Characterization of an H3N2 canine influenza virus isolated from Tibetan mastiffs in China

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Ten 3-month-old Tibetan mastiffs became ill 2 days after they were bought from a Tibetan mastiff exhibition, and 4 of them died 2 weeks later. A canine influenza virus (ZJ0110) was isolated from the lung of a deceased Tibetan mastiff and was characterized in detail. Sequence analysis indicated that the 8 genes of the canine isolate were most similar to those of avian-origin canine influenza viruses (H3N2) isolated in South Korea in 2007, with which they shared 98% sequence identity. ZJ0110 could experimentally infect 6-month-old beagles by intranasal inoculation and by airborne transmission, causing severe respiratory syndrome. Moreover, ZJ0110 could replicate in the upper respiratory tracts of mice and guinea pigs, and the virus titer was comparable to that in the upper respiratory tracts of dogs. Although the virus was genetically of avian origin, ZJ0110 could not experimentally infect chicken or ducks by intranasal inoculation. These results suggest that dogs might be an intermediary host in which avian influenza viruses adapt to replicate in mammals.

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1. Introduction

Dogs were not considered a reservoir species for influenza A viruses until January 2004, when H3N8 canine influenza virus was first isolated from racing greyhounds affected with respiratory disease in Florida (Crawford et al., 2005). Since then, the H3N8 virus has been identified in most states in the contiguous USA, affecting both racing greyhounds and pet dogs (Gibbs and Anderson, 2010). The transmission of H3N8 equine influenza viruses to dogs has been reported in Australia and the United Kingdom (Kirkland et al., 2010; Newton et al., 2007; Daly et al., 2008). In 2007, canine influenza caused by an H3N2 influenza virus was reported in South Korea (Song et al., 2008). Between May 2006 and October 2007, 4 H3N2 canine influenza virus isolates were identified from nasal swabs collected from sick dogs at animal clinics in Guangdong province in China (Li et al., 2010).

Phylogenetic analysis indicated that the H3N2 canine influenza viruses belonged to a different cluster than those of the H3N8 equine and canine influenza viruses (Song et al., 2008). All 8 genes of these H3N2 canine influenza viruses were ≥94.7% homologous to Asian H3N2 avian influenza viruses (Li et al., 2010). It was hypothesized that virus transmission occurred through feeding dogs infected poultry products or by aerosol transmission (Gibbs and Anderson, 2010). However, although virus isolates that are genetically similar to H3N2 canine viruses have been detected in birds, it is unclear whether the H3N2 canine viruses can re-infect birds, or be transmitted from birds to other dogs.

A serologic survey of H3N2 canine influenza virus was conducted in farmed and pet dogs in South Korea from June to December 2007, and the results indicated that the virus
had spread rapidly through the local dog population (Lee et al., 2009). The transmission of H3N2 canine viruses from dog to dog by contact was proven through experimentally infected and contact-exposed dogs (Song et al., 2009). Airborne transmission through aerosols and large droplets plays an important role in the spread of human influenza, but it is unknown whether airborne transmission of H3N2 canine influenza virus occurs between dogs.

In 2010, we isolated an H3N2 canine influenza virus from the lung of a Tibetan mastiff bought from a recent dog exhibition in eastern China. Herein, we describe the biological properties of this canine influenza virus isolate.

2. Materials and methods

2.1. Outbreaks

In March 2010, ten 3-month-old Tibetan mastiffs started to have fever, cough, purulent nasal discharge, and loss of appetite 2 days after they were bought from 3 different exhibit areas in a Tibetan mastiff exhibition in Zhejiang province of China. Two weeks later, 4 of them died; the others recovered. Nasal swabs were collected from these Tibetan mastiffs 7 days after they became ill. Lung samples were collected from the dogs that died.

2.2. Virus isolation

The nasal swabs and homogenated lung samples were used to isolate the influenza A virus by inoculation into 10-day-old specific pathogen-free (SPF) embryonated chicken eggs. After 3 days of incubation, the allantoic fluids were collected, and the hemagglutination (HA) test was performed using chicken erythrocytes (Rivailier et al., 2010). The fluids shown to agglutinate chicken erythrocytes were stored at –80 °C until use for virologic and molecular analyses.

The allantoic fluid from samples capable of agglutinating chicken erythrocytes was tested using a commercial rapid influenza virus antigen detection kit (Quicking Biotech Co., Ltd, Shanghai, China). Hemagglutinin inhibition (HI) tests were performed according to the recommendations of the World Organization for Animal Health.

2.3. RT-PCR and sequencing

Viral RNAs were extracted from allantoic fluid using the Trizol reagent (Invitrogen, Carlsbad, CA) according to the manufacturer’s instructions. Reverse transcription was performed under standard conditions using 12 bp primers (AGCAAAAGCAGG). PCR amplification was performed using universal primers for influenza A virus (Hoffmann et al., 2001). PCR products were directly sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (ABI, Foster, CA, USA) and an ABI 3500 genetic analyzer. To compile and analyze the sequences, we used the SEQMAN program (DNASTAR, Madison, WI). The sequences of reference viruses were obtained from GenBank using the basic local alignment search tool (BLAST) at the National Center for Biotechnology Information (NCBI). The nucleotide sequences were compared and phylogenetic trees were generated using the MEGALIGN program (DNASTAR) with the Clustal alignment algorithm. The sequences of canine, avian, equine, human, and swine influenza viruses selected from GenBank were used as references (data not shown). The reliability of phylogenetic inference at each tree node was estimated by the bootstrap method with 1000 replications, using the MEGA package. In addition, the signal peptide was predicted using the signalP version 2.0 (SignalP–NN and SignalP–HMM software) of SignalP World Wide Web server (Bendtsen et al., 2004).

2.4. Animal experiments

All the animal experiments were approved by the Review Board of Shanghai Veterinary Research Institute and by the Animal Care and Use Committee of Shanghai Veterinary Research Institute. All the data was analyzed using the PASW Statistics 18.0 software.

2.5. Dogs

To determine the pathogenicity of the virus isolate, six 10-week-old beagle puppies (Pet Kennel Breeding Center, Suzhou) free of influenza virus infection (group I) were inoculated intranasally with 10^2 50% infectious doses for eggs (EID_{50}) ZJ0110 virus in a volume of 1 mL of allantoic fluids. Animals were housed in a very large cage in a biosafety level II room. Three beagles inoculated intranasally with PBS in a volume of 1 mL were used as negative controls (group II) and housed separately. Before inoculation, the beagles were sedated by intramuscular injection of 0.1 mg/kg acepromazine malate (Shengda Animal Medicine Company, Jilin, China). Four days after inoculation, 3 beagles in group I were euthanized. Samples of nasal tissue, trachea, lung, kidney, brain, liver, pancreas, spleen, and blood were collected and homogenized in 1 mL of cold phosphate-buffered saline per gram of organs. Solid debris was pelleted by centrifugation. Virus titers of undiluted and 10-fold serially diluted supernatants were determined in 9–11-day-old embryonated eggs, and 3 eggs were used per dilution. The HA titer of allantoic fluid was tested 72 h post inoculation, and eggs with an HA titer of 8 or more were considered positive. On days 2, 4, and 6 after inoculation, the other 3 beagles in group I and all 3 beagles in group II were sedated, and their nasal tracts were washed using 5 mL PBS. The nasal washes were collected, and virus titers were determined using 9-day-old SPF embryonated chicken eggs. The beagles in groups I and II were monitored daily for 14 days for signs of disease, and these beagles were euthanized on day 14 post inoculation. Samples of nasal tissue, trachea, and lung were collected for virus titration and blood samples were collected for HI test.

To test the airborne transmissibility of the virus, 3 naïve dogs (group III) were put into a cage that was placed 0.5 m away from the cage in which the 6 intranasally inoculated beagles were housed. This exposure took place on the day that the 6 beagles were inoculated with ZJ0110. On days 2, 4, and 6 after exposure, the beagles in group III were sedated, and their nasal tracts were washed using 5 mL PBS. The nasal washes were collected, and virus titers were
determined using 9-day-old SPF embryonated chicken eggs. Six days after exposure, all 3 beagles in group III were euthanized. Samples were collected, and virus titers were determined. For histopathological study, samples were fixed in 10% neutral buffered formalin solution, sectioned, and stained with hematoxylin and eosin. Alternatively, immunohistochemical analysis was conducted with a monoclonal antibody against the NP protein of influenza A viruses. To avoid transmission of the virus from inoculated dogs to naïve dogs via investigators, the beagles in group III were treated first each time, and all the personal protective equipment (PPE) was autoclaved after investigators had contacted with the inoculated dogs.

To verify the airborne transmissibility of ZJ0110, 2 beagles were inoculated intranasally with \(^{10^7}\) EID\(_{50}\) ZJ0110 viruses in a volume of 1 mL of allantoic fluids and housed in a large cage. Two days later, 3 naïve beagles were housed in another cage, which was placed 0.5 m away from the cage in which the 2 intranasally inoculated beagles were housed. The beagles were monitored for clinical symptoms, and seroconversions were tested by analyzing hemagglutination inhibition against ZJ0110 on day 21 after exposure. To avoid transmission of the virus from inoculated dogs to naïve dogs via investigators, measures were taken as described previously.

### 2.6. Mice and guinea pigs

To test the infectivity of ZJ0110 virus in mice, eleven 6-week-old SPF female BALB/c mice (Shanghai Slac Laboratory Animal Co. Ltd, Shanghai) were lightly anesthetized with CO\(_2\) and inoculated intranasally with \(^{10^6}\) EID\(_{50}\) of ZJ0110 virus in a volume of 50 μL. Three mice were euthanized on days 4 and 6 for virus titration of the lung, nasal turbinate, kidneys, spleen, and brain. Organs were collected, homogenized, and the virus titrated as previously described. The remaining 5 mice were monitored daily for 14 days for weight loss and mortality. As a negative control, 5 mice were inoculated with PBS to compare weight loss and mortality with infected mice.

To investigate the infectivity of ZJ0110 virus in guinea pigs, six 8-week-old guinea pigs free of influenza virus (Shanghai Slac Laboratory Animal Co. Ltd) were lightly anesthetized by intramuscular injection of 0.1 mg/kg acepromazine malate (Shengda Animal Medicine Company, Jilin, China) and inoculated intranasally with \(^{10^6}\) EID\(_{50}\) of ZJ0110 virus in a volume of 100 μL. Three guinea pigs were euthanized on days 4 and 14 for determination of virus titers in the lungs, nasal turbinate, kidneys, spleen, and brain. As a negative control, 3 guinea pigs were inoculated with PBS and euthanized on day 14.

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Amino acid residue (single-letter code) and position in the mature proteins of H3 subtype influenza viruses.
Organs were collected, homogenized, and virus titers determined as mentioned above.

2.7. Chickens and ducks

To investigate the infectivity of ZJ0110 virus in chickens and ducks, eleven 6-week-old SPF chickens and 9-week-old ducks (a local outbreed strain of shelducks), which were proved to be free of either the influenza A virus or the antibody against the virus, were inoculated intranasally with 10<sup>3</sup> EID<sub>50</sub> of ZJ0110 virus in a volume of 100 μL and housed in different isolators. Three chickens and 3 ducks were euthanized on days 4 and 6 for determination of virus titers in the lungs, trachea, liver, pancreas, kidneys, spleen, and brain. In addition, as a negative control, 5 chickens and 5 ducks were inoculated with PBS. The remaining infected and control chickens and ducks were continually monitored for 14 days for clinical symptoms and seroconversions were analyzed on day 14 after inoculation. During these days, pharyngeal and cloacal swabs were obtained for virus titer determination on days 3, 5, 7, 10, and 14 after infection.

3. Results

3.1. Virus isolation

Influenza A virus was isolated from nasal swabs of sick Tibetan mastiffs and homogenated lungs of deceased Tibetan mastiffs by inoculation into SPF embryonated chicken eggs. The allantoic fluids of the eggs inoculated with virus induced agglutination of chicken erythrocytes. Allantoic fluids were positive for influenza A virus in assays using a commercial rapid influenza virus antigen detection kit. The virus was identified as H3 subtype influenza virus by the HI test. The virus isolate from homogenated lung tissue of a deceased dog was named as A/canine/Zhejiang/01/2010 (ZJ0110) and was selected for further characterization.

3.2. Sequence analysis

Eight gene segments of ZJ0110 were amplified by RT-PCR using the universal primers for influenza A viruses, and the PCR products were sequenced. The sequences (GenBank accession JF714149–JF714156) were submitted to GenBank at NCBI. The HA and NA genes of the virus isolate shared a high degree of homology with avian-origin H3N2 canine influenza viruses when analyzed using BLAST at the NCBI. The influenza virus isolate was designated A/canine/Zhejiang/01/2010(H3N2) (ZJ0110).

The critical determinants of host species specificity of influenza virus are distributed over several genes (Suzuki et al., 2000). To identify residues within the 8 genes that may be associated with adaptation of a bird virus to the canine host, we compared the amino acid sequences of canine H3N2 influenza virus with those of other H3 viruses. Thirty amino acid substitutions differentiate the

![Phylogenetic trees](image)

**Fig. 1.** Phylogenetic trees for the HA genes of H3N2 influenza A viruses. The tree was generated using MEGALIGN software (DNASTAR) on the basis of the HA gene sequences: nucleotides 30–1731 (1702 bp) of HA. The length of each pair of branches represents the distance between sequence pairs, and the units at the bottom of the tree indicate the number of substitution events. Virus sequences referred to in the tree were obtained from GenBank.
canine influenza viruses from avian H3N2 influenza viruses (Table 1). In the HA protein, leucine was found at position 238 only in the canine influenza viruses; this observation was true for both the H3N2 and H3N8 subtypes.

3.3. Phylogenetic analysis

Phylogenetic comparison of the H3 nucleotide sequences showed that ZJ0110 was most closely related to the H3N2 influenza virus isolates found in South Korea recently (Fig. 1). ZJ0110 and other canine H3N2 virus isolates formed a sub-clade that was separated from the clades of bird H3 influenza viruses. This clade was 1 of 3 that together included all H3 subtype influenza viruses (Fig. 1). The phylogenetic comparisons of ZJ0110 and A/ canine/Korea/GCVP01/2007 (H3N2; one of the canine influenza virus isolates in South Korea in 2007) with respect to the PA, PB1, PB2, HA, NP, NA, M and NS genes showed 99.0%, 99.2%, 99.0%, 99.2%, 98.7%, 99.1%, and 98.4% identity, respectively.

3.4. Pathogenicity of the ZJ0110 virus in canines

To assess the pathogenicity of the ZJ0110 virus in dogs, 6 beagles were inoculated intranasally with ZJ0110. The dogs presented with sneezing, coughing, and a runny nose 2 days after inoculation; fever began 3 days after inoculation. The body temperature of dogs returned to normal 7 days after inoculation. However, sneezing and cough continued until the dogs were euthanized on day 14 after inoculation (Fig. 2A). The body temperatures of puppies inoculated with CIV were significantly higher than those of control puppies (p < 0.05) on days 3, 4 and 5 post inoculation. Viruses in nasal washes collected from 3 intranasally inoculated beagles were titrated in 9-day-old SPF embryonated chicken eggs (Fig. 2B). The virus titers were 10^{2.75}–10^{3.75} EID_{50}/mL on day 2 after inoculation and increased to 10^{3.5}–10^{4.75} EID_{50}/mL on day 4 after inoculation. On day 6 after inoculation, the virus titer of nasal washes from 1 beagle decreased to 10^3 EID_{50}/mL, and those from the other 2 beagles remained at 10^{3.5} EID_{50}/mL (Fig. 2B). The virus titers in the nasal tract, trachea, and lung tissues of the intranasally inoculated beagles euthanized on days 4 and 14 after inoculation were also determined as previously described. The virus titers were 10^{1.5}–10^{2.5} EID_{50}/g in nasal tissues, 10^{2.5}–10^{3.5} EID_{50}/g in tracheal tissues, and 10^{2.5}–10^{3.5} EID_{50}/g in lung tissues of the beagles euthanized on day 4 after inoculation. Surprisingly, even on day 14 after inoculation, virus infection were significantly lower than the titers in both the inoculated puppies euthanized on day 4 and the indirect-exposure puppies euthanized on day 6 post exposure (p < 0.05). However, the titers of lung tissue from inoculated puppies euthanized on day 14 post infection were significantly higher than those from inoculated puppies euthanized on day 4 post infection (p < 0.01) and the indirect-exposure puppies euthanized on day 6 post exposure. The titers of nasal tissue from inoculated puppies euthanized on day 14 post infection were not decreased compared to those of puppies euthanized on day 4 post infection (p > 0.05).
titers in lungs were high, at $10^{4.25-10^{4.75}} \text{EID}_{50}/\text{g}$. Virus titers in nasal and tracheal tissues decreased to $10^{1.25-10^{1.5}}$ and $0-10^{1.75} \text{EID}_{50}/\text{g}$, respectively (Fig. 2C). No virus was detected in the brain, kidney, liver, or spleen of the infected beagles. Among the blood samples collected on day 14 post inoculation, sera collected from 1 beagle in group I had HI activity and was associated with an HI titer of 1:80. The sera collected from the remaining 2 beagles in group I and the beagles in group II did not show any HI activity.

To test the transmissibility of the virus, 3 naïve beagles were exposed to the virus by indirect contact on the day when the beagles were inoculated with ZJ0110. On days 2, 4, and 6 after contact, nasal washes were collected from these beagles. The virus in nasal washes was not detectable on day 2 after exposure; however, on day 4, virus titers increased to $10^{2.75-10^{3.0}} \text{EID}_{50}/\text{ml}$, and on day 6, virus titers reached $10^{3.0-10^{4.5}} \text{EID}_{50}/\text{ml}$ (Fig. 2B). On day 6 after exposure, virus titers in the nasal tract, trachea, and lung tissues of the beagles with indirect exposure were $10^{3.25-10^{4.5}}, 10^{2.25-10^{2.5}},$ and $10^{3.25-10^{3.5}} \text{EID}_{50}/\text{g}$, respectively (Fig. 2C). To verify the airborne transmissibility of ZJ0110, 3 naïve beagles were exposed indirectly to the dogs that were intranasally inoculated with ZJ0110 before 2 days. The beagles started to present sneezing, coughing, and a runny nose on day 6 after exposure; fever was observed 7 days after exposure. The body temperature of the dogs returned to normal on day 10 after exposure, and the cough lasted for 2 weeks. Serum antibody titers were measured by HI test against ZJ0110 on day 21 after exposure. The HI titers of the 3 dogs reached to 320, 640, and 160, respectively.

Histopathological analysis revealed heterophil granulocyte infiltration, exudative inflammation, and pulmonary congestion in all of the beagles that were inoculated or had indirect exposure (Fig. 3A). Immunohistochemical analysis showed large amounts of viral antigens in the lung tissues of these beagles (Fig. 3C).

### 3.5. Pathogenicity and replication of ZJ0110 virus in mice

Mice inoculated intranasally with $10^6 \text{EID}_{50}$ of ZJ0110 virus lost weight on days 1 and 2 after inoculation, followed by a slow increase in weight and recovery on day 10 after inoculation (Fig. 4A). In mice inoculated intranasally with $10^6 \text{EID}_{50}$ of ZJ0110 virus, virus titers in lungs and nasal turbinates were $10^{4.5-10^{5.75}}$ and $10^{2.5-10^{3.5}} \text{EID}_{50}/\text{ml}$, respectively, on day 4 after inoculation. Even on day 6 after inoculation, the virus titers were still as high as $10^{3.25-10^{5.25}} \text{EID}_{50}/\text{g}$ in lungs and $10^{2.25-10^{4.5}} \text{EID}_{50}/\text{g}$ in nasal turbinates (Fig. 4B). ZJ0110 virus was not detected in other tissues, including the brain, spleen, liver, and kidneys.

### 3.6. Replication of the ZJ0110 virus in guinea pigs

The virus replicated well in the nasal turbinates of guinea pigs inoculated intranasally with $10^6 \text{EID}_{50}$ of

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Fig. 3. The Image of lesion found on the lung of puppies infected by ZJ0110. Histopathological studies showed (A) severe pneumonia in the lung tissue of infected puppies and (B) normal lung section in uninfected puppies. The lung sections of (C) infected or (D) uninfected puppies were subjected to immunohistochemistry with a monoclonal antibody against NP of influenza A virus. Viral antigens were detected in the lung tissue of infected puppies, but no antigens were detected in lung tissue of uninfected puppies.
ZJ0110 virus. The virus titers ranged from $10^{4.25}$ to $10^{4.5}$ on day 4 after inoculation. Surprisingly, on day 14 after inoculation, the virus titers remained at $10^{2.75}$–$10^{3.75}$ EID$_{50}$/g in nasal turbinates of guinea pigs (Fig. 5). However, the virus was not detected in the lungs or other organs.

3.7. Pathogenicity of the ZJ0110 virus in chickens and ducks

To investigate the infectivity of ZJ0110 virus in chickens and ducks, the virus titers in the lung, trachea, liver, pancreas, kidneys, spleen, and brain of chickens and ducks inoculated intranasally with $10^6$ EID$_{50}$ of ZJ0110 virus were analyzed. ZJ0110 virus was not found in any of these organs, neither on day 4 nor day 6 after inoculation. In addition, no virus was detected in the pharyngeal or cloacal swabs collected on days 3, 5, 7, 10, and 14 after infection, and no seroconversion was observed in the samples from the inoculated chickens or ducks by performing the HI test. These results suggested that the ZJ0110 virus had lost the ability to replicate in chickens and ducks.

4. Discussion

Influenza viruses that cause illness in canines are a concern for canine health worldwide (Gibbs and Anderson, 2010). Since the isolation of H3N8 canine influenza virus from racing greyhounds in 2004, the virus has reportedly caused respiratory outbreaks among pet dogs in the USA (Crawford et al., 2005). The transmission of H3N8 equine influenza viruses to dogs has been reported in Australia and the United Kingdom (Kirkland et al., 2010; Newton et al.,
In 2007, the H3N2 influenza virus was first isolated from miniature schnauzer, cocker spaniel, and Jindo dogs in South Korea (Song et al., 2008). In this study, we isolated the H3N2 influenza virus from the Tibetan mastiff, which is an ancient breed of domestic dog (Canis lupus familiaris) that originated with nomadic cultures of Central Asia. Host species specificity of influenza virus has been attributed to multiple amino acids in several genes (Suzuki et al., 2000). The receptor-binding sites on HA and sites 627 and 701 on PB2 have proven to be important in interspecies transmission of avian influenza virus to mammals (Hatta et al., 2001; Li et al., 2005; Skehel and Wiley, 2000). In this study, we identified 30 amino acids that distinguish the canine H3N2 influenza viruses from other H3 viruses (Table 1). In the HA protein, leucine was present at position 238 only in the canine influenza viruses, including both the H3N2 and H3N8 subtypes. Whether these amino acids play an important role in the adaptation of avian influenza virus to dogs is unclear.

Dogs infected with canine influenza virus excrete virus in nasal discharge but not in feces (Song et al., 2008), and the virus can be transmitted to susceptible dogs through direct contact with dogs who are experimentally infected (Song et al., 2009). These findings suggested that dog-to-dog transmission of subtype H3N2 occurs through the nasal route. In the present study, we clearly demonstrated that the H3N2 canine influenza virus is transmissible to susceptible dogs through airborne transmission. Influenza A viruses are reported to be shed for 2–4 weeks by ducks, and 45 days by pheasants (Humberd et al., 2007; Webster et al., 1992), but are rarely reported to be shed for more than 10 days post-infection in mammals. In this study, we found that ZJ0110 could exist at a high titer for up to 14 days in the lungs of infected dogs. The reason for this viral persistence in the lungs should be investigated in future studies.

Influenza viruses are transmitted from the avian reservoir to other birds and mammals relatively frequently, yet they do not typically establish permanent lineages in these new hosts (Webster, 2002). However, we know little about transmission of canine influenza viruses to birds and mammals. Experimental induction of the disease caused by this isolate resulted in severe pathologic changes and showed that the virus titers remained high from 4 to 14 days after infection in the lungs of infected dogs. These findings indicate that the infected dogs excreted influenza virus (H3N2) for a long period of time. Influenza viruses of the H3 subtype have proven to be highly adaptable and are able to recruit avian, mammalian, and human hosts (Harder and Vahlenkamp, 2009). In this study, we showed that the canine H3N2 influenza virus was able to infect mice and guinea pigs but not ducks or chickens. This finding suggests that canine H3N2 influenza virus has adapted to replicate in different mammal species. Although the canine H3N2 virus is genetically and antigenically different from strains currently circulating in humans, we should remain aware of the potential of this virus to be transmitted to the human population from pet dogs.

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