together with those of the currently circulating avian-origin H3N2 CIVs in South Korea and China and those of the pandemic H1N1 viruses, respectively (Table 1; Supplementary Fig. S1, available in JGV Online) (Li et al., 2010). The phylogenetic topologies of the other genes of the H3N1 CIV isolate paralleled that of the NA gene (data not shown). The genetic and phylogenetic analyses suggested that the novel H3N1 CIV isolate might be a natural reassortant between the H3N2 canine virus and the pandemic H1N1 virus.

To compare the pathogenicity of the H3N1 CIV isolate with those of the two putative parent viruses [A/canine/Korea/GCVP01/2007 (GCVP01/07; H3N2) and A/California/04/2009 (CA/04; H1N1)] in dogs, we experimentally inoculated 5-month-old beagle dogs (two dogs per group; three groups) by intranasal instillation with 10^6.5 50 % egg infectious dose (EID_{50}) of each virus ml^{-1}. All animal experiments were conducted in accordance with guidelines established by the NVRQS and GCVP committee. Dogs infected with the H3N2 GCVP01/07 CIV showed archetypal symptoms of disease, such as fever, cough, sneezing and lethargy, whereas dogs infected with the H3N1 CIV or the H1N1 CA/04 virus did not show any notable symptoms of disease. Neither H3N1-infected dogs nor ferrets presented fever (Fig. 1a; Supplementary Fig. S2, available in JGV Online). Higher viral loads were detected in the upper respiratory tract of dogs infected with the H3N2 GCVP01/07 CIV (mean titre, 10^{6.8} EID_{50} ml^{-1}) or the H1N1 CA/04 virus (mean titre, 10^{6.5} EID_{50} ml^{-1}). In contrast, the reassortant H3N1 CIV-infected dogs shed lower titres of virus (mean titre, 10^{2.5} EID_{50} ml^{-1}) than those infected with the two putative parent viruses (Fig. 1b). Seroconversion was observed in all CIV-infected animals (Fig. 1c). Additionally, the macroscopic and microscopic lung lesions of the dogs infected with the H3N1 CIV isolate were characterized by mild reddish consolidation with focal lesions and mild to no consolidation in the lungs. Histology indicated mild lung consolidation and mild to no inflammation. In contrast, dogs infected with H3N2 GCVP01/07 CIV showed archetypal symptoms of disease, such as fever, cough, sneezing and lethargy, whereas dogs infected with the H3N1 CIV or the H1N1 CA/04 virus did not show any notable symptoms of disease. Neither H3N1-infected dogs nor ferrets presented fever (Fig. 1a; Supplementary Fig. S2, available in JGV Online). Higher viral loads were detected in the upper respiratory tract of dogs infected with the H3N2 GCVP01/07 CIV (mean titre, 10^{6.8} EID_{50} ml^{-1}) or the H1N1 CA/04 virus (mean titre, 10^{6.5} EID_{50} ml^{-1}). In contrast, the reassortant H3N1 CIV-infected dogs shed lower titres of virus (mean titre, 10^{2.5} EID_{50} ml^{-1}) than those infected with the two putative parent viruses (Fig. 1b). Seroconversion was observed in all CIV-infected animals (Fig. 1c). Additionally, the macroscopic and microscopic lung lesions of the dogs infected with the H3N1 CIV isolate were characterized by mild reddish consolidation with focal lesions and mild to

Table 1. Percentage identity of the genes of the A/canine/Korea/01/2010 influenza virus (H3N1) isolated in South Korea to related sequences in GenBank

<table>
<thead>
<tr>
<th>Gene</th>
<th>Virus with highest identity</th>
<th>Origin</th>
<th>Identity (%)</th>
<th>GenBank accession no.</th>
</tr>
</thead>
<tbody>
<tr>
<td>HA</td>
<td>A/canine/Korea/GCVP01/2007</td>
<td>A-O CIV* (H3N2)</td>
<td>99.6</td>
<td>EU127500</td>
</tr>
<tr>
<td>NA</td>
<td>A/Finland/631/2009</td>
<td>Pandemic (H1N1)</td>
<td>99.9</td>
<td>HQ247664</td>
</tr>
<tr>
<td>PB1</td>
<td>A/Russia/171/2009</td>
<td>Pandemic (H1N1)</td>
<td>99.7</td>
<td>CY054668</td>
</tr>
<tr>
<td>PB2</td>
<td>A/Madrid/INS130/2009</td>
<td>Pandemic (H1N1)</td>
<td>99.7</td>
<td>CY083744</td>
</tr>
<tr>
<td>PA</td>
<td>A/Malaysia/12617/2009</td>
<td>Pandemic (H1N1)</td>
<td>99.9</td>
<td>CY055278</td>
</tr>
<tr>
<td>NP</td>
<td>A/swine/Korea/SCJ41/2010</td>
<td>Pandemic (H1N1)</td>
<td>99.7</td>
<td>HM189646</td>
</tr>
<tr>
<td>M</td>
<td>A/California/VRDL4/2010</td>
<td>Pandemic (H1N1)</td>
<td>99.9</td>
<td>CY086883</td>
</tr>
<tr>
<td>NS</td>
<td>A/Zhejiang/DTID-ZJU03/2009</td>
<td>Pandemic (H1N1)</td>
<td>99.8</td>
<td>GU189653</td>
</tr>
</tbody>
</table>

*A-O CIV, Avian-origin canine influenza virus.
moderate focal interstitial pneumonia (Supplementary Fig. S3, available in JGV Online), whilst only benign histopathological lung lesions were observed in dogs infected with the H1N1 CA/04 virus (Fig. 2). These virological analyses indicated that the novel CIV H3N1 was not pathogenic, as the H3N2 canine donor virus was, but could be shed through the respiratory tract and cause moderate lung lesions. Our previous study presented the close relationship between virus shedding and body temperature in CIV infection (Song et al., 2011). The lower virus shedding in H3N1 CIV-infected animals could be related to asymptomatic infection or relatively mild pathogenicity.

In this study, we found evidence of reassortment between the pandemic H1N1 virus and the H3N2 CIV. Although human-to-animal transmission of the pandemic H1N1 virus has been reported in several animal species, there are limited natural cases of reassortment between the pandemic H1N1 virus and other virus strains (Ducatez et al., 2011; Ma et al., 2010; Tremblay et al., 2011; Vijaykrishna et al., 2010).

Unfortunately, although the presence of the pandemic H1N1 virus and H3N2 CIV in the same dog or sample was not confirmed in this study, genetic analyses showed that the H3N1 CIV isolate possessed an HA gene segment from avian-origin H3N2 CIV and the other seven gene segments from the pandemic H1N1 virus, indicating natural reassortment between the pandemic H1N1 virus and H3N2 CIV. In animal experiments, even though the titre of virus shedding in the H3N1-inoculated group was lower than those in the other two groups, it demonstrated the potential ability of the new reassortant H3N1 to adapt to dog populations. A possible explanation is that the emergence of the novel H3N1 CIV in this study could have resulted from recombination in another dog that was co-infected with H3N2 CIV and pandemic H1N1 virus, or singly infected with H3N1 CIV. The nucleotide similarity analysis revealed that all gene segments of the reassortant H3N1 CIV were highly similar (>99 %) to the corresponding gene segments of the donor viruses, implying that the reassortant H3N1 CIV might be generated by co-infection of dogs with the two donor viruses without any significant mutations. Ilyushina et al. (2010) reported that reassortant viruses could be generated by co-infection of the seasonal H1N1 virus (A/New Jersey/15/2007) and the pandemic H1N1 virus (A/Tennessee/1-560/2009) under

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**Fig. 1.** (a) Body temperature of each of the virus-inoculated groups. Fever (>39.5 °C) was not observed in the H3N1- or H1N1-inoculated groups (●, ▲, respectively); however, fever lasted for 5 days in the H3N2-inoculated group (■). (b) Virus shedding of each of the virus-inoculated groups. Mean titres of the H3N1-, H3N2- and H1N1-inoculated groups were $10^{2.5}$, $10^{3.8}$ and $10^{3.5}$ EID$_{50}$ ml$^{-1}$ (●, ■, ▲), respectively. The duration of virus shedding was 1–3 days in the H3N1- and H1N1-inoculated groups. (c) Serological analysis in H3N2 CIV-, H3N1 CIV- and H1N1 CA/04-infected dogs. NC, Negative control. All of the infected dogs showed seroconversion 7 days after infection.

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**Fig. 2.** Histopathological lesions in lungs from dogs infected with H3N2 CIV, H3N1 CIV and H1N1 CA/04. (a) Mild focal supplicative necrotizing bronchointerstitial pneumonia and mild diffuse supplicative necrotizing tracheitis. (b) Mild diffuse interstitial pneumonia with lymphohistiocytic perivascular and peribronchiolar cuffing. (c) Focal mild to moderate interstitial pneumonia.
experimental conditions. When the two strains of influenza virus were co-infected in vitro, most of the dominant progeny viruses were reassortants containing the HA gene from the seasonal strain and the remaining genes from the pandemic virus, which was consistent with the genetic characteristics of the novel H3N1 CIV.

This possible reassortment event between the pandemic H1N1 virus and the H3N2 CIV in dogs suggests that the behaviour of companion animals may be a critical determinant of their ability to act as intermediate hosts for influenza viruses. Therefore, intensive monitoring for influenza infection in companion animals is an area that needs further research.

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**References**


