

## Transmission of Equine Influenza Virus to Dogs

P. C. Crawford,<sup>1</sup> Edward J. Dubovi,<sup>2</sup> William L. Castleman,<sup>1</sup> Iain Stephenson,<sup>3</sup> E.P.J. Gibbs,<sup>1</sup> Limei Chen,<sup>3</sup> Catherine Smith,<sup>3</sup> Richard C. Hill,<sup>1</sup> Pamela Ferro,<sup>4</sup> Justine Pompey,<sup>3</sup> Rick A. Bright,<sup>3</sup> Marie-Jo Medina,<sup>3</sup> Influenza Genomics Group,<sup>3\*</sup> Calvin M. Johnson,<sup>5</sup> Christopher W. Olsen,<sup>6</sup> Nancy J. Cox,<sup>3</sup> Alexander I. Klimov,<sup>3</sup> Jackie M. Katz,<sup>3</sup> Ruben O. Donis<sup>3†</sup>

<sup>1</sup>College of Veterinary Medicine, University of Florida, Gainesville, FL, USA. <sup>2</sup>College of Veterinary Medicine, Cornell University, Ithaca, NY, USA. <sup>3</sup>Division of Viral and Rickettsial Diseases, Centers for Disease Control and Prevention, Atlanta, GA, USA. <sup>4</sup>College of Veterinary Medicine, Texas A&M University, College Station, TX, USA. <sup>5</sup>College of Veterinary Medicine, Auburn University, Auburn, AL, USA. <sup>6</sup>School of Veterinary Medicine, University of Wisconsin-Madison, Madison, WI, USA.

\*Influenza Genomics Group members are listed in the Supporting Online Material.

†To whom correspondence should be addressed. E-mail: rvd6@cdc.gov.

**Molecular and antigenic analyses of three influenza viruses isolated from outbreaks of severe respiratory disease in racing greyhounds revealed that they are closely related to H3N8 equine influenza virus. Phylogenetic analysis indicated that the canine influenza virus genomes form a monophyletic group, consistent with a single interspecies virus transfer. Molecular changes in the hemagglutinin suggested adaptive evolution in the new host. The etiologic role of this virus in respiratory disease was supported by the temporal association of rising antibody titers with disease and by experimental inoculation studies. The geographic expansion of the infection and its persistence for several years indicates efficient transmission of canine influenza virus among greyhounds. Evidence of infection in pet dogs suggests that this infection may also become enzootic in this population.**

Transmission of virus from one host species to another is a crucial feature of the ecology and epidemiology of influenza virus (1). Two basic mechanisms of interspecies transmission of influenza virus are possible (2, 3). One is the direct transfer of an essentially unaltered virus from one species to another. Examples of this mechanism include the recent human infections with the H5N1 subtype of avian influenza virus (4–6). The second mechanism is a consequence of the segmented nature of the influenza genome. Simultaneous co-infection of a host with viruses from different species can result in reassortment of the segmented viral genes and the generation of a reassortant virus with the ability to infect other species. For example, novel viruses generated by gene reassortment between avian and human influenza viruses resulted in influenza pandemics in 1957 and 1968 (2, 3, 7).

Most direct transmissions of whole influenza viruses from the natural host species to a different one do not result in sustained transmission in the new host species. Multiple virus-host interactions are necessary for replication and horizontal transmission and provide a barrier to perpetuation of influenza viruses in the new host (8). Therefore, establishment of new long-lived host-specific lineages of influenza virus is uncommon and has only occurred in domestic poultry, pigs, horses, and humans (2, 3). In this report, we describe an unprecedented interspecies transfer of a complete equine influenza virus to the dog, and the emergence of a new canine-specific influenza virus associated with acute respiratory disease.

In January 2004, an outbreak of respiratory disease occurred in 22 racing greyhounds at a Florida racetrack (supporting online text). Two clinical syndromes were evident: a milder illness characterized by initial fever and then cough for 10–14 days with subsequent recovery (14 dogs), or a peracute death associated with hemorrhage in the respiratory tract (8 dogs for a case-fatality rate of 36%). Postmortem examinations were performed on 6 of the 8 fatal cases (9). All dogs had extensive hemorrhage in the lungs, mediastinum, and pleural cavity. Histological examination of the respiratory tract revealed tracheitis, bronchitis, bronchiolitis, and suppurative bronchopneumonia (fig. S1). The epithelial lining and airway lumens in these tissues were infiltrated by neutrophils and macrophages. Lung homogenates prepared from these dogs were inoculated into a variety of monkey, human, bovine, and canine cell lines for virus culture (9). The lung homogenate from one dog caused cytopathic effects in Madin-Darby canine kidney epithelial cells (MDCK) cultured in the presence of trypsin, and the cell culture supernatant agglutinated chicken red blood cells (9).

Preliminary evidence of an influenza type A virus was provided by a commercial ELISA for detection of the nucleoprotein of influenza A and B viruses, and by PCR analysis using primers specific for the matrix gene of influenza A viruses (9). In addition, the hemagglutinating activity was inhibited by reference antisera to the equine influenza A H3 subtype, but not by antisera specific for avian, swine, and human influenza A subtypes H1-H11 and H13 (table S1) (9). To characterize the molecular properties of the virus, we determined the nucleotide sequences of the 8 RNA segments of the viral genome (9). Sequence comparisons with known influenza virus genes and phylogenetic analyses indicated that the 8 genes of the canine isolate were most similar to those from contemporary equine influenza A (H3N8) viruses, with which they shared >96% sequence identity (Fig. 1A, table S2). In contrast, representative genes from avian, swine, and human influenza A isolates had 80-94% identity with the canine isolate (table S2). These data identified the canine isolate, named A/canine/Florida/43/2004 (canine/FL/04), as an influenza A H3N8 virus closely related to contemporary equine influenza viruses. Since all genes of the canine isolate were of equine influenza virus origin, we concluded that the entire genome of an equine influenza virus had been transmitted to the dog.

To investigate the role of the canine/FL/04 virus in the clinical and pathological observations in the greyhounds, we performed immunohistochemical staining (IHC) on lung tissues using a monoclonal antibody to influenza A H3 (9). Viral H3 antigen was consistently detected in the cytoplasm of bronchial and bronchiolar epithelial cells, bronchial gland epithelial cells, and macrophages in airway lumens and alveolar spaces (Fig. 2A). These data supported a diagnosis of pulmonary infection with influenza virus of the H3 subtype in the dogs.

To determine involvement of a canine/FL/04-like virus in the etiology of the respiratory disease outbreak, we analyzed paired acute and convalescent sera from 11 sick dogs and 16 asymptomatic contacts for virus-specific antibodies using hemagglutination inhibition (HI) and microneutralization (MN) assays (9). Seroconversion, defined as a  $\geq 4$ -fold rise in antibody titers to canine/FL/04 from the acute to convalescent phase, occurred in 8 of 11 (73%) sick dogs in both assays (table S3). Seroconversion occurred in 6 of 16 (38%) asymptomatic contacts in the HI assay, while 8 of 16 (50%) seroconverted in the MN assay (table S3). The seroconversion data demonstrated infection of the dogs with a canine/FL/04-like virus which coincided temporally with the onset of respiratory disease in most animals.

Single serum samples were collected 3 months after the outbreak from an additional 46 asymptomatic dogs housed with the sick dogs. Of these, 43 (93%) were seropositive in both assays. For the total population of 73 dogs tested, 93%

were seropositive in both assays, including 82% (9/11) of the sick dogs and 95% (59/62) of the healthy contacts. The high seroprevalence in dogs with no history of respiratory disease indicates that infections with canine influenza virus can be subclinical and suggests efficient spread of the virus among dogs.

To better understand the capacity of the canine/FL/04 virus to infect dogs, four 6-month old purpose-bred beagles were each inoculated with  $10^{6.6}$  median tissue culture infectious doses (TCID<sub>50</sub>) by the intratracheal and intranasal routes (9). All dogs developed a fever (rectal temperature  $\geq 39^\circ\text{C}$ ) for the first 2 days postinoculation (p.i.), but none exhibited respiratory signs such as cough or nasal discharge over a 14-day observation period. Virus shedding was examined by quantification of virus in nasal and oropharyngeal swabs (9). Only 2 of the 4 dogs shed detectable amounts of virus. One dog shed virus on days 1 and 2 p.i. ( $1.0$ - $2.5 \log_{10}$  PFU per swab), whereas the other dog shed virus for 4 consecutive days after inoculation ( $1.4$ - $4.5 \log_{10}$  PFU per swab). Postmortem examination of 2 dogs on day 5 p.i. revealed necrotizing and hyperplastic tracheitis, bronchitis, and bronchiolitis similar to that found in the greyhounds, but there was no pulmonary hemorrhage or bronchopneumonia (9). Viral H3 antigen was detected in epithelial cells of bronchi, bronchioles, and bronchial glands of both dogs by IHC (Fig. 2B). Infectious virus was recovered from the lung tissue of one of the dogs (9). Postmortem examination of the remaining 2 dogs on day 14 p.i. showed minimal histological changes in respiratory tissues, no viral H3 antigen by IHC, and no recovery of virus from lung homogenates. Seroconversion in these latter 2 dogs was detected in MN assays by day 7 p.i., with a further 2-to 3-fold increase in antibody titers by day 14. These results established the susceptibility of dogs to infection with canine/FL/04, as evidenced by the febrile response, virus shedding from the upper respiratory tract, presence of viral antigen and infectious virus in the lungs, histopathological findings typical for influenza, and seroconversion. The failure to reproduce severe disease and death in the experimentally inoculated beagles is not surprising since a large proportion of the naturally infected greyhounds were asymptomatic.

To investigate whether a canine/FL/04-like influenza virus had circulated among greyhound populations in Florida prior to the January 2004 outbreak, archival sera from 65 racing greyhounds were tested for the presence of antibodies to canine/FL/04 using the HI and MN assays (9). There were no detectable antibodies in 33 dogs sampled from 1996 to 1998. Of 32 dogs sampled between 2000 and 2003, 9 were seropositive in both assays - 1 in 2000, 2 in 2002, and 6 in 2003 (table S4). The seropositive dogs were located at Florida tracks involved in outbreaks of respiratory disease of unknown etiology from 1999 to 2003, suggesting that a

canine/FL/04-like virus may have been the causative agent of those outbreaks. To investigate this possibility further, we examined archival tissues from greyhounds that died from hemorrhagic bronchopneumonia in March 2003 (9). Lung homogenates from one dog inoculated into MDCK cells yielded H3N8 influenza virus, termed A/canine/Florida/242/2003 (canine/FL/03), in the first passage (9). Sequence analysis of the complete genome of canine/FL/03 revealed >99% identity to canine/FL/04 (table S5), indicating that canine/FL/04-like viruses had infected greyhounds prior to 2004.

From June to August 2004, respiratory disease outbreaks occurred at 14 tracks in 6 states (Florida, Texas, Alabama, Arkansas, West Virginia, and Kansas) with a combined population of ~10,000 racing greyhounds (supporting online text). We collected paired acute and convalescent sera from 94 dogs located at 4 Florida tracks: 56% of these dogs had ≥4-fold rises in antibody titers to canine/FL/04, and 100% were seropositive (Table 1 and table S6). Convalescent sera from 29 dogs in West Virginia and Kansas also had antibodies to canine/FL/04.

From January to May 2005, respiratory disease outbreaks occurred at 20 tracks in 11 states (Florida, Texas, Arkansas, Arizona, West Virginia, Kansas, Iowa, Colorado, Rhode Island, and Massachusetts) with a combined population of ~20,000 greyhounds (supporting online text). We collected paired acute and convalescent sera from 96 dogs located at 7 Florida tracks: 58% of these dogs seroconverted to canine/FL/04 and 100% were seropositive (Table 1 and table S7). For paired samples from 25 dogs at a West Virginia track, 84% of the dogs seroconverted and 100% were seropositive. For paired samples from 10 dogs at a Wisconsin track, 50% of the dogs seroconverted and 100% were seropositive. Convalescent sera from an additional 13 dogs at this track and 115 dogs at 2 Arizona tracks contained antibody to canine/FL/04.

We isolated influenza A (H3N8) virus from the lung of a greyhound that died of hemorrhagic bronchopneumonia at a Texas track in July 2004. Sequence analysis of the HA and NA of this isolate, named A/canine/Texas/1/2004 (canine/TX/04), revealed ≥99% identity to canine/FL/04 (table S5). The isolation of three closely related influenza viruses from fatal canine cases over a 16-month period and from different geographic locations, together with the substantial serological evidence of widespread infection among racing greyhounds, suggested sustained circulation of a canine/FL/04-like virus in this population.

The high prevalence of canine influenza infection in racing greyhounds suggested that the pet dog population might be at risk of infection. Serological tests were performed on 70 dogs with respiratory disease in a shelter facility in northeast Florida, 4 veterinary clinics located in the northeast, north

central, south, and southwest regions of Florida, and one veterinary clinic located in New York (supplemental online text). Ninety-seven percent of the shelter and pet dogs were positive for antibody to canine/FL/04 (Table 1 and table S8). The serologic evidence of influenza virus infection associated with respiratory disease in shelter and pet dogs of various breeds indicated the lack of genetic barriers to infection in the dog population and the spread of the virus to pet populations of regions of the country without greyhound racing.

Phylogenetic analysis (9) of the HA genes of canine/FL/03, canine/FL/04, and canine/TX/04 showed that they constitute a monophyletic group with robust bootstrap support. The canine influenza genes were most closely related to the equine H3 “Florida lineage” that emerged in the early 1990s (Fig. 1B) (10). Phylogenetic analysis and pairwise nucleotide sequence comparisons (9) of the other 7 genomic segments supported the segregation of the canine genes as a distinct sub-lineage most closely related to the equine virus lineage (table S2 and table S9). Together with identification of infected dogs in widespread geographical locations from 2003 to 2005, these data are most consistent with a single virus transmission event from horses to dogs with subsequent horizontal spread of the adapted virus in the canine population. However, repeated introductions of this unique lineage of influenza virus from an unidentified reservoir species cannot be formally excluded, unlikely as it may be. The presence of virus in the nasal passages and oropharynx of the experimentally inoculated dogs indicate that shedding is possible, and that dog-to-dog transmission of virus by large droplet aerosols, fomites, or direct mucosal contact could play a role in the epizootiology of the disease.

The viral HA is a critical determinant of host species specificity of influenza virus (11). To identify residues within HA that may be associated with adaptation of an equine virus to the canine host, we compared the amino acid sequence of canine HAs to those of contemporary equine viruses. Four amino acid changes differentiate the equine and canine HA consensus amino acid sequences: N83S, W222L, I328T, and N483T (Table 2). The substitution of serine for asparagine at position 83 is a change of unknown functional significance since various polar residues are found in H3 molecules from other species. The strictly conserved isoleucine at position 328 near the cleavage site of the H3 HA has been replaced by threonine. The pivotal role of HA cleavage by host proteases in pathogenesis suggests that this change merits further study. The substitution of leucine for tryptophan at position 222 is quite remarkable because it represents a non-conservative change adjacent to the sialic acid binding pocket which could modulate receptor function (12). Interestingly, leucine at position 222 is not unique to canine H3 HA since it is typically found in the H4, H8, H9, and H12 HA subtypes (13, 14). The leucine substitution may be more compatible with

virus specificity for certain mammalian hosts since infections of swine with subtype H4 (15) and humans and swine with subtype H9 (16) viruses have been reported. The substitution of asparagine with threonine at position 483 resulted in the loss of a glycosylation site in the HA2 subunit that is conserved in all HA subtypes (17). Although the importance of these amino acid changes in the HA for adaptation of an equine virus to dogs remains to be determined, similar amino acid changes have been observed previously in association with interspecies transfer (18, 19).

The interspecies transfer of a whole mammalian influenza virus to an unrelated mammal species is a relatively rare event. Previous studies have provided limited serological or virological evidence, but not both, of transient infection of dogs with human influenza A (H3N2) viruses (20–23). However, there was no evidence of sustained circulation in the canine host. Although direct transfer of swine influenza viruses from pigs to people is well-documented (24–27), there is no evidence for adaptation of the swine viruses in human hosts. In this report, we provide virological, serological, and molecular evidence for interspecies transmission of an entire equine influenza A (H3N8) virus to another mammalian species, the dog. Unique amino acid substitutions in the canine virus HA, coupled with serological confirmation of infection of dogs in multiple states in the U.S., were correlated with sustained circulation of the virus in the canine population. Evidence of canine influenza infection in pet dogs, a primary companion animal for humans, raises the possibility that dogs may provide a new source for transmission of novel influenza A viruses to humans.

## References and Notes

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## Supporting Online Material

[www.sciencemag.org/cgi/content/full/1117950/DC1](http://www.sciencemag.org/cgi/content/full/1117950/DC1)

Materials and Methods

SOM Text

Fig S1

Tables S1 to S9

References

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**Fig. 1.** Phylogenetic relationships among the hemagglutinin genes. (A) Tree of HA genes from representative canine,

human, avian, swine, and equine isolates, using A/Budgerigar/Hokkaido/1/77 (H4) as the outgroup (indicated by arrowhead and H4). **(B)** Tree of the canine influenza virus HA genes with contemporary and older equine HA genes, using A/Duck/Ukraine/63 (H3) as the outgroup. Phylogenetic trees were inferred from nucleotide sequences by the neighbor joining method, and bootstrap analysis values  $\geq 90\%$  are shown. The bar denotes the number of nucleotide changes per unit length of the horizontal tree branches.

**Fig. 2.** Immunohistochemical detection of influenza H3 antigen in the lungs. Lung tissue sections were probed with a mouse monoclonal antibody to H3 hemagglutinin and binding was detected by immunoperoxidase reaction (brown precipitate). **(A)** Bronchial epithelium from a greyhound with disease. Viral H3 antigen was detected in bronchial epithelial cell cytoplasm and in macrophages in airway lumens and in alveolar spaces. **(B)** Bronchial epithelium from a beagle dog 5 days after inoculation with A/canine/Florida/43/04 (H3N8). Viral H3 antigen was detected in bronchial epithelial cell cytoplasm. Scale bar, 66  $\mu\text{m}$ .

**Table 1.** HI antibody responses to A/canine/Florida/43/04 (H3N8) in racing greyhounds and pet dogs with respiratory disease.

| HI Response <sup>a</sup>        | Greyhounds<br>2004 | Greyhounds<br>2005 | Pet dogs        |
|---------------------------------|--------------------|--------------------|-----------------|
| Number of dogs tested           | 94 <sup>b</sup>    | 96 <sup>c</sup>    | 70 <sup>d</sup> |
| Seroconversion (%) <sup>e</sup> | 56                 | 58                 | ND <sup>f</sup> |
| Seropositive (%) <sup>g</sup>   | 100                | 100                | 97              |
| GMT <sup>h</sup>                | 381                | 389                | 428             |

<sup>a</sup>Dogs with respiratory disease were tested by HI using A/canine/Florida/43/04 (H3N8).

<sup>b</sup>Number of dogs tested at 4 different tracks in Florida from June to August 2004.

<sup>c</sup>Number of dogs tested at 7 different tracks in Florida from January to April 2005.

<sup>d</sup>Number of pet dogs tested at 1 shelter and 4 veterinary clinics in Florida, and 1 veterinary clinic in New York. <sup>e</sup>Percentage of dogs with  $\geq 4$ -fold increase in antibody titer between paired acute and convalescent sera. <sup>f</sup>Convalescent serum samples only were collected from the pet dogs. <sup>g</sup>Percentage of dogs with a positive antibody titer (HI titer  $\geq 32$ ) in the convalescent sera. <sup>h</sup>Geometric mean antibody titer for the convalescent sera.

**Table 2.** Amino acid differences between the canine and equine H3 hemagglutinins.

| Equine H3 consensus | canine/FL/03 | canine/FL/04   | canine/TX/04 | Potential functional significance  |
|---------------------|--------------|----------------|--------------|--|
| G7*                 | D            | - <sup>†</sup> | -            | D also found in duck and human H3 HA   |
| I29                 | -            | M              | M            | I is conserved in H3 HAs from all species  |
| N83                 | S            | S              | S            | Various polar amino acids present at this position in H3 HAs of other species                  |
| 92                  | -            | N              | -            | N is present in some duck H3 HAs   |
| L118                | -            | -              | V            | L is conserved in all H3 HAs   |
| W222                | L            | L              | L            | W is conserved in most H3 HAs of all species; located near the receptor binding site           |
| I328                | T            | T              | T            | T is strictly conserved in all avian, swine or human H3 HAs                                    |
| G479                | G            | G              | E            | E present in equine isolates in 1970s  |
| N483                | T            | T              | T            | N occurs in all H3 and other HA subtypes. Replacement results in loss of a glycosylation site. |
| K541                | -            | R              | -            | Basic amino acid conservative change   |

\*Amino acid residue (single-letter code) and position in the mature H3 HA. The amino acid code is A, alanine; D, aspartic acid; G, glycine; I, isoleucine; K, lysine; L, leucine; M, methionine; N, asparagine; R, arginine; S, serine; T, threonine; V, valine; W, tryptophan. <sup>†</sup>Denotes no change from the consensus equine H3 HAs.



