Natural and experimental infection of dogs with pandemic H1N1/2009 influenza virus

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Evidence of H1N1/2009 influenza virus infection was identified in two domestic dogs in China in November 2009. Virus isolation and sequence analysis of all eight genes of the two isolates showed that they were related closely to the H1N1/2009 influenza virus circulating in humans, indicating that they were probably acquired from humans. To determine the pathogenicity and transmissibility of H1N1/2009 influenza virus in dogs, experimental infection and transmission were performed. Inoculated dogs were able to shed virus in nasal secretions, but symptoms were very mild. Uninoculated dogs were co-mingled to determine the transmissibility of the isolate, and one of three exposed dogs was shown to develop infection. The present findings indicate that human H1N1/2009 can infect dogs, but is transmitted inefficiently between dogs.

Since the pandemic H1N1/2009 influenza outbreak in Mexico and the USA in April 2009, the responsible virus spread across the globe by human-to-human transmission at an unprecedented rate (Neumann et al., 2009). Subsequently, H1N1/2009 viruses were also detected in other mammals, including pigs, cats and cheetahs (http://vetmedicine.about.com/od/zoonotic/tp/H1N1news.htm). This could pose a more important public-health concern, whereby H1N1/2009 influenza virus could recombine with other influenza viruses prevailing in these animals and generate a novel virus that might cause another pandemic (Lam et al., 2011; Vijaykrishna et al., 2010; Zhu et al., 2011).

Previous reports have shown that racing greyhound dogs can be infected by equine H3N8 influenza virus, and this was speculated to be the result of a single cross-species transmission event directly from horses (Equis caballus), possibly facilitated by direct contact at racing environments (Crawford et al., 2005). Subsequent seroepidemiological investigations indicated that H3N8 viruses not only continued to circulate in racing greyhounds, but were also transmitted to pet dogs in the USA (Payungporn et al., 2008). Pet dogs are frequently exposed to influenza viruses due to close contact with their hosts, who may be infected with influenza virus. Serological and virological data have documented sporadic transmission and subclinical infection of dogs with human influenza subtype H3N2 viruses (Chang et al., 1976; Houser & Heuschele, 1980; Kilbourne & Kehoe, 1975/1976; Nikitin et al., 1972). Previous studies in Korea have reported transmission to dogs of avian-origin influenza A viruses (H3N2) that spread across South Korea from May to December in 2007 (Song et al., 2008), and determined experimentally that the virus can be transmitted directly between dogs (Song et al., 2009). Despite evidence of H3 subtype influenza infections in dogs, other subtypes of great risk to human health have also been found in domestic dogs. Songserm et al. (2006) reported a dog that died from naturally acquired H5N1 influenza virus infection and found systemic replication of the virus in the animal. Experimentally, Chen et al. (2010) found that dogs are highly susceptible to H5N1 virus and showed obvious symptoms. However, other studies showed that experimentally infected dogs developed only mild disease, and virus could not be recovered from all of the inoculated animals (Giese et al., 2008; Maas et al., 2007). These conflicting studies indicate that the pathogenicity of H5N1 virus in dogs should be correlated with the virus strain(s) or the individual animal. Although there is no evidence for sustained transmission of human H3N2 and avian H5N1 influenza viruses to dogs, at least two virus lineages of H3N8 and avian-origin H3N2 influenza viruses are capable of adapting to canine populations, and are currently or have been circulating in America, Europe and Asia (Harder & Vahlenkamp, 2010).

During the outbreak of pandemic H1N1/2009 influenza virus in humans towards the end of 2009, cases of respiratory disease occurred in dogs at the Veterinary Teaching Hospital of China Agricultural University (CAU).

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The first case, presented on 7 November 2009, was identified in a 9-year-old female Pekingese dog, which exhibited severe cough with sputum, mild depression and anorexia for 3 days. The owner mentioned that family members had experienced an influenza-like illness prior to the dog becoming ill. A grade II/VI heart murmur, a slight arrhythmia and lung crackles were detected. Complete blood count (CBC) showed that the dog had increased neutrophilic granulocytes and lymphocytes with a descending white blood cell (WBC) count. Thoracic radiographs showed some degree of whole-heart enlargement, and increased bronchial markings. Echocardiograms showed abnormal systolic mitral-valve motion and thickened mitral leaflets. On 20 November 2009, a 7-year-old female Dalmatian dog was brought to the Veterinary Teaching Hospital of CAU. The dog presented with severe cough with sputum, nasal discharge, enlargement of the mandibular lymph node, and pyrexia with sputum of 4 days duration. CBC showed that lymphocytes and neutrophils were moderately increased, while the WBC count was normal. Lobar pneumonia was observed by radiography. The dog was then hospitalized and, after treatment with broad-spectrum antibiotics and blood transfusion, the clinical status of the dog improved.

Nasal swabs of these two dogs were collected and were found to be positive for H1N1/2009 influenza virus by RT-PCR, according to the directions for rapid identification of the H1N1/2009 influenza virus distributed by the Chinese Center for Disease Control and Prevention (CCDC, 2009). Sequence analysis of the haemagglutinin (HA) genes indicated that the two viruses shared 98.6–98.9 % nucleotide identity with human H1N1/2009 virus (A/California/04/2009). Virus isolation was then performed. Nasal swabs of these two dogs were placed in 1 ml cold PBS containing 2000 U penicillin ml⁻¹, 400 µg streptomycin ml⁻¹ and 800 µg gentamicin ml⁻¹. The specimens were subsequently used to isolate virus by inoculation of 10-day-old embryonated specific pathogen-free chicken eggs and incubation at 37 °C for 72 h. Harvested allantoic fluids tested positive by haemagglutination assay and viral RNA was extracted by using TRIzol reagent (Invitrogen). Testing by PCR amplification indicated that the isolates were indeed positive for H1N1/2009 virus and negative for other pathogens, such as canine distemper virus and canine parainfluenza virus 2. The two isolates were named A/canine/Beijing/cau2/2009 and A/canine/Beijing/cau9/2009, respectively. Follow-up serological survey of these two dogs demonstrated that their sera reacted well with H1N1/2009 antigens and showed haemagglutination-inhibition (HI) titres of 1:128 and 1:256, respectively, confirming that the dogs were infected with H1N1/2009 virus.

To study the genome of these canine isolates further, reverse transcription for viral RNA was performed using the influenza universal primer Uni12 (5′-AGCAAAAGCAGG-3′; Hoffmann et al., 2001). PCR amplification was performed using fragment-specific primers (primer sequences available on request) and amplification products were sequenced as described previously (Sun et al., 2010) (PB2, nt 1318–2200; PB1, nt 1138–1783; PA, nt 756–1228; HA, nt 10–1701; NP, nt 457–1462; NA, nt 28–1387; M, nt 1–982; and NS, nt 1–838). A BLAST search (http://blast.ncbi.nlm.nih.gov/Blast.cgi) was used to determine the most similar sequences to those of the isolates. Analyses showed that all of the eight gene segments have a close relationship to H1N1/2009 influenza virus circulating in humans, with nucleotide identities of 98.9–100 % to those of a human representative H1N1/2009 strain, A/California/04/2009, suggesting that a human H1N1/2009 influenza virus was transmitted directly to each dog without recombination. Phylogenetic analysis of the HA gene, using the neighbour-joining method with 1000 bootstrap replicates using MEGA4.1 (Tamura et al., 2007), showed that both canine strains were associated with the H1N1/2009 clade of influenza viruses (Fig. 1).

To evaluate the pathogenicity and transmissibility of H1N1/2009 virus in dogs, we experimentally infected healthy, influenza virus-negative, 10-week-old beagle dogs with A/canine/Beijing/cau9/2009 virus and observed whether un inoculated dogs co-housed with infected animals became ill. Dogs were anaesthetized by intramuscular injection of Zoletil 100 (tiletamine–zolazepam; Virbac; 10–15 mg kg⁻¹) prior to all procedures, including inoculation, nasal-wash collection, and collection of blood. Six dogs were inoculated with virus [10⁻³ 50 % egg infectious dose (EID₅₀) per ml] via the intranasal route. At 1 day post-inoculation (p.i.), three naïve dogs were introduced into the inoculated groups and housed together in a new room. Another three dogs were housed separately as negative controls. Animals were observed daily for clinical signs, including ocular and nasal discharge, body temperature, sneezing, coughing, dyspnoea and depression, as described by Deshpande et al. (2009a, b). Nasal and anal swabs were collected at 0–14 days p.i. Blood samples were collected at 0–14 days p.i. to determine influenza virus-specific antibodies by HI assay. All animal research was approved by the Beijing Association for Science and Technology [approval reference SYXK (Beijing) 2007-0023], and complied with the laboratory-animal welfare and ethical guidelines of the Beijing Administration Committee of Laboratory Animals. The statistical significance of differences between the virus titres of different tissues was determined by using Student’s t-test. P<0.05 was considered statistically significant.

Our studies revealed that the inoculated dogs had a mild cough during days 1–7 p.i., and two of the six dogs exhibited nasal discharge on days 2–4 p.i. The rectal temperatures of all inoculated dogs were elevated (39.1–39.4 °C) at 2 days p.i. and declined to ≤39 °C from days 8 to 9 p.i. (Fig. 2a). In the un inoculated contact group, no obvious signs were observed except for a slightly elevated rectal temperature (39.1–39.2 °C) in one of the three dogs during days 4–8 p.i. Virus shedding in the nasal discharge of dogs in the inoculated group began on day 1 p.i. and persisted up to 5 days p.i. (Fig. 2b). At 4 days p.i., the three dogs from the inoculated group were euthanized humanely by intravenous anaesthetic. No obvious gross lesions were observed, and then tracheas, lungs and tonsils...
were collected for virus detection and titration in eggs. A portion of the excised tonsils and lungs of euthanized dogs was deposited in 10% phosphate-buffered formalin. Samples were then processed for paraffin embedding and cut into 5 μm sections. One section from each tissue sample was stained with haematoxylin and eosin (H&E) and another section was processed for immunohistological staining with a mouse mAb (AA5H; Abcam) specifically against influenza A virus nucleoprotein and a goat anti-mouse IgG biotin-conjugated antibody (Millipore). Specific antigen–antibody reactions were visualized using diaminobenzidine tetrahydrochloride (DAB) (Sigma) plus substrate and chromogen staining, and counterstained with haematoxylin. Viruses were detected in tonsils, tracheas and lungs.

**Fig. 1.** Phylogenetic tree of HA genes from H1N1/2009 viruses isolated from canine, human, swine, turkey and ferret hosts. The unrooted phylogenetic tree was generated by the distance-based neighbour-joining method using MEGA 4.1. The reliability of the tree was assessed by bootstrap analysis with 1000 replications; numbers above branches indicate bootstrap values. Analysis was based on nt 11–1700 of the HA gene. Bar, 0.05 substitutions per site.

**Fig. 2.** Experimental infection of dogs with A/canine/Beijing/cau9/2009 virus. (a) Body temperature, (b) virus shedding in nose and (c) antibody seroconversion of three inoculated dogs (filled symbols and solid lines) and three exposed dogs (empty symbols and dotted lines). The limit of virus detection was 10^1.0 EID_{50} ml^{-1}.
on day 4 p.i., and the virus titres in these organs were significantly higher than those detected in the nasal discharge (P<0.01) (Fig. 3a). No virus was detected in anal swabs. The HI titres in the inoculated group increased on days 5–6 p.i. with titres of 1:32–1:64 (Fig. 2c). Negative-control animals showed no evidence of disease or virus infection. Further histopathological examination by H&E staining of tissue sections revealed no apparent histopathological lesion in the tonsils; however, virus antigen was observed (Fig. 3b, c). Lung tissue showed desquamation of epithelial cells of the mucosa in the bronchial lumen and interstitial oedema, and a small number of inflammatory-cell infiltrates around the blood vessels were observed (Fig. 3d). Virus antigen was observed in alveolar cells (Fig. 3e).

One of three uninoculated but exposed dogs developed nasal discharge on day 5 post-exposure and virus assays on the nasal discharge were positive (Fig. 2b). Seroconversion in this animal further confirmed influenza virus infection (Fig. 2c); however, the other two exposed dogs did not show any evidence of infection and did not record an HI antibody titre. These results indicated that, although transmission of H1N1/2009 virus did occur directly between dogs, the transmissibility was low.

The present study confirmed H1N1/2009 influenza virus infection in dogs by RT-PCR, virus isolation and nucleotide sequencing. As the two cases occurred during the H1N1/2009 influenza outbreak in the winter of 2009 and the dogs’ owners also had ‘flu-like disease prior to the dogs becoming sick, we suggest that these dogs acquired influenza virus from their owners. Moreover, infections of dogs with human H1N1/2009 influenza virus in the USA have been reported previously (http://vetmedicine.about.com/b/2009/12/22/first-confirmed-case-of-h1n1-in-a-dog.htm). Compared with the large number of H1N1/2009 virus infections in humans, the number of dog influenza cases is significantly lower, indicating that transmission is possible, but rare (Dundon et al., 2010). H1N1/2009 virus infection in dogs may pose a potential public-health threat, as multiple subtypes of influenza virus, such as H3N2, H5N1 and H3N8 (Chang et al., 1976; Crawford et al., 2003; Houser & Heuschele, 1980; Kilbourne & Kehoe, 1975/1976; Nikitin et al., 1972; Payungporn et al., 2008), occasionally infect domestic dogs, possibly generating novel, reassortant influenza viruses. However, as influenza virus infections in dogs seem to be sporadic and the sustained circulation of influenza viruses in dogs is rare and regional, the possibility of recombination of influenza viruses in dogs is lower than that in swine.

Fig. 3. Virus replication, histopathological changes and immunohistochemical staining in dogs infected with A/canine/Beijing/Can9/2009 virus. (a) Virus titres (mean ± SD) in tonsil, trachea and lung. (b) Histopathology of the tonsil; no apparent histopathological lesion can be seen. (c) Immunohistochemistry of the tonsil. Positive virus antigen can be seen scattered in the tonsil. (d) Histopathology of the lung. Desquamation of epithelial cells of mucosa can be seen in the bronchial lumen (black arrows), as well as widened perivascular space and a small number of inflammatory-cell infiltrates around blood vessels (white arrows). (e) Immunohistochemistry of the lung. Virus antigen staining can be seen scattered in alveolar cells. Bars, 50 μm.
Experimental infection of dogs with one of the virus isolates obtained in the current study showed that the clinical response of infected dogs to H1N1/2009 virus was mild, producing only a low fever and occasional cough. Such mild or subclinical infections are likely to be overlooked in the clinic. Although only two dogs were found to be clinically infected with H1N1/2009 virus, we speculate that it is possible that many cases of H1N1/2009 virus infections are ignored due to these mild, inapparent symptoms. A serological survey conducted from October to December 2009 in Italy investigated pandemic H1N1/2009 infection in dogs and demonstrated that seven of 964 specimens showed evidence of exposure to the H1N1/2009 virus (Dundon et al., 2010). Further, the collection time of positive serum specimens coincided with the peak number of human cases of H1N1/2009 infection (Dundon et al., 2010). Thus, further serological and virological surveys should be performed to better understand the prevalence of H1N1/2009 influenza virus in dogs.

In summary, we found clinical evidence of subtype H1N1/2009 influenza virus infection in dogs and determined experimentally that dogs can be infected with H1N1/2009 virus and that transmission among dogs can occur, albeit with low transmissibility. The present findings further stress the importance of surveillance, epidemiological data and molecular epidemiology of H1N1/2009 virus in dogs, due to the potential to develop strains of influenza virus that could cause a new influenza pandemic.

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References


