

DESCRIPTION

INFLUENZA VIRUSES ABLE TO INFECT CANIDS, USES THEREOF

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CROSS-REFERENCE TO RELATED APPLICATION

This application is a continuation-in-part of application U.S. Serial No. 11/409,416, filed April 21, 2006; and this application claims priority to U.S. Serial Nos. 60/728,449, filed October 19, 2005; 60/754,881, filed December 29, 2005; 60/759,162, filed January 14, 2006; 10 60/761,451, filed January 23, 2006; and 60/779,080, filed March 3, 2006, the disclosure of each of which is hereby incorporated by reference herein in its entirety, including any brief summary, detailed descriptions of the invention, examples, claims, abstract, figures, tables, nucleic acid sequences, amino acid sequences, and drawings.

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BACKGROUND OF THE INVENTION

“Kennel cough” or infectious tracheobronchitis (ITB) is an acute, contagious respiratory infection in dogs characterized mainly by coughing (Ford *et al*, 1998). Canine ITB is considered one of the most prevalent infectious respiratory diseases of dogs worldwide, and outbreaks can reach epidemic proportions when dogs are housed in high- 20 density population environments such as kennels. Most outbreaks are due to direct dog-to-dog contact or aerosolization of respiratory secretions (Ford *et al*, 1998). The clinical signs are caused by infection with one or a combination of bacterial and viral agents that colonize the epithelium of the upper and lower respiratory tract. Canine parainfluenza virus (CPiV) and *Bordetella bronchiseptica* bacteria are the most common organisms isolated from 25 affected dogs, but several other viruses such as canine distemper virus (CDV) and canine adenoviruses-1 and -2 (CAV-1, CAV-2), along with bacteria such as *Streptococcus sp.*, *Pasteurella multocida* and *Escherichia coli*, can influence the clinical course and outcome (Ford *et al*, 1998). While outbreaks occur most efficiently and rapidly in high-density populations with high morbidity, complicated respiratory infections and death are 30 uncommon. Although life-threatening secondary bacterial pneumonia can develop, the majority of ITB cases are self-limiting and resolve without any treatment (Ford *et al*, 1998).

In July 1992, a respiratory infection presumed to be “kennel cough” became epidemic at several greyhound tracks in New England, Florida, West Virginia, Wisconsin, Kansas,

Colorado, Oklahoma and Arizona. According to veterinarians, most of the affected dogs had a mild cough that resolved, but more than a dozen greyhounds developed an acute hemorrhagic pneumonia followed by rapid death (Greyhound Daily News, 1999).

5 In late 1998 to early 1999, several outbreaks of "kennel cough" occurred in racing greyhound kennels across the country, resulting in mandatory closure of tracks and quarantine of all racing greyhounds in the U.S. for several weeks (Greyhound Daily News, 1999). At one track in Florida (Palm Beach Kennel Club), coughing was recorded in nearly 40% of the dog population on a single day (Personal communication from Dr. William Duggar). Similar to the outbreak in 1992, the coughing resolved in most greyhounds, but 10 dogs in Florida died from a hemorrhagic pneumonia syndrome uncharacteristic of "kennel cough" (Putnam, 1999).

15 In March-April 2003, another outbreak of "kennel cough" occurred at greyhound tracks in the eastern U.S. The outbreak is believed to have originated in kennels at four tracks in Florida and caused the suspension of racing and quarantine of dogs for almost three weeks. Nearly 25% of the dogs at the track in West Palm Beach were affected, while almost 50% of the 1400 dogs at Derby Lane in St. Petersburg developed coughing. Again, most dogs recovered, but several dogs have died from the respiratory infection. The estimated economic impact of the respiratory outbreak at the Derby Lane track alone was \$2 million.

20 There are no published reports documenting the etiology or clinicopathology of the "kennel cough" epidemics in racing greyhound kennels in 1992, 1998-1999, or 2003. The assumption has been that the infections were due to CPiV and/or *B. bronchiseptica*, the two most common causes of kennel cough. Unsubstantiated communications such as web sites have attributed the fatal hemorrhagic pneumonias reported in some of the coughing dogs to infection with β -hemolytic *Streptococcus equi* subspecies *zooepidemicus*, and refer to the syndrome as "canine streptococcal toxic shock."

25 Transmission of virus from one host species to another is a crucial feature of the ecology and epidemiology of influenza virus (Webster, 1998). Two basic mechanisms of interspecies transmission of influenza virus are possible (Webster *et al.*, 1992; Lipatov *et al.*, 2004). One is the direct transfer of an essentially unaltered virus from one species to another. 30 Examples of this mechanism include the recent human infections with the H5N1 subtype of avian influenza virus (Subbarao *et al.*, 1998; Peiris *et al.*, 2004; Guan *et al.*, 2004) and possibly the pandemic of 1918, known as Spanish flu (Reid *et al.*, 2004). The second mechanism is a consequence of the segmented nature of the influenza genome. Co-infection

of a host with viruses from different species can result in reassortment of the segmented viral genes and the generation of a recombinant with the ability to infect other species. For example, novel viruses generated by gene reassortment between avian and human influenza viruses resulted in human influenza pandemics in 1957 and 1968 (Webster *et al.*, 1992; Lipatov *et al.*, 2004; Kawaoka *et al.*, 1989).

Most direct transmissions of unaltered influenza viruses from the natural host species to a different species are terminal events because sustained transmission between individuals of the new species fails to occur. Multiple virus-host interactions are necessary for replication and horizontal transmission and provide a formidable barrier to perpetuation of influenza viruses in the new host (Webster *et al.*, 2004). Therefore, establishment of new host-specific lineages of influenza virus is uncommon and has only occurred in domestic poultry, pigs, horses, and humans (Webster *et al.*, 1992; Lipatov *et al.*, 2004).

Because of the serious nature of influenza virus infection, there remains a need for methods for diagnosing, preventing, and treating infection by influenza virus.

BRIEF SUMMARY OF THE INVENTION

The subject invention pertains to isolated influenza virus that is capable of infecting canids and causing respiratory disease in the canid. The subject invention also pertains to compositions and methods for inducing an immune response against an influenza virus of the present invention. The subject invention also pertains to compositions and methods for identifying a virus of the invention and diagnosing infection of an animal with a virus of the invention.

One aspect of the invention relates to vaccines and methods for protecting canines from canine influenza, kits comprising such vaccines, and methods for using such vaccines. This protection includes preventing, reducing the risk of, delaying the onset of, reducing the spread of, ameliorating, suppressing, and/or eradicating the influenza and/or one or more (typically two or more) of its symptoms. It is believed that the vaccines, kits, and methods of this invention are generally suitable for use with canines. Canines include wild, zoo, and domestic canines, such as wolves, coyotes, and foxes. Canines also include dogs, particularly domestic dogs, such as, for example, pure-bred and/or mongrel companion dogs, show dogs, working dogs, herding dogs, hunting dogs, guard dogs, police dogs, racing dogs, and/or laboratory dogs.

This invention is also directed, in part, to a method for protecting a canine from an influenza virus infection (*i.e.*, preventing, reducing the risk of, delaying the onset of, suppressing, ameliorating, or eradicating an influenza virus infection). The method comprises administering a therapeutically effective amount of a vaccine that comprises at least one equine influenza virus antigen, at least one H3 influenza virus antigen, and/or at least one H7 influenza virus antigen.

This invention also is directed, in part, to a method for protecting a canine from respiratory lesions (*i.e.*, preventing, reducing the risk of, delaying the onset of, suppressing, ameliorating, or eradicating respiratory lesions) caused by canine influenza virus. The method comprises administering to the canine a therapeutically effective amount of a vaccine that comprises at least one equine influenza virus antigen, at least one H3 influenza virus antigen, and/or at least one H7 influenza virus antigen.

This invention also is directed, in part, to a method for protecting a canine from having canine influenza virus in nasal or oral secretion (*i.e.*, preventing, reducing the risk of, delaying the onset of, suppressing, ameliorating, or eradicating canine influenza virus in nasal or oral secretion) caused by canine influenza virus infection. The method comprises administering to the canine a therapeutically effective amount of a vaccine that comprises at least one equine influenza virus antigen, at least one H3 influenza virus antigen, and/or at least one H7 influenza virus antigen.

This invention also is directed, in part, to a canine influenza vaccine. In some embodiments, for example, the vaccine comprises a therapeutically effective amount of at least one equine influenza virus antigen, at least one H3 influenza virus antigen, and/or at least one H7 influenza virus antigen.

This invention also is directed, in part, to a kit for protecting a canine from influenza virus infection. The kit comprises a therapeutically effective amount of a vaccine that comprises at least one equine influenza virus antigen, at least one H3 influenza virus antigen, and/or at least one H7 influenza virus antigen. In addition, the kit comprises at least one of the following:

an apparatus for administering the vaccine to the canine,

a pharmaceutically acceptable excipient that aids in administering the vaccine to the canine,

a pharmaceutically acceptable excipient that enhances the canine's immune response to the vaccine,

a food to be consumed by the canine simultaneously with the vaccine, and/or
a treat to be consumed by the canine simultaneously with the vaccine.

Further benefits of Applicants' invention will be apparent to one skilled in the art
from reading this specification.

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BRIEF DESCRIPTION OF THE DRAWINGS

Figures 1A-1B show phylogenetic relationships among the hemagglutinin genes.
Figure 1A shows a tree of HA genes from representative canine, human, avian, swine, and
equine isolates, including A/Budgerigar/Hokkaido/1/77 (H4) as outgroup. **Figure 1B** shows
a tree of the canine influenza virus HA genes with contemporary and older equine HA genes,
using A/Duck/Ukraine/63 (H3) as 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.

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Figures 2A-2B show 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).
Figure 2A shows bronchial epithelium from a greyhound with spontaneous disease. Viral
H3 antigen was detected in bronchial epithelial cell cytoplasm and in macrophages in airway
lumens and in alveolar spaces. **Figure 2B** shows bronchial epithelium from a dog 5 days
after inoculation with A/canine/Florida/43/2004 (H3N8). Viral H3 antigen was detected in
bronchial epithelial cell cytoplasm. Scale bar, 66 μm .

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Figure 3 shows the characteristic histological changes in the bronchi of greyhounds
that died from hemorrhagic pneumonia associated with influenza virus infection. The tissues
are stained with H&E. Upper panel: Normal bronchus with ciliated epithelial cells, mucous
cells, and basal cells. Lower panel: Bronchus from a greyhound with spontaneous influenza.
There is necrosis and erosion of the bronchial ciliated epithelial cells. Scale bar, 100 μm .

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Figures 4A-4B shows phylogenetic relationships among the H3 hemagglutinin genes.
Figure 4A shows a phylogenetic tree of the canine influenza virus HA genes with
contemporary and older equine HA genes. **Figure 4B** shows a phylogenetic tree of the
canine influenza virus HA protein with contemporary and older equine HA. Phylogenetic
trees were inferred from genetic or amino acid sequences by the neighbor joining method and

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bootstrap analysis values $\geq 80\%$ are shown. The bar denotes the number of amino acid changes per unit length of the horizontal tree branches.

Figure 5 shows Influenza virus H3 protein in epithelial cells of bronchi and bronchial glands in lungs of dogs that died of pneumonia associated with influenza virus infection. Upper panels: Erosion of ciliated bronchial epithelial cells in bronchi. Tissues were stained with H&E. Lower panels: Influenza virus H3 protein in the cytoplasm of bronchial (left) and bronchial gland (right) epithelial cells. Tissues were stained with a monoclonal antibody to influenza H3 detected by immunoperoxidase reaction (brown precipitate) and counterstained with hematoxylin.

Figures 6A-6D show amplification plots of H3 and Matrix genes (**Figure 6A** and **Figure 6B**) obtained from the amplification of 10-fold serially diluted *in vitro* transcribed RNA standards. Standard curves of H3 and Matrix genes (**Figure 6C** and **Figure 6D**) constructed by plotting the log of starting RNA concentrations against the threshold cycle (Ct) obtained from each dilution.

Figure 7 shows sensitivity of Directigen Flu A was tested with 10-fold serially diluted virus stocks including A/Wyoming/3/2003 and A/canine/FL/242/2003. The purple triangle indicates positive result.

BRIEF DESCRIPTION OF THE SEQUENCES

SEQ ID NO: 1 is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding a PB2 protein that can be used according to the present invention.

SEQ ID NO: 2 is the amino acid sequence encoded by SEQ ID NO: 1.

SEQ ID NO: 3 is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding a PB1 protein that can be used according to the present invention.

SEQ ID NO: 4 is the amino acid sequence encoded by SEQ ID NO: 3.

SEQ ID NO: 5 is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding a PA protein that can be used according to the present invention.

SEQ ID NO: 6 is the amino acid sequence encoded by SEQ ID NO: 5.

SEQ ID NO: 7 is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding an NS protein that can be used according to the present invention.

SEQ ID NO: 8 is the amino acid sequence encoded by SEQ ID NO: 7.

SEQ ID NO: 9 is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding an NP protein that can be used according to the present invention.

SEQ ID NO: 10 is the amino acid sequence encoded by **SEQ ID NO: 9**.

SEQ ID NO: 11 is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding an NA protein that can be used according to the present invention.

SEQ ID NO: 12 is the amino acid sequence encoded by **SEQ ID NO: 11**.

5 **SEQ ID NO: 13** is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding an MA protein that can be used according to the present invention.

SEQ ID NO: 14 is the amino acid sequence encoded by **SEQ ID NO: 13**.

SEQ ID NO: 15 is a nucleotide sequence of a canine influenza virus (Florida/43/04) encoding an HA protein that can be used according to the present invention.

10 **SEQ ID NO: 16** is the amino acid sequence encoded by **SEQ ID NO: 15**.

SEQ ID NO: 17 is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding a PB2 protein that can be used according to the present invention.

SEQ ID NO: 18 is the amino acid sequence encoded by **SEQ ID NO: 17**.

15 **SEQ ID NO: 19** is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding a PB1 protein that can be used according to the present invention.

SEQ ID NO: 20 is the amino acid sequence encoded by **SEQ ID NO: 19**.

SEQ ID NO: 21 is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding a PA protein that can be used according to the present invention.

SEQ ID NO: 22 is the amino acid sequence encoded by **SEQ ID NO: 21**.

20 **SEQ ID NO: 23** is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding an NS protein that can be used according to the present invention.

SEQ ID NO: 24 is the amino acid sequence encoded by **SEQ ID NO: 23**.

SEQ ID NO: 25 is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding an NP protein that can be used according to the present invention.

25 **SEQ ID NO: 26** is the amino acid sequence encoded by **SEQ ID NO: 25**.

SEQ ID NO: 27 is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding an NA protein that can be used according to the present invention.

SEQ ID NO: 28 is the amino acid sequence encoded by **SEQ ID NO: 27**.

30 **SEQ ID NO: 29** is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding an MA protein that can be used according to the present invention.

SEQ ID NO: 30 is the amino acid sequence encoded by **SEQ ID NO: 29**.

SEQ ID NO: 31 is a nucleotide sequence of a canine influenza virus (FL/242/03) encoding an HA protein that can be used according to the present invention.

SEQ ID NO: 32 is the amino acid sequence encoded by SEQ ID NO: 31.

SEQ ID NO: 33 is the mature form of the HA protein shown in SEQ ID NO: 16 wherein the N-terminal 16 amino acid signal sequence has been removed.

5 SEQ ID NO: 34 is the mature form of the HA protein shown in SEQ ID NO: 32 wherein the N-terminal 16 amino acid signal sequence has been removed.

SEQ ID NO: 35 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 36 is an oligonucleotide that can be used according to the present invention.

10 SEQ ID NO: 37 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 38 is an oligonucleotide that can be used according to the present invention.

15 SEQ ID NO: 39 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 41 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 42 is an oligonucleotide that can be used according to the present invention.

20 SEQ ID NO: 43 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 44 is an oligonucleotide that can be used according to the present invention.

25 SEQ ID NO: 45 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 46 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 47 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding a PB2 protein that can be used according to the present invention.

30 SEQ ID NO: 48 is the amino acid sequence encoded by SEQ ID NO: 47.

SEQ ID NO: 49 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding a PB1 protein that can be used according to the present invention.

SEQ ID NO: 50 is the amino acid sequence encoded by SEQ ID NO: 49.

SEQ ID NO: 51 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding a PA protein that can be used according to the present invention.

SEQ ID NO: 52 is the amino acid sequence encoded by **SEQ ID NO: 51**.

SEQ ID NO: 53 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding an NS protein that can be used according to the present invention.

SEQ ID NO: 54 is the amino acid sequence encoded by **SEQ ID NO: 53**.

SEQ ID NO: 55 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding an NP protein that can be used according to the present invention.

SEQ ID NO: 56 is the amino acid sequence encoded by **SEQ ID NO: 55**.

SEQ ID NO: 57 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding an NA protein that can be used according to the present invention.

SEQ ID NO: 58 is the amino acid sequence encoded by **SEQ ID NO: 57**.

SEQ ID NO: 59 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding an MA protein that can be used according to the present invention.

SEQ ID NO: 60 is the amino acid sequence encoded by **SEQ ID NO: 59**.

SEQ ID NO: 61 is a nucleotide sequence of a canine influenza virus (Miami/2005) encoding an HA protein that can be used according to the present invention.

SEQ ID NO: 62 is the amino acid sequence encoded by **SEQ ID NO: 61**.

SEQ ID NO: 63 is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding a PB2 protein that can be used according to the present invention.

SEQ ID NO: 64 is the amino acid sequence encoded by **SEQ ID NO: 63**.

SEQ ID NO: 65 is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding a PB1 protein that can be used according to the present invention.

SEQ ID NO: 66 is the amino acid sequence encoded by **SEQ ID NO: 65**.

SEQ ID NO: 67 is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding a PA protein that can be used according to the present invention.

SEQ ID NO: 68 is the amino acid sequence encoded by **SEQ ID NO: 67**.

SEQ ID NO: 69 is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding an NS protein that can be used according to the present invention.

SEQ ID NO: 70 is the amino acid sequence encoded by **SEQ ID NO: 69**.

SEQ ID NO: 71 is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding an NP protein that can be used according to the present invention.

5 **SEQ ID NO: 72** is the amino acid sequence encoded by **SEQ ID NO: 71**.

SEQ ID NO: 73 is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding an NA protein that can be used according to the present invention.

SEQ ID NO: 74 is the amino acid sequence encoded by **SEQ ID NO: 73**.

10 **SEQ ID NO: 75** is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding an MA protein that can be used according to the present invention.

SEQ ID NO: 76 is the amino acid sequence encoded by **SEQ ID NO: 75**.

15 **SEQ ID NO: 77** is a nucleotide sequence of a canine influenza virus (Jacksonville/2005) encoding an HA protein that can be used according to the present invention.

SEQ ID NO: 78 is the amino acid sequence encoded by **SEQ ID NO: 77**.

SEQ ID NO: 79 is an oligonucleotide that can be used according to the present invention.

20 **SEQ ID NO: 80** is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 81 is an oligonucleotide that can be used according to the present invention.

25 **SEQ ID NO: 82** is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 83 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 84 is an oligonucleotide that can be used according to the present invention.

30 **SEQ ID NO: 85** is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 86 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 87 is an oligonucleotide that can be used according to the present invention.

SEQ ID NO: 88 is an oligonucleotide that can be used according to the present invention.

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DETAILED DISCLOSURE OF THE INVENTION

The subject invention concerns isolated influenza virus that is capable of infecting canids and causing respiratory disease. In one embodiment, an influenza virus of the invention comprises a polynucleotide which encodes a protein having an amino acid
10 sequence shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional and/or immunogenic fragment or variant thereof. In a specific embodiment, the polynucleotide comprises the nucleotide sequence shown in any of SEQ ID Nos: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73,
15 75, or 77, or a fragment or variant thereof. Influenza virus of the present invention can have an HA subtype of H1, H2, H3, H4, H5, H6, H7, H8, and H9, H10, H11, H12, H13, H14, H15, or H16 or an NA subtype of N1, N2, N3, N4, N5, N6, N7, N8, OR N9. In a specific embodiment, an influenza virus of the present invention is a subtype H3. Virus can be isolated from infected dogs and cultured in cells or eggs according to methods described
20 herein. In an exemplified embodiment, the influenza virus is an influenza A virus.

The subject invention also concerns polynucleotides that comprise all or part of a gene or genes or a genomic segment of an influenza virus of the present invention. In one embodiment, a polynucleotide of the invention comprises an influenza hemagglutinin (HA) gene, neuraminidase (NA) gene, nucleoprotein (NP) gene, matrix protein (MA or M) gene,
25 polymerase basic (PB) protein gene, polymerase acidic (PA) protein gene, non-structural (NS) protein gene, or a functional fragment or variant of any of these genes. In a specific embodiment, a polynucleotide of the invention comprises the hemagglutinin (HA) gene, or a functional fragment or variant thereof. In a further embodiment, the HA gene encodes a hemagglutinin protein having one or more of the following: a serine at position 83; a leucine
30 at position 222; a threonine at position 328; and/or a threonine at position 483, versus the amino acid sequence of equine H3 consensus sequence. In one embodiment, the HA gene encodes a polypeptide having an amino acid sequence shown in SEQ ID NOs: 16, 32, 62, or 78, or a functional and/or immunogenic fragment or variant thereof. In a specific

embodiment, the HA gene comprises a nucleotide sequence shown in SEQ ID NOs: 15, 31, 61, or 77.

In one embodiment, a polynucleotide of the invention encodes a polypeptide having the amino acid sequence shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional and/or immunogenic fragment or variant thereof. In a specific embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, comprises the nucleotide sequence shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, respectively, or a sequence encoding a functional and/or immunogenic fragment or variant of any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78. Thus, the subject invention concerns polynucleotide sequences comprising the nucleotide sequence shown in any of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, or a fragment or variant, including a degenerate variant, of any of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77. In a further specific embodiment, a polynucleotide of the invention can comprise: Nucleotides 1-2271 of SEQ ID NO: 3; Nucleotides 1-2148 of SEQ ID NO: 5; Nucleotides 1-657 of SEQ ID NO: 7; Nucleotides 1-1494 of SEQ ID NO: 9; Nucleotides 1-1410 of SEQ ID NO: 11; Nucleotides 1-756 of SEQ ID NO: 13; Nucleotides 1-1695 of SEQ ID NO: 15; Nucleotides 1-2271 of SEQ ID NO: 19; Nucleotides 1-2148 of SEQ ID NO: 21; Nucleotides 1-657 of SEQ ID NO: 23; Nucleotides 1-1494 of SEQ ID NO: 25; Nucleotides 1-756 of SEQ ID NO: 29; Nucleotides 1-1695 of SEQ ID NO: 31; Nucleotides 1-2277 of SEQ ID NO: 47; Nucleotides 1-2271 of SEQ ID NO: 49; Nucleotides 1-2148 of SEQ ID NO: 51; Nucleotides 1-690 of SEQ ID NO: 53; Nucleotides 1-1494 of SEQ ID NO: 55; Nucleotides 1-1410 of SEQ ID NO: 57; Nucleotides 1-756 of SEQ ID NO: 59; Nucleotides 1-1695 of SEQ ID NO: 61; Nucleotides 1-2277 of SEQ ID NO: 63; Nucleotides 1-2271 of SEQ ID NO: 65; Nucleotides 1-2148 of SEQ ID NO: 67; Nucleotides 1-690 of SEQ ID NO: 69; Nucleotides 1-1494 of SEQ ID NO: 71; Nucleotides 1-1410 of SEQ ID NO: 73; Nucleotides 1-756 of SEQ ID NO: 75; and Nucleotides 1-1695 of SEQ ID NO: 77. Nucleotide and amino acid sequences of viral polynucleotide and polypeptide sequences contemplated within the scope of the present

invention have also been deposited with GenBank at accession Nos. DQ124147 through DQ124161 and DQ124190, the disclosure of which is incorporated herein by reference.

The subject invention also concerns polypeptides encoded by polynucleotides of an influenza virus of the present invention. The subject invention also concerns functional and/or immunogenic fragments and variants of the subject polypeptides. Polypeptides contemplated include HA protein, NA protein, NS protein, nucleoprotein, polymerase basic protein, polymerase acidic protein, and matrix protein of an influenza virus of the invention. In an exemplified embodiment, a polypeptide of the invention has an amino acid sequence shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional and/or immunogenic fragment or variant thereof.

The subject invention also concerns polynucleotide expression constructs comprising a polynucleotide sequence of the present invention. In one embodiment, an expression construct of the invention comprises a polynucleotide sequence encoding a polypeptide comprising an amino acid sequence shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional and/or immunogenic fragment or variant thereof. In a specific embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78 comprises the nucleotide sequence shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, respectively, or a sequence encoding a functional and/or immunogenic fragment or variant of any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78. Thus, the subject invention concerns expression constructs comprising a polynucleotide sequence comprising the nucleotide sequence shown in any of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, or a fragment or variant, including a degenerate variant, of any of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77. In a preferred embodiment, an expression construct of the present invention provides for overexpression of an operably linked polynucleotide of the invention.

Expression constructs of the invention generally include regulatory elements that are functional in the intended host cell in which the expression construct is to be expressed.

Thus, a person of ordinary skill in the art can select regulatory elements for use in, for example, human host cells, mammalian host cells, insect host cells, yeast host cells, bacterial host cells, and plant host cells. In one embodiment, the regulatory elements are ones that are functional in canine cells. Regulatory elements include promoters, transcription termination sequences, translation termination sequences, enhancers, and polyadenylation elements. As used herein, the term "expression construct" refers to a combination of nucleic acid sequences that provides for transcription of an operably linked nucleic acid sequence. As used herein, the term "operably linked" refers to a juxtaposition of the components described wherein the components are in a relationship that permits them to function in their intended manner. In general, operably linked components are in contiguous relation.

An expression construct of the invention can comprise a promoter sequence operably linked to a polynucleotide sequence encoding a polypeptide of the invention. Promoters can be incorporated into a polynucleotide using standard techniques known in the art. Multiple copies of promoters or multiple promoters can be used in an expression construct of the invention. In a preferred embodiment, a promoter can be positioned about the same distance from the transcription start site in the expression construct as it is from the transcription start site in its natural genetic environment. Some variation in this distance is permitted without substantial decrease in promoter activity. A transcription start site is typically included in the expression construct. Preferably, the promoter associated with an expression construct of the invention provides for overexpression of an operably linked polynucleotide of the invention.

Promoters for use with an expression construct of the invention in eukaryotic cells can be of viral or cellular origin. Viral promoters include, but are not limited to, cytomegalovirus (CMV) gene promoters, SV40 early or late promoters, or Rous sarcoma virus (RSV) gene promoters. Promoters of cellular origin include, but are not limited to, desmin gene promoter and actin gene promoter. Promoters suitable for use with an expression construct of the invention in yeast cells include, but are not limited to, 3-phosphoglycerate kinase promoter, glyceraldehyde-3-phosphate dehydrogenase promoter, metallothionein promoter, alcohol dehydrogenase-2 promoter, and hexokinase promoter.

If the expression construct is to be provided in or introduced into a plant cell, then plant viral promoters, such as, for example, a cauliflower mosaic virus (CaMV) 35S (including the enhanced CaMV 35S promoter (see, for example U.S. Patent No. 5,106,739 and An, 1997)) or a CaMV 19S promoter can be used. Other promoters that can be used for expression constructs in plants include, for example, prolifera promoter, Ap3 promoter, heat

shock promoters, T-DNA 1'- or 2'-promoter of *A. tumefaciens*, polygalacturonase promoter, chalcone synthase A (CHS-A) promoter from petunia, tobacco PR-1a promoter, ubiquitin promoter, actin promoter, alcA gene promoter, pin2 promoter (Xu *et al.*, 1993), maize WipI promoter, maize trpA gene promoter (U.S. Patent No. 5,625,136), maize CDPK gene promoter, and RUBISCO SSU promoter (U.S. Patent No. 5,034,322) can also be used. Root-specific promoters, such as any of the promoter sequences described in U.S. Patent No. 6,455,760 or U.S. Patent No. 6,696,623, or in published U.S. patent application Nos. 20040078841; 20040067506; 20040019934; 20030177536; 20030084486; or 20040123349, can be used with an expression construct of the invention. Constitutive promoters (such as the CaMV, ubiquitin, actin, or NOS promoter), developmentally-regulated promoters, and inducible promoters (such as those promoters that can be induced by heat, light, hormones, or chemicals) are also contemplated for use with polynucleotide expression constructs of the invention. Tissue-specific promoters, for example fruit-specific promoters, such as the E8 promoter of tomato (accession number: AF515784; Good *et al.* (1994)) can also be used. Seed-specific promoters such as the promoter from a β -phaseolin gene (for example, of kidney bean) or a glycinin gene (for example, of soybean), and others, can also be used.

For expression in prokaryotic systems, an expression construct of the invention can comprise promoters such as, for example, alkaline phosphatase promoter, tryptophan (trp) promoter, lambda P_L promoter, β -lactamase promoter, lactose promoter, phoA promoter, T3 promoter, T7 promoter, or tac promoter (de Boer *et al.*, 1983).

Expression constructs of the invention may optionally contain a transcription termination sequence, a translation termination sequence, a sequence encoding a signal peptide, and/or enhancer elements. Transcription termination regions can typically be obtained from the 3' untranslated region of a eukaryotic or viral gene sequence. Transcription termination sequences can be positioned downstream of a coding sequence to provide for efficient termination. A signal peptide sequence is a short amino acid sequence typically present at the amino terminus of a protein that is responsible for the relocation of an operably linked mature polypeptide to a wide range of post-translational cellular destinations, ranging from a specific organelle compartment to sites of protein action and the extracellular environment. Targeting gene products to an intended cellular and/or extracellular destination through the use of an operably linked signal peptide sequence is contemplated for use with the polypeptides of the invention. Classical enhancers are cis-acting elements that increase gene transcription and can also be included in the expression construct. Classical enhancer

elements are known in the art, and include, but are not limited to, the CaMV 35S enhancer element, cytomegalovirus (CMV) early promoter enhancer element, and the SV40 enhancer element. Intron-mediated enhancer elements that enhance gene expression are also known in the art. These elements must be present within the transcribed region and are orientation
5 dependent.

DNA sequences which direct polyadenylation of mRNA transcribed from the expression construct can also be included in the expression construct, and include, but are not limited to, an octopine synthase or nopaline synthase signal.

Expression constructs can also include one or more dominant selectable marker genes,
10 including, for example, genes encoding antibiotic resistance and/or herbicide-resistance for selecting transformed cells. Antibiotic-resistance genes can provide for resistance to one or more of the following antibiotics: hygromycin, kanamycin, bleomycin, G418, streptomycin, paromomycin, neomycin, and spectinomycin. Kanamycin resistance can be provided by neomycin phosphotransferase (NPT II). Herbicide-resistance genes can provide for
15 resistance to phosphinothricin acetyltransferase or glyphosate. Other markers used for cell transformation screening include, but are not limited to, genes encoding β -glucuronidase (GUS), β -galactosidase, luciferase, nopaline synthase, chloramphenicol acetyltransferase (CAT), green fluorescence protein (GFP), or enhanced GFP (Yang *et al.*, 1996).

The subject invention also concerns polynucleotide vectors comprising a
20 polynucleotide sequence of the invention that encodes a polypeptide of the invention. Unique restriction enzyme sites can be included at the 5' and 3' ends of an expression construct or polynucleotide of the invention to allow for insertion into a polynucleotide vector. As used herein, the term "vector" refers to any genetic element, including for example, plasmids, cosmids, chromosomes, phage, virus, and the like, which is capable of replication when
25 associated with proper control elements and which can transfer polynucleotide sequences between cells. Vectors contain a nucleotide sequence that permits the vector to replicate in a selected host cell. A number of vectors are available for expression and/or cloning, and include, but are not limited to, pBR322, pUC series, M13 series, pGEM series, and pBLUESCRIPT vectors (Stratagene, La Jolla, CA and Promega, Madison, WI).

30 The subject invention also concerns oligonucleotide probes and primers, such as polymerase chain reaction (PCR) primers, that can hybridize to a coding or non-coding sequence of a polynucleotide of the present invention. Oligonucleotide probes of the invention can be used in methods for detecting influenza virus nucleic acid sequences.

Oligonucleotide primers of the invention can be used in PCR methods and other methods involving nucleic acid amplification. In a preferred embodiment, a probe or primer of the invention can hybridize to a polynucleotide of the invention under stringent conditions. Probes and primers of the invention can optionally comprise a detectable label or reporter molecule, such as fluorescent molecules, enzymes, radioactive moiety, and the like. Probes and primers of the invention can be of any suitable length for the method or assay in which they are being employed. Typically, probes and primers of the invention will be 10 to 500 or more nucleotides in length. Probes and primers that are 10 to 20, 21 to 30, 31 to 40, 41 to 50, 51 to 60, 61 to 70, 71 to 80, 81 to 90, 91 to 100, or 101 or more nucleotides in length are contemplated within the scope of the invention. In one embodiment, probes and primers are any of 9, 10, 11, 12, 13, 14, 15, 16, 17, 18, 19, 20, 21, 22, 23, 24, 25, 26, 27, 28, 29, or 30 nucleotides in length. Probes and primers of the invention can have complete (100%) nucleotide sequence identity with the polynucleotide sequence, or the sequence identity can be less than 100%. For example, sequence identity between a probe or primer and a sequence can be 99%, 98%, 97%, 96%, 95%, 90%, 85%, 80%, 75%, 70% or any other percentage sequence identity so long as the probe or primer can hybridize under stringent conditions to a nucleotide sequence of a polynucleotide of the invention. Exemplified probes and primers of the invention include those having the nucleotide sequence shown in any of SEQ ID NO: 35, SEQ ID NO: 36, SEQ ID NO: 37, SEQ ID NO: 38, SEQ ID NO: 39, SEQ ID NO: 40, SEQ ID NO: 41, SEQ ID NO: 42, SEQ ID NO: 43, SEQ ID NO: 44, SEQ ID NO: 45, and SEQ ID NO: 46, or a functional fragment or variant of any of the SEQ ID NOs: 35-46.

As used herein, the terms "nucleic acid," "polynucleotide," and "oligonucleotide" refer to a deoxyribonucleotide, ribonucleotide, or a mixed deoxyribonucleotide and ribonucleotide polymer in either single- or double-stranded form, and unless otherwise limited, would encompass known analogs of natural nucleotides that can function in a similar manner as naturally-occurring nucleotides. Polynucleotide sequences include the DNA strand sequence that can be transcribed into RNA and the RNA strand that can be translated into protein. The complementary sequence of any nucleic acid, polynucleotide, or oligonucleotide of the present invention is also contemplated within the scope of the invention. Polynucleotide sequences also include both full-length sequences as well as shorter sequences derived from the full-length sequences. The subject invention also encompasses those polynucleotides that are complementary in sequence to the

polynucleotides disclosed herein. Polynucleotides and polypeptides of the invention can be provided in purified or isolated form.

Because of the degeneracy of the genetic code, a variety of different polynucleotide sequences can encode a polypeptide of the present invention. A table showing all possible triplet codons (and where U also stands for T) and the amino acid encoded by each codon is described in Lewin (1985). In addition, it is well within the skill of a person trained in the art to create alternative polynucleotide sequences encoding the same, or essentially the same, polypeptides of the subject invention. These degenerate variant and alternative polynucleotide sequences are within the scope of the subject invention. As used herein, references to "essentially the same" sequence refers to sequences which encode amino acid substitutions, deletions, additions, or insertions which do not materially alter the functional and/or immunogenic activity of the polypeptide encoded by the polynucleotides of the present invention.

The subject invention also concerns variants of the polynucleotides of the present invention that encode polypeptides of the invention. Variant sequences include those sequences wherein one or more nucleotides of the sequence have been substituted, deleted, and/or inserted. The nucleotides that can be substituted for natural nucleotides of DNA have a base moiety that can include, but is not limited to, inosine, 5-fluorouracil, 5-bromouracil, hypoxanthine, 1-methylguanine, 5-methylcytosine, and tritylated bases. The sugar moiety of the nucleotide in a sequence can also be modified and includes, but is not limited to, arabinose, xylulose, and hexose. In addition, the adenine, cytosine, guanine, thymine, and uracil bases of the nucleotides can be modified with acetyl, methyl, and/or thio groups. Sequences containing nucleotide substitutions, deletions, and/or insertions can be prepared and tested using standard techniques known in the art.

Substitution of amino acids other than those specifically exemplified or naturally present in a polypeptide of the invention are also contemplated within the scope of the present invention. For example, non-natural amino acids can be substituted for the amino acids of a polypeptide, so long as the polypeptide having the substituted amino acids retains substantially the same functional activity as the polypeptide in which amino acids have not been substituted. Examples of non-natural amino acids include, but are not limited to, ornithine, citrulline, hydroxyproline, homoserine, phenylglycine, taurine, iodotyrosine, 2,4-diaminobutyric acid, α -amino isobutyric acid, 4-aminobutyric acid, 2-amino butyric acid, γ -amino butyric acid, ϵ -amino hexanoic acid, 6-amino hexanoic acid, 2-amino isobutyric acid,

3-amino propionic acid, norleucine, norvaline, sarcosine, homocitrulline, cysteic acid, τ -butylglycine, τ -butylalanine, phenylglycine, cyclohexylalanine, β -alanine, fluoro-amino acids, designer amino acids such as β -methyl amino acids, C-methyl amino acids, N-methyl amino acids, and amino acid analogues in general. Non-natural amino acids also include amino acids having derivatized side groups. Furthermore, any of the amino acids in the protein can be of the D (dextrorotary) form or L (levorotary) form. Allelic variants of a protein sequence of a polypeptide of the present invention are also encompassed within the scope of the invention.

Amino acids can be generally categorized in the following classes: non-polar, uncharged polar, basic, and acidic. Conservative substitutions whereby a polypeptide of the present invention having an amino acid of one class is replaced with another amino acid of the same class fall within the scope of the subject invention so long as the polypeptide having the substitution still retains substantially the same functional activity as the polypeptide that does not have the substitution. Polynucleotides encoding a polypeptide having one or more amino acid substitutions in the sequence are contemplated within the scope of the present invention. Table 11 below provides a listing of examples of amino acids belonging to each class. Single letter amino acid abbreviations are defined in Table 12.

Fragments and variants of polypeptides of influenza virus of the present invention can be generated using standard methods known in the art and tested for the presence of function or immunogenicity using standard techniques known in the art. For example, for testing fragments and/or variants of a neuraminidase polypeptide of the invention, enzymatic activity can be assayed. Thus, an ordinarily skilled artisan can readily prepare and test fragments and variants of a polypeptide of the invention and determine whether the fragment or variant retains activity relative to full-length or a non-variant polypeptide.

Polynucleotides and polypeptides contemplated within the scope of the subject invention can also be defined in terms of more particular identity and/or similarity ranges with those sequences of the invention specifically exemplified herein. The sequence identity will typically be greater than 60%, preferably greater than 75%, more preferably greater than 80%, even more preferably greater than 90%, and can be greater than 95%. The identity and/or similarity of a sequence can be 49, 50, 51, 52, 53, 54, 55, 56, 57, 58, 59, 60, 61, 62, 63, 64, 65, 66, 67, 68, 69, 70, 71, 72, 73, 74, 75, 76, 77, 78, 79, 80, 81, 82, 83, 84, 85, 86, 87, 88, 89, 90, 91, 92, 93, 94, 95, 96, 97, 98, or 99% as compared to a sequence exemplified herein. Unless otherwise specified, as used herein percent sequence identity and/or similarity

of two sequences can be determined using the algorithm of Karlin and Altschul (1990), modified as in Karlin and Altschul (1993). Such an algorithm is incorporated into the NBLAST and XBLAST programs of Altschul *et al.* (1990). BLAST searches can be performed with the NBLAST program, score = 100, wordlength = 12, to obtain sequences with the desired percent sequence identity. To obtain gapped alignments for comparison purposes, Gapped BLAST can be used as described in Altschul *et al.* (1997). When utilizing BLAST and Gapped BLAST programs, the default parameters of the respective programs (NBLAST and XBLAST) can be used. See NCBI/NIH website.

The subject invention also contemplates those polynucleotide molecules having sequences which are sufficiently homologous with the polynucleotide sequences exemplified herein so as to permit hybridization with that sequence under standard stringent conditions and standard methods (Maniatis *et al.*, 1982). As used herein, "stringent" conditions for hybridization refers to conditions wherein hybridization is typically carried out overnight at 20-25 C below the melting temperature (T_m) of the DNA hybrid in 6x SSPE, 5x Denhardt's solution, 0.1% SDS, 0.1 mg/ml denatured DNA. The melting temperature, T_m , is described by the following formula (Beltz *et al.*, 1983):

$$T_m = 81.5^\circ\text{C} + 16.6 \log[\text{Na}^+] + 0.41(\%G+C) - 0.61(\% \text{ formamide}) - 600/\text{length of duplex in base pairs.}$$

Washes are typically carried out as follows:

- (1) Twice at room temperature for 15 minutes in 1x SSPE, 0.1% SDS (low stringency wash).
- (2) Once at $T_m - 20^\circ\text{C}$ for 15 minutes in 0.2x SSPE, 0.1% SDS (moderate stringency wash).

The subject invention also concerns viral proteins and peptides encoded by the genes of an influenza virus of the present invention. In one embodiment, the viral protein is a mature HA protein. In a specific embodiment, the mature HA protein comprises one or more of the following: a serine at position 82; a leucine at position 221; a threonine at position 327; and/or a threonine at position 482. In an exemplified embodiment, the mature HA protein has an amino acid sequence shown in SEQ ID NO: 33 or SEQ ID NO: 34, or a functional and/or immunogenic fragment or variant of SEQ ID NO: 33 or SEQ ID NO: 34. In another embodiment, the viral protein is an NA protein, NS protein, PB protein, PA protein, or MA protein. Viral proteins and peptides of the invention can be used to generate

antibodies that bind specifically to the protein or peptide. Viral proteins and peptides of the present invention can also be used as immunogens and in vaccine compositions.

The subject invention also concerns compositions and methods for inducing an immune response against an influenza virus that is capable of infecting a susceptible host animal and causing respiratory disease. The invention can be used to induce an immune response against an influenza virus of any subtype in a susceptible host animal. For example, the influenza virus can be an HA subtype of H1, H2, H3, H4, H5, H6, H7, H8, H9, H10, H11, H12, H13, H14, H15, or H16, and an NA subtype of N1, N2, N3, N4, N5, N6, N7, N8, or N9. In one embodiment, the HA subtype is H3 or H5. In a further embodiment, the NA subtype is N7 or N8. In a specific embodiment, an immune response is induced against an influenza virus of subtype H3N8. In one embodiment, the host animal is a canid. Canines include wild, zoo, and domestic canines, such as wolves, coyotes, and foxes. Canines also include dogs, particularly domestic dogs, such as, for example, pure-bred and/or mongrel companion dogs, show dogs, working dogs, herding dogs, hunting dogs, guard dogs, police dogs, racing dogs, and/or laboratory dogs. In a specific embodiment, the host animal is a domesticated dog, such as a greyhound. In one embodiment, an animal is administered an effective amount of an immunogenic composition of the present invention sufficient to induce an immune response against an influenza virus of the invention. The immune response can be a humoral and/or cellular immune response. In a specific embodiment, the immune response is a protective immune response that is capable of preventing or minimizing viral infection in the immunized host animal for some period of time subsequent to the immunization. Thus, the subject invention also concerns vaccine compositions and methods that can provide a vaccinated animal with a protective immune response to a virus of the present invention.

As described herein, the vaccine or immunogenic compositions of the subject invention may comprise cell-free whole virus, including attenuated or inactivated virus, or portions of the virus, including subviral particles (including "split vaccine" wherein a virion is treated to remove some or all viral lipids), viral proteins (including individual proteins and macromolecular complexes of multiple proteins), polypeptides, and peptides, as well as virus-infected cell lines, or a combination of any of these. Vaccine or immunogenic compositions comprising virus-infected cell lines may comprise multiple cell lines, each infected with a different viral strain.

In one embodiment of the invention, a canine may be immunized with one or more inactivated (*i.e.*, killed) and/or live attenuated influenza virus vaccines or vaccines

comprising one or a multiplicity of influenza virus antigens from one or more virus isolates. In one embodiment, the influenza virus is a canine influenza virus. In another embodiment, the influenza virus is an equine influenza virus that encodes or expresses a polypeptide that has at least about 90%, or at least about 95%, or at least about 96%, or 97%, or 98%, or 99% or more amino acid sequence identity with a canine influenza virus polypeptide. In one embodiment, an influenza antigen used in a vaccine of the present invention has at least about 96% sequence identity with an HA antigen and/or NA antigen of a canine influenza virus.

An example of an inactivated vaccine is EQUICINE IITM, which has been marketed by Intervet Inc. (Millsboro, DE, USA) as a liquid vaccine. EQUICINE IITM contains inactivated A/Pennsylvania/63 influenza virus ("A/Pa/63") and A/equine/Kentucky/93 influenza virus ("A/KY/93") with carbopol (*i.e.*, HAVLOGEN® (Intervet Inc.)). More specifically, a dose of EQUICINE IITM contains: inactivated A/Pa/63 at 10^{6.0} EID₅₀, inactivated A/KY/93 at 10^{6.7} EID₅₀, 0.25% by volume carbopol, and sufficient PBS to create a total volume of 1 ml.

Another example of an inactivated vaccine is equine flu virus A/equine/Ohio/03 ("Ohio 03"). In some embodiments, such a vaccine contains CARBIGENTM, which is an emulsified polymer-based adjuvant commercially available from MVP Laboratories, Inc. (Ralston, NE). In such vaccines, a dosage unit typically comprises at least about 250 HA units of the virus, from about 250 to about 12,500 HA units of the virus, or from about 1000 to about 6200 HA units of the virus. The recommended concentration of CARBIGENTM is from about 5 to about 30% (by mass).

An example of a live attenuated vaccine is modified live equine/Kentucky/91 ("A/KY/91") influenza in the form of a freeze-dried vaccine that may be reconstituted with water. In some embodiments, this reconstitution is conducted using vaccine-grade water sufficient to bring the vaccine dosage to a total volume of 1 ml. Aspects of such vaccines are discussed in, for example, U.S. Patent Nos. 6,436,408; 6,398,774; and 6,177,082, which are incorporated by reference in their entirety into this patent. When reconstituted, a dose of such a vaccine may, for example, contain A/KY/91 at 10^{7.2} TCID₅₀ per ml, 0.015 grams N-Z AMINE ASTM per ml, 0.0025 grams gelatin per ml, and 0.04 grams D lactose per ml. N-Z AMINE ASTM is a refined source of amino acids and peptides produced by enzymatic hydrolysis of casein. N-Z AMINE ASTM is marketed by Kerry Bio-Science (Norwich, NY, USA).

In a preferred embodiment, the vaccine comprises an H3 influenza antigen having at least about 93% homology with Florida/43/2004 in HA coding sequences, such as, for example, the equine/New Market/79 strain. Preferred homology is at least about 96%, such as, for example, the equine/Alaska/1/91 and equine/Santiago/85 strains. In the examples that follow, the equine/Kentucky/ 91, equine-2/Kentucky/93, equine-1/Pennsylvania/63, and equine Ohio/03 influenza antigens were incorporated into vaccines. Preferred vaccines also include vaccines comprising equine/Wisconsin/03, equine/Kentucky/02, equine/Kentucky/93, and equine/New Market 2/93. In the examples that follow, H3N8 viruses were used. It is believed, however, that other H3 influenza viruses can be used in accordance with this invention.

Live attenuated vaccines can be prepared by conventional means. Such means generally include, for example, modifying pathogenic strains by *in vitro* passaging, cold adaptation, modifying the pathogenicity of the organism by genetic manipulation, preparation of chimeras, insertion of antigens into viral vectors, selecting non-virulent wild type strains, etc.

In some embodiments, the live attenuated virus strain is derived by serial passage of the wild-type virus through cell culture, laboratory animals, non-host animals, or eggs. The accumulation of genetic mutation during such passage(s) typically leads to progressive loss of virulence of the organism to the original host.

In some embodiments, the live attenuated virus strain is prepared by co-infection of permissible cells with an attenuated mutant virus and pathogenic virus. The desired resultant recombinant virus has the safety of the attenuated virus with genes coding for protective antigens from the pathogenic virus.

In some embodiments, the live attenuated virus strain is prepared by cold adaptation. A cold-adapted virus has an advantage of replicating only at the temperature found in upper respiratory tract. A method of generation of a cold-adapted equine influenza virus has been described in U.S. Patent No. 6,177,082. A desired resulting cold-adapted virus confers one or more of the following phenotypes: cold adaptation, temperature sensitivity, dominant interference, and/or attenuation.

In some embodiments, the live attenuated virus strain is prepared by molecular means, such as point mutation, deletion, or insertion to convert a pathogenic virus to a non-pathogenic or less-pathogenic virus compared to the original virus, while preserving the protective properties of the original virus.

In some embodiments, the live attenuated virus is prepared by cloning the candidate of genes of protective antigens into a genome of a non-pathogenic or less-pathogenic virus or other organism.

5 Inactivated (*i.e.*, "killed") virus vaccines may be prepared by inactivating the virus using conventional methods. Typically, such vaccines include excipients that may enhance an immune response, as well as other excipients that are conventionally used in vaccines. For example, in the examples that follow, EQUICINE II™ comprises HAVLOGEN®. Inactivation of the virus can be accomplished by treating the virus with inactivation chemicals (*e.g.*, formalin, beta propiolactone ("BPL"), bromoethylamine ("BEA"), and binary
10 ethylenimine ("BEI")) or by non-chemical methods (*e.g.*, heat, freeze/thaw, or sonication) to disable the replication capacity of the virus.

In the examples that follow, equine/Ohio/03 was used as a challenge virus. It is known to have about 99% homology with Florida/43/04 isolates, and has been shown to induce symptoms of infection and seroconversion in dogs. Example 18 illustrates the
15 efficacy of equine influenza vaccine in dogs, showing hemagglutination inhibition (or "HI" or "HAI") titers in dogs vaccinated with inactivated Ohio 03 antigen in a vaccine composition comprising CARBIGEN™ adjuvant. Table 29 shows titers pre-vaccination, post-vaccination, and post-second vaccination, as well as post-challenge. The results indicate HI titers at each stage post-vaccination for the vaccinated dogs, with little or no increase for
20 controls. Table 30 illustrates the clinical signs, virus isolation, and histopathology results from the same study. Although challenged animals did not show clinical signs, virus shedding, or positive histopathology, the positive HI titers (Table 29) indicate significant antibody titers in immunized animals.

It should be noted that other H3 influenza virus antigen vaccines are encompassed by
25 this invention as well. Those described in this specification and the following examples are provided to illustrate the invention and its preferred embodiments, and not to limit the scope of the invention claimed.

It should further be noted that influenza antigens other than H3 influenza virus antigens may be used in accordance with this invention. Such antigens include, for example,
30 those from equine/PA/63, which is an equine A1 subtype (H7N7). It is contemplated that one or more of such antigens may be used with or without one or more H3 influenza antigens.

In general, the vaccine is administered in a therapeutically effective amount. A "therapeutically effective amount" is an amount sufficient to induce a protective response in

the canine patient against the target virus. Typically, a dosage is "therapeutically effective" if it prevents, reduces the risk of, delays the onset of, reduces the spread of, ameliorates, suppresses, or eradicates the influenza or one or more (typically two or more) of its symptoms. Typical influenza symptoms include, for example, fever (for dogs, typically $\geq 103.0^{\circ}\text{F}$; $\geq 39.4^{\circ}\text{C}$), cough, sneezing, histopathological lesions, ocular discharge, nasal discharge, vomiting, diarrhea, depression, weight loss, gagging, hemoptysis, and/or audible rales. Other often more severe symptoms may include, for example, hemorrhage in the lungs, mediastinum, or pleural cavity; tracheitis; bronchitis; bronchiolitis; supportive bronchopneumonia; and/or infiltration of the epithelial lining and airway lumens of the lungs with neutrophils and/or macrophages.

The vaccine may be administered as part of a combination therapy, *i.e.*, a therapy that includes, in addition to the vaccine itself, administering one or more additional active agents, adjuvants, therapies, etc. In that instance, it should be recognized the amount of vaccine that constitutes a "therapeutically effective" amount may be less than the amount of vaccine that would constitute a "therapeutically effective" amount if the vaccine were to be administered alone. Other therapies may include those known in the art, such as, for example, anti-viral medications, analgesics, fever-reducing medications, expectorants, anti-inflammation medications, antihistamines, antibiotics to treat bacterial infection that results from the influenza virus infection, rest, and/or administration of fluids. In some embodiments, the vaccine of this invention is administered in combination with a bordetella vaccine, adenovirus vaccine, and/or parainfluenza virus vaccine.

In some embodiments, for example, a typical dose for a live attenuated vaccine is at least about 10^3 pfu/canine; and more typically from about 10^3 to about 10^9 pfu/canine. In this patent, "pfu" means "plaque forming units". In some embodiments, a typical dose for a live attenuated vaccine is at least about 10^3 TCID₅₀/canine, and more typically from about 10^3 to about 10^9 TCID₅₀/canine. In some embodiments, a typical dose for a live attenuated vaccine is at least about 10^3 EID₅₀/canine, and more typically from about 10^3 to about 10^9 EID₅₀/canine. In some embodiments, a typical dose for a killed vaccine is at least about 40 HA units, typically from about 40 to about 10,000 HA units, and more typically from about 500 to about 6200 HA units. In some embodiments, the dose is from about 6100 to about 6200 HA units.

In some preferred embodiments, the vaccine comprises a live attenuated vaccine at a concentration which is at least about $10^{0.5}$ pfu/canine greater than the immunogenicity level.

In some preferred embodiments, the vaccine comprises a live attenuated vaccine at a concentration which is at least about $10^{0.5}$ TCID₅₀/canine greater than the immunogenicity level. In some preferred embodiments, the vaccine comprises a live attenuated vaccine at a concentration which is at least about $10^{0.5}$ EID₅₀/canine greater than the immunogenicity level.

The immunogenicity level may be determined experimentally by challenge dose titration study techniques generally known in the art. Such techniques typically include vaccinating a number of canines with the vaccine at different dosages, and then challenging the canines with the virulent virus to determine the minimum protective dose.

Factors affecting the preferred dosage regimen may include, for example, the type (e.g., species and breed), age, weight, sex, diet, activity, lung size, and condition of the subject; the route of administration; the efficacy, safety, and duration-of-immunity profiles of the particular vaccine used; whether a delivery system is used; and whether the vaccine is administered as part of a drug and/or vaccine combination. Thus, the dosage actually employed can vary for specific animals, and, therefore, can deviate from the typical dosages set forth above. Determining such dosage adjustments is generally within the skill of those in the art using conventional means. It should further be noted that live attenuated viruses are generally self-propagating; thus, the specific amount of such a virus administered is not necessarily critical.

It is contemplated that the vaccine may be administered to the canine patient a single time; or, alternatively, two or more times over days, weeks, months, or years. In some embodiments, the vaccine is administered at least two times. In some such embodiments, for example, the vaccine is administered twice, with the second dose (e.g., the booster) being administered at least about 2 weeks after the first. In some embodiments, the vaccine is administered twice, with the second dose being administered no greater than 8 weeks after the first. In some embodiments, the second dose is administered at from about 2 weeks to about 4 years after the first dose, from about 2 to about 8 weeks after the first dose, or from about 3 to about 4 weeks after the first dose. In some embodiments, the second dose is administered about 4 weeks after the first dose. In the above embodiments, the first and subsequent dosages may vary, such as, for example, in amount and/or form. Often, however, the dosages are the same as to amount and form. When only a single dose is administered, the amount of vaccine in that dose alone generally comprises a therapeutically effective

amount of the vaccine. When, however, more than one dose is administered, the amounts of vaccine in those doses together may constitute a therapeutically effective amount.

In some embodiments, the vaccine is administered before the canine recipient is infected with influenza. In such embodiments, the vaccine may, for example, be administered to prevent, reduce the risk of, or delay the onset of influenza or one or more (typically two or more) influenza symptoms.

In some embodiments, the vaccine is administered after the canine recipient is infected with influenza. In such embodiments, the vaccine may, for example, ameliorate, suppress, or eradicate the influenza or one or more (typically two or more) influenza symptoms.

The preferred composition of the vaccine depends on, for example, whether the vaccine is an inactivated vaccine, live attenuated vaccine, or both. It also depends on the method of administration of the vaccine. It is contemplated that the vaccine will comprise one or more conventional pharmaceutically acceptable carriers, adjuvants, other immune-response enhancers, and/or vehicles (collectively referred to as "excipients"). Such excipients are generally selected to be compatible with the active ingredient(s) in the vaccine. Use of excipients is generally known to those skilled in the art.

The term "pharmaceutically acceptable" is used adjectivally to mean that the modified noun is appropriate for use in a pharmaceutical product. When it is used, for example, to describe an excipient in a pharmaceutical vaccine, it characterizes the excipient as being compatible with the other ingredients of the composition and not disadvantageously deleterious to the intended recipient canine.

The vaccines may be administered by conventional means, including, for example, mucosal administration, (such as intranasal, oral, intratracheal, and ocular), and parenteral administration. Mucosal administration is often particularly advantageous for live attenuated vaccines. Parenteral administration is often particularly advantageous for inactivated vaccines.

Mucosal vaccines may be, for example, liquid dosage forms, such as pharmaceutically acceptable emulsions, solutions, suspensions, syrups, and elixirs. Excipients suitable for such vaccines include, for example, inert diluents commonly used in the art, such as, water, saline, dextrose, glycerol, lactose, sucrose, starch powder, cellulose esters of alkanolic acids, cellulose alkyl esters, talc, stearic acid, magnesium stearate, magnesium oxide, sodium and calcium salts of phosphoric and sulfuric acids, gelatin, acacia gum, sodium alginate,

polyvinylpyrrolidone, and/or polyvinyl alcohol. Excipients also can comprise various wetting, emulsifying, suspending, flavoring (*e.g.*, sweetening), and/or perfuming agents.

Oral mucosal vaccines also may, for example, be tableted or encapsulated for convenient administration. Such capsules or tablets can contain a controlled-release formulation. In the case of capsules, tablets, and pills, the dosage forms also can comprise buffering agents, such as sodium citrate, or magnesium or calcium carbonate or bicarbonate. Tablets and pills additionally can be prepared with enteric coatings.

It is contemplated that the vaccine may be administered via the canine patient's drinking water and/or food. It is further contemplated that the vaccine may be administered in the form of a treat or toy.

"Parenteral administration" includes subcutaneous injections, submucosal injections, intravenous injections, intramuscular injections, intrasternal injections, transcutaneous injections, and infusion. Injectable preparations (*e.g.*, sterile injectable aqueous or oleaginous suspensions) can be formulated according to the known art using suitable excipients, such as vehicles, solvents, dispersing, wetting agents, emulsifying agents, and/or suspending agents. These typically include, for example, water, saline, dextrose, glycerol, ethanol, corn oil, cottonseed oil, peanut oil, sesame oil, benzyl alcohol, benzyl alcohol, 1,3-butanediol, Ringer's solution, isotonic sodium chloride solution, bland fixed oils (*e.g.*, synthetic mono- or diglycerides), fatty acids (*e.g.*, oleic acid), dimethyl acetamide, surfactants (*e.g.*, ionic and non-ionic detergents), propylene glycol, and/or polyethylene glycols. Excipients also may include small amounts of other auxiliary substances, such as pH buffering agents.

The vaccine may include one or more excipients that enhance a canine patient's immune response (which may include an antibody response, cellular response, or both), thereby increasing the effectiveness of the vaccine. Use of such excipients (or "adjuvants") may be particularly beneficial when using an inactivated vaccine. The adjuvant(s) may be a substance that has a direct (*e.g.*, cytokine or Bacillé Calmette-Guerin ("BCG")) or indirect effect (liposomes) on cells of the canine patient's immune system. Examples of often suitable adjuvants include oils (*e.g.*, mineral oils), metallic salts (*e.g.*, aluminum hydroxide or aluminum phosphate), bacterial components (*e.g.*, bacterial liposaccharides, Freund's adjuvants, and/or MDP), plant components (*e.g.*, Quil A), and/or one or more substances that have a carrier effect (*e.g.*, bentonite, latex particles, liposomes, and/or Quil A, ISCOM). As noted above, adjuvants also include, for example, CARBIGENTM and carbopol. It should be

recognized that this invention encompasses both vaccines that comprise an adjuvant(s), as well as vaccines that do not comprise any adjuvant.

It is contemplated that the vaccine may be freeze-dried (or otherwise reduced in liquid volume) for storage, and then reconstituted in a liquid before or at the time of administration.

5 Such reconstitution may be achieved using, for example, vaccine-grade water.

The present invention further comprises kits that are suitable for use in performing the methods described above. The kit comprises a dosage form comprising a vaccine described above. The kit also comprises at least one additional component, and, typically, instructions for using the vaccine with the additional component(s). The additional component(s) may, 10 for example, be one or more additional ingredients (such as, for example, one or more of the excipients discussed above, food, and/or a treat) that can be mixed with the vaccine before or during administration. The additional component(s) may alternatively (or additionally) comprise one or more apparatuses for administering the vaccine to the canine patient. Such an apparatus may be, for example, a syringe, inhaler, nebulizer, pipette, forceps, or any 15 medically acceptable delivery vehicle. In some embodiments, the apparatus is suitable for subcutaneous administration of the vaccine. In some embodiments, the apparatus is suitable for intranasal administration of the vaccine.

Other excipients and modes of administration known in the pharmaceutical or biologics arts also may be used.

20 The vaccine or immunogenic compositions of the subject invention also encompass recombinant viral vector-based constructs that may comprise, for example, genes encoding HA protein, NA protein, nucleoprotein, polymerase basic protein, polymerase acidic protein, and/or matrix protein of an influenza virus of the present invention. Any suitable viral vector that can be used to prepare a recombinant vector/virus construct is contemplated for use with 25 the subject invention. For example, viral vectors derived from adenovirus, avipox, herpesvirus, vaccinia, canarypox, entomopox, swinepox, West Nile virus and others known in the art can be used with the compositions and methods of the present invention. Recombinant polynucleotide vectors that encode and express components can be constructed using standard genetic engineering techniques known in the art. In addition, the various 30 vaccine compositions described herein can be used separately and in combination with each other. For example, primary immunizations of an animal may use recombinant vector-based constructs, having single or multiple strain components, followed by secondary boosts with vaccine compositions comprising inactivated virus or inactivated virus-infected cell lines.

Other immunization protocols with the vaccine compositions of the invention are apparent to persons skilled in the art and are contemplated within the scope of the present invention.

The subject invention also concerns reassortant virus comprising at least one gene or genomic segment of an influenza virus of the present invention and the remainder of viral genes or genomic segments from a different influenza virus of the invention or from an influenza virus other than a virus of the present invention. Reassortant virus can be produced by genetic reassortant of nucleic acid of a donor influenza virus of the present invention with nucleic acid of a recipient influenza virus and then selecting for reassortant virus that comprises the nucleic acid of the donor virus. Methods to produce and isolate reassortant virus are well known in the art (Fields *et al.*, 1996). In one embodiment, a reassortant virus of the invention comprises genes or genomic segments of a human, avian, swine, or equine influenza virus. A reassortant virus of the present invention can include any combination of nucleic acid from donor and recipient influenza virus so long as the reassortant virus comprises at least one gene or genomic segment from a donor influenza virus of the present invention. In one embodiment, a recipient influenza virus can be an equine influenza virus.

Natural, recombinant or synthetic polypeptides of viral proteins, and peptide fragments thereof, can also be used as vaccine compositions according to the subject methods. In one embodiment, a vaccine composition comprises a polynucleotide or a polypeptide of a canine influenza virus. In one embodiment, a vaccine composition comprises a polynucleotide encoding a polypeptide having the amino acid sequence shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional and/or immunogenic fragment or variant thereof. In a specific embodiment, the polynucleotide encoding the amino acid sequence shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, comprises the nucleotide sequence shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, respectively, or a sequence encoding a functional and/or immunogenic fragment or variant of any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78. In a further specific embodiment, a polynucleotide of the invention can comprise: Nucleotides 1-2271 of SEQ ID NO: 3; Nucleotides 1-2148 of SEQ ID NO: 5; Nucleotides 1-657 of SEQ ID NO: 7; Nucleotides 1-1494 of SEQ ID NO: 9; Nucleotides 1-1410 of SEQ ID NO: 11; Nucleotides 1-756 of SEQ ID NO: 13; Nucleotides

1-1695 of SEQ ID NO: 15; Nucleotides 1-2271 of SEQ ID NO: 19; Nucleotides 1-2148 of SEQ ID NO: 21; Nucleotides 1-657 of SEQ ID NO: 23; Nucleotides 1-1494 of SEQ ID NO: 25; Nucleotides 1-756 of SEQ ID NO: 29; Nucleotides 1-1695 of SEQ ID NO: 31; Nucleotides 1-2277 of SEQ ID NO: 47; Nucleotides 1-2271 of SEQ ID NO: 49; Nucleotides 1-2148 of SEQ ID NO: 51; Nucleotides 1-690 of SEQ ID NO: 53; Nucleotides 1-1494 of SEQ ID NO: 55; Nucleotides 1-1410 of SEQ ID NO: 57; Nucleotides 1-756 of SEQ ID NO: 59; Nucleotides 1-1695 of SEQ ID NO: 61; Nucleotides 1-2277 of SEQ ID NO: 63; Nucleotides 1-2271 of SEQ ID NO: 65; Nucleotides 1-2148 of SEQ ID NO: 67; Nucleotides 1-690 of SEQ ID NO: 69; Nucleotides 1-1494 of SEQ ID NO: 71; Nucleotides 1-1410 of SEQ ID NO: 73; Nucleotides 1-756 of SEQ ID NO: 75; and Nucleotides 1-1695 of SEQ ID NO: 77. In another embodiment, a vaccine composition comprises a polypeptide having the amino acid sequence shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional and/or immunogenic fragment or variant thereof. In a further embodiment, a vaccine composition comprises a polynucleotide or a polypeptide of an equine influenza virus wherein the polynucleotide or polypeptide has at least about 90%, or at least about 95%, or at least about 96%, or 97%, or 98%, or 99% or more sequence identity with a canine influenza polynucleotide or polypeptide. In one embodiment, viral polypeptides derived from multiple strains can be combined in a vaccine composition and are used to vaccinate a host animal. For example, polypeptides based on the viral HA protein from at least two different strains of influenza virus of the invention can be combined in the vaccine. The polypeptides may be homologous to one strain or may comprise "hybrid" or "chimeric" polypeptides whose amino acid sequence is derived from joining or linking polypeptides from at least two distinct strains. Procedures for preparing viral polypeptides are well known in the art. For example, viral polypeptides and peptides can be synthesized using solid-phase synthesis methods (Merrifield, 1963). Viral polypeptides and peptides can also be produced using recombinant DNA techniques wherein a polynucleotide molecule encoding an viral protein or peptide is expressed in a host cell, such as bacteria, yeast, or mammalian cell lines, and the expressed protein purified using standard techniques of the art.

Vaccine compositions of the present invention also include naked nucleic acid compositions. In one embodiment, a nucleic acid may comprise a nucleotide sequence encoding an HA and/or an NA protein of an influenza virus of the present invention. Methods for nucleic acid vaccination are known in the art and are described, for example, in

U.S. Patent Nos. 6,063,385 and 6,472,375. The nucleic acid can be in the form of a plasmid or a gene expression cassette. In one embodiment, the nucleic acid is provided encapsulated in a liposome which is administered to an animal.

Vaccine compositions and immunogens, such as polypeptides and nucleic acids, that can be used in accordance with the present invention can be provided with a pharmaceutically-acceptable carrier or diluent. Compounds and compositions useful in the subject invention can be formulated according to known methods for preparing pharmaceutically useful compositions. Formulations are described in detail in a number of sources which are well known and readily available to those skilled in the art. For example, *Remington's Pharmaceutical Science* by E.W. Martin, Easton Pennsylvania, Mack Publishing Company, 19th ed., 1995, describes formulations which can be used in connection with the subject invention. In general, the compositions of the subject invention will be formulated such that an effective amount of an immunogen is combined with a suitable carrier in order to facilitate effective administration of the composition. The compositions used in the present methods can also be in a variety of forms. These include, for example, solid, semi-solid, and liquid dosage forms, such as tablets, pills, powders, liquid solutions or suspension, suppositories, injectable and infusible solutions, and sprays. The preferred form depends on the intended mode of administration and therapeutic application. The compositions also preferably include conventional pharmaceutically acceptable carriers and diluents which are known to those skilled in the art. Examples of carriers or diluents for use with the subject peptidomimetics include, but are not limited to, water, saline, oils including mineral oil, ethanol, dimethyl sulfoxide, gelatin, cyclodextrans, magnesium stearate, dextrose, cellulose, sugars, calcium carbonate, glycerol, alumina, starch, and equivalent carriers and diluents, or mixtures of any of these. Formulations of an immunogen of the invention can also comprise suspension agents, protectants, lubricants, buffers, preservatives, and stabilizers. To provide for the administration of such dosages for the desired therapeutic treatment, pharmaceutical compositions of the invention will advantageously comprise between about 0.1% and 45%, and especially, 1 and 15% by weight of the immunogen or immunogens based on the weight of the total composition including carrier or diluent.

The vaccine and immunogenic compositions of the subject invention can be prepared by procedures well known in the art. For example, the vaccine or immunogens are typically prepared as injectables, e.g., liquid solutions or suspensions. The vaccine or immunogens are administered in a manner that is compatible with dosage formulation, and in such amount as

will be therapeutically effective and immunogenic in the recipient. The optimal dosages and administration patterns for a particular vaccine or immunogens formulation can be readily determined by a person skilled in the art.

Peptides and/or polypeptides of the present invention can also be provided in the form of a multiple antigenic peptide (MAP) construct. The preparation of MAP constructs has been described in Tam (1988). MAP constructs utilize a core matrix of lysine residues onto which multiple copies of an immunogen are synthesized (Posnett *et al.*, 1988). Multiple MAP constructs, each containing the same or different immunogens, can be prepared and administered in a vaccine composition in accordance with methods of the present invention.

In one embodiment, a MAP construct is provided with and/or administered with one or more adjuvants. Influenza polypeptides of the invention can also be produced and administered as macromolecular protein structures comprising one or more polypeptides. Published U.S. Patent Application US2005/0009008 describes methods for producing virus-like particles as a vaccine for influenza virus.

According to the methods of the subject invention, the vaccine and immunogenic compositions described herein are administered to susceptible hosts, typically canids, and more typically domesticated dogs, in an effective amount and manner to induce protective immunity against subsequent challenge or infection of the host by virus. In one embodiment, the host animal is a canid. Canines include wild, zoo, and domestic canines, such as wolves, coyotes, and foxes. Canines also include dogs, particularly domestic dogs, such as, for example, pure-bred and/or mongrel companion dogs, show dogs, working dogs, herding dogs, hunting dogs, guard dogs, police dogs, racing dogs, and/or laboratory dogs. In a specific embodiment, the host animal is a domesticated dog, such as a greyhound. The vaccines or immunogens are typically administered parenterally, by injection, for example, either subcutaneously, intraperitoneally, or intramuscularly. Other suitable modes of administration include oral or nasal administration. Usually, the vaccines or immunogens are administered to an animal at least two times, with an interval of one or more weeks between each administration. However, other regimens for the initial and booster administrations of the vaccine or immunogens are contemplated, and may depend on the judgment of the practitioner and the particular host animal being treated.

Virus and virus-infected cells in a vaccine formulation may be inactivated or attenuated using methods known in the art. For example, whole virus and infected cells can be inactivated or attenuated by exposure to paraformaldehyde, formalin, beta propiolactone

(BPL), bromoethylamine (BEA), binary ethylenimine (BEI), phenol, UV light, elevated temperature, freeze thawing, sonication (including ultrasonication), and the like. The amount of cell-free whole virus in a vaccine dose can be in the range from about 0.1 mg to about 5 mg, and more usually being from about 0.2 mg to about 2 mg. The dosage for vaccine
5 formulations comprising virus-infected cell lines will usually contain from about 10^6 to about 10^8 cells per dose, and more usually from about 5×10^6 to about 7.5×10^7 cells per dose. The amount of protein or peptide immunogen in a dose for an animal can vary from about 0.1 μ g to 10000 μ g, or about 1 μ g to 5000 μ g, or about 10 μ g to 1000 μ g, or about 25 μ g to 750 μ g, or about 50 μ g to 500 μ g, or 100 μ g to 250 μ g, depending upon the size, age, *etc.*, of the
10 animal receiving the dose.

An immunogenic or vaccine composition of the invention, such as virus or virus-infected cells or viral proteins or peptides, can be combined with an adjuvant, typically just prior to administration. Adjuvants contemplated for use in the vaccine formulations include threonyl muramyl dipeptide (MDP) (Byars *et al.*, 1987), saponin, *Cornebacterium parvum*,
15 Freund's complete and Freund's incomplete adjuvants, aluminum, or a mixture of any of these. A variety of other adjuvants suitable for use with the methods and vaccines of the subject invention, such as alum, are well known in the art and are contemplated for use with the subject invention.

The subject invention also concerns antibodies that bind specifically to a protein or a
20 peptide of the present invention. Antibodies of the subject invention include monoclonal and polyclonal antibody compositions. Preferably, the antibodies of the subject invention are monoclonal antibodies. Whole antibodies and antigen binding fragments thereof are contemplated in the present invention. Thus, for example, suitable antigen binding fragments include Fab₂, Fab and Fv antibody fragments. Antibodies of the invention can be labeled
25 with a detectable moiety, such as a fluorescent molecule (*e.g.*, fluorescein or an enzyme).

The subject invention also concerns methods and compositions for detection and identification of an influenza virus of the invention and for diagnosis of infection of an animal with an influenza virus of the present invention. The methods of the invention include detection of the presence of canine influenza, in a biological sample from an animal. The
30 detection of canine influenza in a sample, is useful to diagnose canine influenza in an animal. In turn, this information can provide the ability to determine the prognosis of an animal based on distinguishing levels of canine influenza present over time, and can assist in selection of therapeutic agents and treatments for the animal, and assist in monitoring therapy. The

method also provides the ability to establish the absence of canine influenza in an animal tested.

The ability to detect canine influenza in an animal permits assessment of outbreaks of canine influenza in different geographical locations. This information also permits early
5 detection so that infected animals can be isolated, to limit the spread of disease, and allows early intervention for treatment options. In addition, having this information available can provide direction to medical personnel for preparing to treat large numbers of ill animals, including assembling medical supplies, and, if available, vaccines.

In one embodiment, a method of the present invention involves the collection of a
10 biological sample from a test animal, such as a canine. The biological sample may be any biological material, including, cells, tissue, hair, whole blood, serum, plasma, nipple aspirate, lung lavage, cerebrospinal fluid, saliva, sweat and tears.

The animal test sample may come from an animal suspected of having canine influenza virus, whether or not the animal exhibits symptoms of the disease. Control samples
15 can also be provided or collected from animals known to be free of canine influenza. Additional controls may be provided, *e.g.*, to reduce false positive and false negative results, and verify that the reagents in the assay are actively detecting canine influenza A virus.

In addition to detecting the presence or absence of canine influenza in a biological sample, the methods of detection used in the invention can detect mutations in canine
20 influenza virus, such as changes in nucleic acid sequence, that may result from the environment, drug treatment, genetic manipulations or mutations, injury, change in diet, aging, or any other characteristic(s) of an animal. Mutations may also cause canine influenza A to become resistant to a drug that was formerly effective, or to enable the virus to infect and propagate in a different species of animal, or human. For example, avian influenza A
25 virus has been shown to infect other animals and humans.

In one embodiment for detecting an influenza virus in an animal, diagnosis is facilitated by the collection of high-quality specimens, their rapid transport to a testing facility, and appropriate storage, before laboratory testing. Virus is best detected in specimens containing infected cells and secretions. In one embodiment, specimens for the
30 direct detection of viral antigens and/or for nucleic acids and/or virus isolation in cell cultures are taken during the first 3 days after onset of clinical symptoms. A number of types of specimens are suitable to diagnose virus infections of the upper respiratory tract, including, but not limited to, nasal swab, nasopharyngeal swab, nasopharyngeal aspirate, nasal wash and

throat swabs. In addition to swabs, samples of tissue or serum may be taken, and invasive procedures can also be performed.

In one embodiment, respiratory specimens are collected and transported in 1-5 ml of virus transport media. A number of media that are satisfactory for the recovery of a wide variety of viruses are commercially available. Clinical specimens are added to transport medium. Nasal or nasopharyngeal swabs can also be transported in the virus transport medium. One example of a transport medium is 10 gm of veal infusion broth and 2 gm of bovine albumin fraction V, added to sterile distilled water to 400 m. Antibiotics such as 0.8 ml gentamicin sulfate solution (50 mg/ml) and 3.2 ml amphotericin B (250 µg/ml) can also be added. The medium is preferably sterilized by filtration. Nasal washes, such as sterile saline (0.85% NaCl), can also be used to collect specimens of respiratory viruses.

In one embodiment, sera is collected in an amount of from 1-5 ml of whole blood from an acute-phase animal, soon after the onset of clinical symptoms, and preferably not later than 7 days. A convalescent-phase serum specimen can also be collected, for example at about 14 days after onset of symptoms. Serum specimens can be useful for detecting antibodies against respiratory viruses in a neutralization test.

In some instances, samples may be collected from individual animals over a period of time (*e.g.*, once a day, once a week, once a month, biannually or annually). Obtaining numerous samples from an individual animal, over a period of time, can be used to verify results from earlier detections, and/or to identify response or resistance to a specific treatment, *e.g.*, a selected therapeutic drug.

The methods of the present invention can be used to detect the presence of one or more pathological agents in a test sample from an animal, and the level of each pathological agent. Any method for detecting the pathological agent can be used, including, but not limited to, antibody assays including enzyme-linked immunosorbent assays (ELISAs), indirect fluorescent antibody (IFA) tests, hemagglutinating, and inhibition of hemagglutination (HI) assays, and Western Blot. Known cell-culture methods can also be used. Positive cultures can be further identified using immunofluorescence of cell cultures or HI assay of the cell culture medium (supernatant).

In addition, methods for detecting nucleic acid (DNA or RNA) or protein can be used. Such methods include, but are not limited to, polymerase chain reaction (PCR), and reverse transcriptase (RT) PCR tests and real time tests, and quantitative nuclease protection assays.

There are commercially available test kits available to perform these assays. For example, QIAGEN (Valencia, CA) sells a one step RT-PCR kit, and viral RNA extraction kit.

In one embodiment, the method utilizes an antibody specific for a virus or viral protein of the invention. In a specific embodiment, an antibody specific for an HA protein of a virus of the invention is utilized. In another embodiment, an antibody specific for an NP protein of a virus of the invention is used. A suitable sample, such as from the nasal or nasopharyngeal region, is obtained from an animal and virus or viral protein is isolated therefrom. The viral components are then screened for binding of an antibody specific to a protein, such as HA or NP, of a virus of the invention. In another embodiment, a serum sample (or other antibody containing sample) is obtained from an animal and the serum screened for the presence of antibody that binds to a protein of a virus of the invention. For example, an ELISA assay can be performed where the plate walls have HA and/or NP protein, or a peptide fragment thereof, bound to the wall. The plate wall is then contacted with serum or antibody from a test animal. The presence of antibody in the animal that binds specifically to the HA and/or NP protein is indicative that the test animal is infected or has been infected with an influenza virus of the present invention.

In one embodiment, the presence of a pathological agent is detected by determining the presence or absence of antibodies against the agent, in a biological sample. It can take some time (*e.g.* months) after an animal is infected before antibodies can be detected in a blood test. Once formed, antibodies usually persist for many years, even after successful treatment of the disease. Finding antibodies to canine influenza A may not indicate whether the infection was recent, or sometime in the past.

Antibody testing can also be done on fluid(s). Antibody assays include enzyme-linked immunosorbent assays (ELISAs), indirect fluorescent antibody (IFA) assays, and Western Blot. Preferably, antibody testing is done using multiple assays, for example ELISA or IFA followed by Western blot. Antibody assays can be done in a two-step process, using either an ELISA or IFA assay, followed by a Western blot assay. ELISA is considered a more reliable and accurate assay than IFA, but IFA may be used if ELISA is not available. The Western blot test (which is a more specific test) can also be done in all animals, particularly those that have tested positive or borderline positive (equivocal) in an ELISA or IFA assay.

Other antibody-based tests that can be used for detection of influenza virus include hemagglutination inhibition assays. Hemagglutination activity can be detected in a biological

sample from an animal, using chicken or turkey red blood cells as described (Burleson *et al.*, 1992) and Kendal *et al.*, 1982). In one embodiment, an influenza or an HA protein or peptide of the invention is contacted with a test sample containing serum or antibody. Red blood cells (RBC) from an animal, such as a bird, are then added. If antibody to HA is present, then the RBC will not agglutinate. If antibody to HA is not present, the RBC will agglutinate in the presence of HA. Variations and modifications to standard hemagglutination inhibition assays are known in the art and contemplated within the scope of the present invention.

Infection of an animal can also be determined by isolation of the virus from a sample, such as a nasal or nasopharyngeal swab. Viral isolation can be performed using standard methods, including cell culture and egg inoculation.

In a further embodiment, a nucleic acid-based assay can be used for detection of a virus of the present invention. In one embodiment, a nucleic acid sample is obtained from an animal and the nucleic acid subjected to PCR using primers that will generate an amplification product if the nucleic acid contains a sequence specific to an influenza virus of the present invention. In a specific embodiment, RT-PCR is used in an assay for the subject virus. In an exemplified embodiment, real-time RT-PCR is used to assay for an influenza virus of the invention. PCR, RT-PCR and real-time PCR methods are known in the art and have been described in U.S. Patent Nos. 4,683,202; 4,683,195; 4,800,159; 4,965,188; 5,994,056; 6,814,934; and in Saiki *et al.* (1985); Sambrook *et al.* (1989); Lee *et al.* (1993); and Livak *et al.* (1995). In one embodiment, the PCR assay uses oligonucleotides specific for an influenza matrix (MA) gene and/or HA gene. The amplification product can also be sequenced to determine if the product has a sequence of an influenza virus of the present invention. Other nucleic acid-based assays can be used for detection and diagnosis of viral infection by a virus of the invention and such assays are contemplated within the scope of the present invention. In one embodiment, a sample containing a nucleic acid is subjected to a PCR-based amplification using forward and reverse primers where the primers are specific for a viral polynucleotide or gene sequence. If the nucleic acid in the sample is RNA, then RT-PCR can be performed. For real-time PCR, a detectable probe is utilized with the primers.

Primer sets specific for the hemagglutinin (HA) gene of many of the circulating influenza viruses are known, and are continually being developed. The influenza virus genome is single-stranded RNA, and a DNA copy (cDNA) must be made using a reverse transcriptase (RT) polymerase. The amplification of the RNA genome, for example using

RT-PCR, requires a pair of oligonucleotide primers, typically designed on the basis of the known HA sequence of influenza A subtypes and of neuraminidase (NM)-1. The primers can be selected such that they will specifically amplify RNA of only one virus subtype. DNAs generated by using subtype-specific primers can be further analyzed by molecular genetic techniques such as sequencing. The test is preferably run with a positive control, or products are confirmed by sequencing and comparison with known sequences. The absence of the target PCR products (*i.e.*, a “negative” result) may not rule out the presence of the virus. Results can then be made available within a few hours from either clinical swabs or infected cell cultures. PCR and RT-PCR tests for influenza A virus are described by Fouchier *et al.*, 2000 and Maertzdorf *et al.*, 2004.

The subject invention also concerns methods for screening for compounds or drugs that have antiviral activity against a virus of the present invention. In one embodiment, cells infected with a virus of the invention are contacted with a test compound or drug. The amount of virus or viral activity following contact is then determined. Those compounds or drugs that exhibit antiviral activity can be selected for further evaluation.

The subject invention also concerns isolated cells infected with an influenza virus of the present invention. In one embodiment, the cell is a canine cell, such as canine kidney epithelial cells.

The subject invention also concerns cells transformed with a polynucleotide of the present invention encoding a polypeptide of the invention. Preferably, the polynucleotide sequence is provided in an expression construct of the invention. More preferably, the expression construct provides for overexpression in the cell of an operably linked polynucleotide of the invention. In one embodiment, the cell is transformed with a polynucleotide sequence comprising a sequence encoding the amino acid sequence shown in any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional fragment or variant thereof. In a specific embodiment, the cell is transformed with a polynucleotide encoding the amino acid sequence shown in SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78 comprises the nucleotide sequence shown in SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, respectively, or a sequence encoding a functional fragment or variant of any of SEQ ID NOs: 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72,

74, 76, or 78. Thus, the subject invention concerns cells transformed with a polynucleotide sequence comprising the nucleotide sequence shown in any of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, or a fragment or variant, including a degenerate variant, of any of SEQ ID NOs: 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77.

The transformed cell can be a eukaryotic cell, for example, a plant cell, including protoplasts, or the transformed cell can be a prokaryotic cell, for example, a bacterial cell such as *E. coli* or *B. subtilis*. Animal cells include human cells, mammalian cells, partially canine cells, avian cells, and insect cells. Plant cells include, but are not limited to, dicotyledonous, monocotyledonous, and conifer cells.

The subject invention also concerns plants, including transgenic plants that express and produce a viral protein or polypeptide of the present invention. Plants, plant tissues, and plant cells transformed with or bred to contain a polynucleotide of the invention are contemplated by the present invention. Preferably, the polynucleotide of the invention is overexpressed in the plant, plant tissue, or plant cell. Plants can be used to produce influenza vaccine compositions of the present invention and the vaccines can be administered through consumption of the plant (see, for example, U.S. Patent Nos. 5,484,719 and 6,136,320).

The subject invention also concerns kits for detecting a virus or diagnosing an infection by a virus of the present invention. In one embodiment, a kit comprises an antibody of the invention that specifically binds to an influenza virus of the present invention, or an antigenic portion thereof. In another embodiment, a kit comprises one or more polypeptides or peptides of the present invention. In a specific embodiment, the polypeptides have an amino acid sequence shown in any of SEQ ID NOs. 2, 4, 6, 8, 10, 12, 14, 16, 18, 20, 22, 24, 26, 28, 30, 32, 33, 34, 48, 50, 52, 54, 56, 58, 60, 62, 64, 66, 68, 70, 72, 74, 76, or 78, or a functional and/or immunogenic fragment or variant thereof. In a further embodiment, a kit comprises one or more polynucleotides or oligonucleotides of the present invention. In a specific embodiment, the polynucleotides have a nucleotide sequence shown in any of SEQ ID NOs. 1, 3, 5, 7, 9, 11, 13, 15, 17, 19, 21, 23, 25, 27, 29, 31, 47, 49, 51, 53, 55, 57, 59, 61, 63, 65, 67, 69, 71, 73, 75, or 77, or a fragment or variant thereof. A kit may optionally comprise one or more control antibody, control polypeptide or peptide, and/or control polynucleotide or oligonucleotide. The antibody, polypeptides, peptides, polynucleotides, and/or oligonucleotides of the kit can be provided in a suitable container or package.

The subject application also concerns the use of mongrel dogs as a model for infection and pathogenesis of influenza virus. In one embodiment, a mongrel dog is inoculated with an influenza virus, such as a canine influenza virus of the present invention. Optionally, the dog can be administered therapeutic agents subsequent to inoculation. The dog can also have
5 been administered a composition for generating an immune response against an influenza virus prior to inoculation with virus. Tissue, blood, serum, and other biological samples can be obtained before and/or after inoculation and examined for the presence of virus and pathogenesis of tissue using methods known in the art including, but not limited to, PCR, RT-PCR, nucleic acid sequencing, and immunohistochemistry.

10 Canine influenza virus strains (designated as "A/canine/Florida/43/2004" and "A/canine/Florida/242/2003") were deposited with American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, on October 9, 2006. Canine influenza virus strains (designated as "canine/Jax/05" and "canine/Miami/05"), were deposited with American Type Culture Collection (ATCC), P.O. Box 1549, Manassas, VA 20108, on
15 October 17, 2006. The subject virus strains have been deposited under conditions that assure that access to the cultures will be available during the pendency of this patent application to one determined by the Commissioner of Patents and Trademarks to be entitled thereto under 37 CFR 1.14 and 35 U.S.C. 122. The deposit will be available as required by foreign patent laws in countries wherein counterparts of the subject application, or its progeny, are filed.
20 However, it should be understood that the availability of a deposit does not constitute a license to practice the subject invention in derogation of patent rights granted by governmental action.

Further, the subject virus deposits will be stored and made available to the public in accord with the provisions of the Budapest Treaty for the Deposit of Microorganisms, i.e., it
25 will be stored with all the care necessary to keep it viable and uncontaminated for a period of at least five years after the most recent request for the furnishing of a sample of the deposit, and in any case, for a period of at least thirty (30) years after the date of deposit or for the enforceable life of any patent which may issue disclosing the culture. The depositor acknowledges the duty to replace the deposit should the depository be unable to furnish a
30 sample when requested, due to the condition of the deposit. All restrictions on the availability to the public of the subject culture deposit will be irrevocably removed upon the granting of a patent disclosing it.

Table 57 illustrates the similarities among the amino acid sequences encoded by the hemagglutinin (or "HA"), neuraminidase (or "NA"), and nucleoprotein (NP) genes of the canine influenza virus identified as A/canine/Florida/43/2004 (Ca/ Fla/43/04) with H3N8 equine isolates, as well as the canine/Florida/242/2003 isolate.

5 Any element of any embodiment disclosed herein can be combined with any other element or embodiment disclosed herein and such combinations are specifically contemplated within the scope of the present invention.

All patents, patent applications, provisional applications, and publications referred to or cited herein are incorporated by reference in their entirety, including all figures and tables,
10 to the extent they are not inconsistent with the explicit teachings of this specification.

MATERIALS AND METHODS FOR EXAMPLES 1-6

Blood and Nasal Swab Collection from Greyhounds.

15 Acute and convalescent blood samples were collected by jugular venipuncture from clinically diseased or normal greyhounds in racing kennels experiencing outbreaks of respiratory disease. Convalescent samples were collected 4 to 12 weeks after the acute sample. Serum was harvested and stored at -80°C. Nasal swabs were collected and placed in Amies transport medium with charcoal (Becton Dickinson Biosciences) pending submission
20 for bacterial isolation.

Postmortem examination of greyhounds.

Complete postmortem examinations were performed by the Anatomic Pathology Service at the University of Florida College of Veterinary Medicine (UF CVM) on 5 of the 8
25 greyhounds that died in the January 2004 outbreak at a Florida track. Postmortem examination of another dog was performed at a private veterinary clinic with submission of tissues to the UF CVM for histopathologic diagnosis. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5-µm sections were either stained with hematoxylin and eosin for histopathologic diagnosis or processed for immunohistochemistry
30 as described below. Unfixed tissues were submitted for bacterial culture and also stored at -80°C.

Serological tests for canine viral respiratory pathogens.

Paired acute and convalescent serum samples were submitted to the Animal Health Diagnostic Laboratory (AHDL) at the Cornell University College of Veterinary Medicine for serum neutralization assays against canine distemper virus, adenovirus type 2, and parainfluenza virus. Antibody titers were expressed as the last dilution of serum that inhibited viral infection of cell cultures. Seroconversion, defined as a ≥ 4 -fold increase in antibody titer between the acute and convalescent sample, indicated viral infection. No seroconversions to these viral pathogens were detected.

Microbial tests for canine bacterial respiratory pathogens.

Paired nasal swabs and postmortem tissues were submitted to the Diagnostic Clinical Microbiology/Parasitology/Serology Service at the UF CVM for bacterial isolation and identification. The samples were cultured on nonselective media as well as media selective for *Bordetella* species (Regan-Lowe; Remel) and *Mycoplasma* species (Remel). All cultures were held for 21 days before reporting no growth. Nasal swabs from some of the greyhounds were also submitted to the Department of Diagnostic Medicine/Pathobiology at the Kansas State University College of Veterinary Medicine for bacterial culture. Of 70 clinically diseased dogs tested, *Bordetella bronchiseptica* was isolated from the nasal cavity of 1 dog, while *Mycoplasma spp.* were recovered from the nasal cavity of 33 dogs. *Pasteurella multocida* was commonly recovered from the nasal cavity of dogs with purulent nasal discharges. Two of the dogs that died in the January 2004 outbreak had scant growth of *Escherichia coli* in the lungs postmortem, one dog had scant growth of *E. coli* and *Streptococcus canis*, and another had scant growth of *Pseudomonas aeruginosa* and a yeast. Neither *Bordetella bronchiseptica* nor *Mycoplasma* was isolated from the trachea or lungs of dogs that died.

Virus isolation from postmortem tissues.

Frozen tissues were thawed and homogenized in 10 volumes of minimum essential medium (MEM) supplemented with 0.5% bovine serum albumin (BSA) and antibiotics. Solid debris was removed by centrifugation and supernatants were inoculated onto cultured cells or into 10-day old embryonated chicken eggs. Tissue homogenates from greyhounds that died were inoculated into diverse cell cultures that supported the replication of a broad range of viral pathogens. The cell cultures included Vero (African green monkey kidney epithelial cells, ATCC No. CCL-81), A-72 (canine tumor fibroblasts, CRL-1542), HRT-18 (human rectal epithelial cells, CRL-11663), MDCK (canine kidney epithelial cells, CCL-34),

primary canine kidney epithelial cells (AHDL, Cornell University), primary canine lung epithelial cells (AHDL), and primary bovine testicular cells (AHDL). MDCK and HRT cells were cultured in MEM supplemented with 2.5 ug/mL TPCK-treated trypsin (Sigma); the remaining cell lines were cultured in MEM supplemented with 10% fetal calf serum and antibiotics. Cells were grown in 25 cm² flasks at 37°C in a humidified atmosphere containing 5% CO₂. A control culture was inoculated with the supplemented MEM. The cultures were observed daily for morphologic changes and harvested at 5 days post inoculation. The harvested fluids and cells were clarified by centrifugation and inoculated onto fresh cells as described for the initial inoculation; two blind passages were performed. Hemagglutination activity in the clarified supernatants was determined using chicken or turkey red blood cells as described (Burleson *et al.*, 1992; Kendal *et al.*, 1982). For virus isolation in chicken embryos, 0.1 mL of tissue homogenate was inoculated into the allantoic sac and incubated for 48 hours at 35°C. After two blind passages, the hemagglutination activity in the allantoic fluids was determined as described (Burleson *et al.*, 1992; Kendal *et al.*, 1982).

RT-PCR, nucleotide sequencing, and phylogenetic analyses.

Total RNA was extracted from tissue culture supernatant or allantoic fluid using the RNeasy kit (Qiagen, Valencia, CA) according to manufacturer's instructions. The total RNA (10 ng) was reverse transcribed to cDNA using a one-step RT-PCR Kit (Qiagen, Valencia, CA) according to manufacturer's instructions. PCR amplification of the coding region of the 8 influenza viral genes in the cDNA was performed as previously described (Klimov *et al.*, 1992a), using universal gene-specific primer sets. The resulting DNA amplicons were used as templates for automated sequencing on an Applied Biosystems 3100 automated DNA sequencer using cycle sequencing dye terminator chemistry (ABI). Nucleotide sequences were analyzed using the GCG Package[®], Version 10.0 (Accelrys) (Womble, 2000). The Phylogeny Inference Package[®] Version 3.5 was used to estimate phylogenies and calculate bootstrap values from the nucleotide sequences (Felsenstein, 1989). Phylogenetic trees were compared to those generated by neighbor-joining analysis with the Tamura-Nei gamma model implemented in the MEGA[®] program (Kumar *et al.*, 2004) and confirmed by the PAUP[®] 4.0 Beta program (Sinauer Associates).

Experimental inoculation of dogs.

Four 6-month old specific pathogen-free beagles [(2 males and 2 females (Liberty Research))] were used. Physical examination and baseline blood tests including complete blood cell count/differential, serum chemistry panel, and urinalysis determined that the animals were healthy. They were housed together in a BSL 2-enhanced facility accredited by the Association for Assessment and Accreditation of Laboratory Animal Care. Baseline rectal temperatures were recorded twice daily for 7 days. The dogs were anesthetized by intravenous injection of propofol (Diprivan®, Zeneca Pharmaceuticals, 0.4 mg/kg body weight to effect) for intubation with endotracheal tubes. Each dog was inoculated with a total dose of $10^{6.6}$ median tissue culture infectious doses (TCID₅₀) of A/Canine/Florida/43/2004 (Canine/FL/04) (H3N8) virus with half the dose administered into the distal trachea through the endotracheal tube and the other half administered into the deep nasal passage through a catheter. Physical examinations and rectal temperature recordings were performed twice daily for 14 days post inoculation (p.i.). Blood samples (4 mL) were collected by jugular venipuncture on days 0, 3, 5, 7, 10, and 14 p.i. Nasal and oropharyngeal specimens were collected with polyester swabs (Fisher Scientific) from each dog on days 0 to 5, 7, 10, and 14 p.i. The swabs were placed in viral transport medium (Remel) and stored at -80°C. Two dogs (1 male and 1 female) were euthanatized by intravenous inoculation of Beuthanasia-D® solution (1 mL/5 kg body weight; Schering-Plough Animal Health Corp) on day 5 p.i. and the remaining 2 dogs on day 14 for postmortem examination. Tissues for histological analysis were processed as described. Tissues for virus culture were stored at -80°C. This study was approved by the University of Florida Institutional Animal Care and Use Committee.

Virus shedding from experimentally inoculated dogs.

Serial dilutions of lung homogenates and swab extracts, prepared by clarification of the swab transport media by centrifugation, were set up in MEM supplemented with 0.5% BSA and antibiotics. Plaque assays were performed as described (Burleson *et al.*, 1992) using monolayers of MDCK cells in 6-well tissue culture plates. Inoculated cell monolayers were overlaid with supplemented MEM containing 0.8% agarose and 1.5 ug/mL of TPCK-trypsin. Cells were cultured for 72 hours at 37°C in a humidified atmosphere containing 5% CO₂ prior to fixation and staining with crystal violet. Virus concentration was expressed as plaque forming units (PFU) per gram of tissue or per swab.

Immunohistochemistry.

Deparaffinized and rehydrated 5- μ m lung tissue sections from the greyhounds and beagles were mounted on Bond-Rite™ slides (Richard-Allan Scientific, Kalamazoo, MI) and subsequently treated with proteinase K (DakoCytomation, Carpinteria, CA) followed by peroxidase blocking reagent (Dako® EnVision™ Peroxidase Kit, Dako Corp.). The sections were incubated with 1:500 dilutions of monoclonal antibodies to canine distemper virus (VMRD, Inc.), canine adenovirus type 2 (VMRD, Inc.), canine parainfluenza virus (VMRD, Inc.), or influenza A H3 (Chemicon International, Inc.) for 2 hours at room temperature. Controls included incubation of the same sections with mouse IgG (1 mg/mL, Serotec, Inc.), and incubation of the monoclonal antibodies with normal canine lung sections. Following treatment with the primary antibodies, the sections were incubated with secondary immunoperoxidase and peroxidase substrate reagents (Dako® EnVision™ Peroxidase Kit, Dako Corp.) according to the manufacturer's instructions. The sections were counterstained with hematoxylin, treated with Clarifier #2 and Bluing Reagent (Richard-Allan Scientific, Kalamazoo, MI), dehydrated, and coverslips applied with Permount (ProSciTech).

Hemagglutination inhibition (HI) assay.

Serum samples were incubated with receptor destroying enzyme (RDE, Denka) (1 part serum: 3 parts RDE) for 16 hours at 37°C prior to heat inactivation for 60 minutes at 56°C. Influenza A/Canine/FL/04 (H3N8) virus was grown in MDCK cells for 36-48 hr at 37°C. Virus culture supernatants were harvested, clarified by centrifugation, and stored at -80°C. The HI assay was performed as described previously (Kendal *et al.*, 1982). Briefly, 4 hemagglutinating units of virus in 25 μ l were added to an equal volume of serially diluted serum in microtiter wells and incubated at room temperature for 30 minutes. An equal volume of 0.5% v/v turkey erythrocytes was added and the hemagglutination titers were estimated visually after 30 minutes. The endpoint HI titer was defined as the last dilution of serum that completely inhibited hemagglutination. Seroconversion was defined as ≥ 4 -fold increase in HI titer between paired acute and convalescent samples. Seropositivity of a single sample was defined as a HI antibody titer $\geq 1:32$.

Microneutralization (MN) assay.

Neutralizing serum antibody responses to A/Canine/FL/04 (H3N8) were detected by a MN assay as described previously (Rowe *et al.*, 1999) except that canine sera were RDE-

treated as described above prior to the assay. The endpoint titer was defined as the highest dilution of serum that gave 50% neutralization of 100 TCID₅₀ of virus. Seroconversion was defined as ≥ 4 -fold increase in MN titer between paired acute and convalescent samples. Seropositivity of a single sample was defined as a MN titer $\geq 1:80$.

5 Following are examples which illustrate procedures for practicing the invention. These examples should not be construed as limiting. All percentages are by weight and all solvent mixture proportions are by volume unless otherwise noted.

EXAMPLE 1

10 In January 2004, an outbreak of respiratory disease occurred in 22 racing greyhounds housed in 2 kennels at a Florida track and the local farm that supplied dogs to these kennels. There were approximately 60 dogs in each kennel building and 300 dogs at the farm. The outbreak occurred over a 6-day period after which no new cases were identified. Fourteen of the 22 dogs had fevers of 39.5 to 41.5°C, a soft, gagging cough for 10 to 14 days, and
15 eventual recovery. Of the remaining 8 dogs, 6 apparently healthy dogs died unexpectedly with hemorrhage from the mouth and nose. Two other dogs were euthanatized within 24 hours of onset of hemorrhage from the mouth and nose due to rapid deterioration. Both of these dogs had fevers of 41°C. Four of the 8 deaths occurred in the kennel buildings and 4 occurred at the farm. Fifty percent of the deaths occurred on day 3 of the outbreak. The 22
20 dogs ranged in age from 17 months to 4 years, but 73% were 17 to 33 months old.

 Two clinical syndromes were evident: a milder illness characterized by initial fever and then cough for 10-14 days (14 dogs) with subsequent recovery, or a peracute death associated with hemorrhage in the respiratory tract (8 dogs for a mortality rate of 36%). Postmortem examinations were performed on 6 of the 8 fatal cases. All dogs had extensive
25 hemorrhage in the lungs, mediastinum, and pleural cavity. Histological examination of the respiratory tract revealed that in addition to pulmonary hemorrhage, all dogs had tracheitis, bronchitis, bronchiolitis, and suppurative bronchopneumonia (Figure 3). 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,
30 bovine, and canine cell lines for virus culture. 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. 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. In addition, the hemagglutinating activity was inhibited by reference antisera to the equine influenza A H3 subtype, but not by antisera specific for human influenza A subtypes H1-H11 and H13 (Table 3). To
5 characterize the molecular properties of the virus, we determined the nucleotide sequences of the 8 RNA segments of the viral genome. 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 -97% sequence identity (Figure 1A, Table 4). In contrast, representative genes
10 from avian, swine, and human influenza A isolates had ≤ 94 % identity with the canine isolate (Table 4). These data identified the canine isolate A/Canine/Florida/43/2004 (Canine/FL/04) as an influenza A H3N8 virus closely related to contemporary lineages of 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
15 dog.

EXAMPLE 2

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
20 tissues using a monoclonal antibody to influenza A H3. 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 (Figure 2A). These data support a diagnosis of pulmonary infection with influenza virus of the H3 subtype in multiple dogs.

EXAMPLE 3

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 by hemagglutination inhibition (HI) and
30 microneutralization (MN). Seroconversion, defined as a ≥ 4 -fold rise in antibody titer to Canine/FL/04 from the acute to convalescent phase, occurred in 8 of 11 (73%) sick dogs in both assays (Table 1). Seroconversion occurred in 6 of 16 (38%) asymptomatic contacts in the HI assay, while 8 of 16 (50%) seroconverted in the MN assay (Table 1). 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 most infections with canine influenza virus are subclinical and suggest efficient spread of the virus among dogs. It is not known if subclinical infections contribute to the spread of the virus.

EXAMPLE 4

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 (TCID₅₀) by the intratracheal and intranasal routes. All dogs developed a fever (rectal temperature $\geq 39^{\circ}\text{C}$) for the first 2 days postinoculation (p.i.), but none exhibited respiratory symptoms 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. 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 log₁₀ PFU per swab), whereas the other dog shed virus for 4 consecutive days after inoculation (1.4-4.5 log₁₀ 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 spontaneous disease in greyhounds, but there was no pulmonary hemorrhage or bronchopneumonia. Viral H3 antigen was detected in the cytoplasm of epithelial cells of bronchi, bronchioles, and bronchial glands by IHC (Figure 2B). Infectious virus was recovered from the lung tissue of one of the dogs. 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, presence of viral antigen and infectious virus in the lung parenchyma, 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.

EXAMPLE 5

5 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. There were no detectable antibodies in 33 dogs sampled from 1996 to 1999. Of 32 dogs sampled between 2000 and 2003, 9 were seropositive in both assays - 1 in 2000, 2
10 in 2002, and 6 in 2003 (Table 5). 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. Lung
15 homogenates inoculated into MDCK cells and chicken embryos from one dog yielded H3N8 influenza virus, termed A/Canine/Florida/242/2003 (Canine/FL/03). Sequence analysis of the complete genome of Canine/FL/03 revealed >99% identity to Canine/FL/04 (Table 4), indicating that Canine/FL/04-like viruses had infected greyhounds prior to 2004.

20 EXAMPLE 6

From June to August 2004, respiratory disease outbreaks occurred in thousands of racing greyhounds at 14 tracks in Florida, Texas, Alabama, Arkansas, West Virginia, and Kansas.

Officials at some of these tracks estimated that at least 80% of their dog population
25 had clinical disease. Most of the dogs had clinical signs of fever ($\geq 39^{\circ}\text{C}$) and cough similar to the dogs in the January 2004 outbreak, but many dogs also had a mucopurulent nasal discharge. Multiple deaths were reported but an accurate mortality rate could not be determined.

We collected paired acute and convalescent sera from 94 dogs located at 4 Florida
30 tracks: 56% of these dogs had ≥ 4 -fold rises in antibody titers to Canine/FL/04, and 100% were seropositive (Table 6). Convalescent sera from 29 dogs in West Virginia and Kansas also had antibodies to Canine/FL/04. We isolated influenza A (H3N8) virus from the lungs of a greyhound that died of hemorrhagic bronchopneumonia at a track in Texas. Sequence

analysis of the entire genome of this isolate, named A/Canine/Texas/1/2004 (Canine/TX/04), revealed $\geq 99\%$ identity to Canine/FL/04 (Table 4). The isolation of three closely related influenza viruses from fatal canine cases over a 13-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 the dog population.

Phylogenetic analysis 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 that was clearly distinct from contemporary H3 genes of equine viruses isolated in 2002 and 2003 (Figure 1B). Phylogenetic analysis and pairwise nucleotide sequence comparisons 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 (data not shown, and Table 4). The clustering of the canine influenza genes as a monophyletic group separate from equine influenza is also supported by the presence of 4 signature amino acid changes in the HA (Table 2). Together with the serological results from 2003 and 2004, these data are consistent with a single virus transmission event from horses to dogs with subsequent horizontal spread of the virus in the greyhound population. However, repeated introductions of this unique lineage of influenza virus from an unidentified reservoir species can not be formally excluded, unlikely as it may be.

The viral HA is a critical determinant of host species specificity of influenza virus (Suzuki *et al.*, 2000). To identify residues within HA that may be associated with adaptation to the canine host, we compared the deduced amino acid sequence of canine HAs to those of contemporary equine viruses. Four amino acid changes differentiate the equine and canine mature HA consensus amino acid sequences: N83S, W222L, I328T, and N483T (see Table 2). The canine viruses have an amino acid deletion when compared to the consensus equine sequences. Therefore, amino acid position 7 in the HA equine sequence is position 6 in the HA canine sequence, amino acid position 29 in the HA equine sequence is position 28 in the HA canine sequence, amino acid position 83 in the HA equine sequence is position 82 in the HA canine sequence, *etc.* Thus, the four substituted amino acids are at position 82, 221, 327, and 482 of the amino acid sequence shown in SEQ ID NO: 33 and SEQ ID NO: 34. The substitution of serine for asparagine at consensus sequence 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 consensus sequence 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 consensus sequence position 222 is quite remarkable because it represents a non-conservative change adjacent to the sialic acid binding pocket which could modulate receptor function (Weis *et al.*, 1988). 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 (Nobusawa *et al.*, 1991; Kovacova *et al.*, 2002). The leucine substitution may be more compatible with virus specificity for mammalian hosts since infections of swine with subtype H4 (Karasin *et al.*, 2000) and humans and swine with subtype H9 (Peiris *et al.*, 1999) viruses have been reported. The substitution of asparagine with threonine at consensus sequence position 483 resulted in the loss of a glycosylation site in the HA2 subunit that is conserved in all HA subtypes (Wagner *et al.*, 2002). 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 (Vines *et al.*, 1998; Matrosovich *et al.*, 2000). Amino acid differences between other influenza viral proteins of the invention and equine consensus sequence are shown in Tables 19 to 25.

The source of the equine influenza virus that initially infected racing greyhounds remains speculative. Kennels at greyhound racetracks are not located near horses or horse racetracks, suggesting that contact between greyhounds and shedding horses is not a sufficient explanation for the multiple outbreaks in different states in 2004. A potential source of exposure to the equine virus is the feeding of horsemeat to greyhounds, whose diet is supplemented with raw meat supplied by packing houses that render carcasses, including horses which could carry influenza. Precedents for this mode of infection include reports of interspecies transmission of H5N1 avian influenza virus to pigs and zoo felids fed infected chicken carcasses (Webster, 1998; Keawcharoen *et al.*, 2004; Kuiken *et al.*, 2004). Although this is a plausible route for the initial introduction of equine influenza into dogs, it does not explain the recent multiple influenza outbreaks in thousands of dogs in different states. Our experimental inoculation study demonstrated the presence of virus in the nasal passages and oropharynx of dogs, albeit at modest titers. Nevertheless, these results 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 interspecies transfer of a whole mammalian influenza virus to an unrelated mammal species is a 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 (Nikitin *et al.*, 1972; Kilbourne, *et al.*, 1975; Chang *et al.*, 1976; Houser *et al.*, 1980). 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 (Dacso *et al.*, 1984; Kimura *et al.*, 1998; Patriarca *et al.*, 1984; Top *et al.*, 1977), 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., suggest adaptation of the virus to the canine host. Since dogs are a primary companion animal for humans, these findings have implications for public health; dogs may provide a new source for transmission of novel influenza A viruses to humans.

Table 1. Antibody response to A/Canine/Florida/43/2004 (H3N8).

Response	Sick Dogs (11) ^a		Healthy Contacts (16) ^b	
	HI ^c	SN ^d	HI	SN
Seroconversion (%) ^e	73	73	38	50
Seropositive (%) ^f	82	82	100	100
Geometric mean titer ^g	329	424	268	431

^a Number of dogs with clinical signs of disease.

^b Number of asymptomatic dogs housed in contact with clinically diseased dogs.

^c Hemagglutination-inhibition (HI) assay using A/Canine/Florida/43/2004 virus.

^d Microneutralization (MN) assay using A/Canine/Florida/43/2004 virus.

^e Percentage of dogs with at least a 4-fold increase in antibody titer in paired acute and convalescent sera.

^f Percentage of dogs with a positive antibody titer (HI titer ≥ 32 ; MN titer ≥ 80) in the convalescent sera.

^g Geometric mean antibody titer for the convalescent sera.

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

Equine H3 consensus	Can/FL/03	Can/FL/04	Can/TX/04	Potential functional significance
G7*	D	- [†]	-	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
S92	-	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
A272	V	A	V	V is present in some recent equine isolates
I328	T	T	T	T is strictly conserved in all avian, swine or humans H3 HAs
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 .

[†] Denotes no change from the consensus equine H3 HAs.

Table 3. Hemagglutination inhibition of a virus isolate by reference antisera to different HA subtypes.		
Reference Antisera	HA Specificity	HI titer^a
Puerto Rico/8/34	H1	5
Swine/Iowa15/30	H1	5
Singapore/01/57	H2	5
Shanghai/11/87	H3 ^b	5
Equine/Miami/1/63	H3	160
Duck/Czechoslovakia/56	H4	5
Tern/South Africa/61	H5	5
Turkey/Massachusetts/65	H6	5
Fowl Plague/Dutch/27	H7	5
Fowl Plague/Rostock/34	H7	5
Equine/Prague/1/56	H7	5
Turkey/Ontario/6118/68	H8	5
Quail/Hong Kong/G1/97	H9 ^b	5
Chicken/Hong Kong/G9/97	H9 ^b	5
Chicken/Germany/49	H10	5
Duck/England/56	H11	5
Gull/Maryland/704/77	H13	5
Normal sheep serum	-	5
Normal ferret serum	-	5

^a Hemagglutination inhibition titer to virus isolate from dog # 43.

^b Polyclonal antisera were produced in ferrets, whereas all other antisera were produced in sheep or goats.

Table 4. Sequence homology of A/Canine/Florida/43/2004 (H3N8) genes to equine, avian, swine, and human strains of influenza A.

Gene	Equine	Avian	Swine	Human
PB2 DQ124147	96.9 (98.7) ^a Eq/Kentucky/2/8 M73526	88.6 (96.8) Mall/Alberta/98/85 AY633315	87.9 (96.8) Sw/Ontario/ 01911-1/99 AF285892	86.2 (96.4) PR/8/34 (HK/213/03) AF389115 (AY576381)
PB1 DQ124148	97.1 (98.8) Eq/Tennessee/5/86 M25929	83.9 (97.1) Ck/BritishColumbia/04 (Gull/Md/704/77) AY61675 (M25933)	83.9 (97.1) Sw/Korea/S109/04 (Sw/Saskatch/ 18789/02) AY790287 (AY619955)	83.9 (97.1) WSN/33 (Sing/1/57) J02178 (M25924)
PA DQ124149	96.3 (97.5) M26082 Eq/Tennessee/5/86	87.0 (94.3) Ck/Chile/4591/02 (Ostrich/SA/08103/95) AY303660(AF508662)	84.3 (94.6) Sw/Hong Kong/ 126/02 M26081	83.8 (93.4) Taiwan/2/70 (Viet Nam/ 1203/04) AY210199 (AY818132)
HA (H3) DQ124190	97.4 (97.1) Eq/FL/1/93 L39916	80.7 (89.0) Dk/Norway/1/03 AJ841293	80.0 (87.7) Sw/Ontario/42729a/01 AY619977	81.8 (87.9) HK/1/68 AF348176
NP DQ124150	96.6 (97.9) Eq/Tennessee/5/86 M30758	87.9 (95.1) Ck/Chile/176822/02 AY303658	85.4 (93.5) Sw/Ontario/42729a/01 (Sw/Fujian/1/2003) AY619974 (AY747611)	84.7 (93.0) HK/1073/99 (Hong Kong /538/97) AF255742 (AF255751)
NA (N8) DQ124151	96.8 (97.0) Eq/Tennessee/5/86 L06583	84.0 (85.2) Dk/NJ/2000 L06583	na ^b	na ^b
M DQ124152	97.9 (95.7) Eq/Tennessee/5/86 (Eq/Kentucky/92) M63529 (AF001683)	94.1 (94.0) Tky/Mn/833/80 AF001683	93.7 (93.5) Sw/Saskatchewan/ 18789/02 M63527	91.2 (95.4) WSN/33 (Hong Kong/ 1073/99) J02177 (AJ278646)
NS DQ124153	97.5 (95.7) Eq/Tn/5/86 (Eq/Kentucky/92) M80973 (AF001671)	92.0 (90.4) Mal/NY/6750/78 M80945	91.1 (89.1) Sw/China/8/78 (Sw/Korea/s452/04) M80968 (AY790309)	91.4 (90.0) Brevig Mission/1/18 AF333238

^a Percent nucleotide and amino acid (in parentheses) sequence identity of A/Canine/Florida/43/2004 (H3N8) genes to the most homologous gene of influenza virus virus isolates from the species, followed by their Genbank sequence database accession numbers.

^b Not applicable: N8 neuraminidase was never reported in human or swine viruses.

Table 5. Antibody titers to A/canine/Florida/43/2004 (H3N8) in greyhound serum collected from 1996 to 2003.						
	Year ^a					
	1996	1997	1998	2000	2002	2003
No. of dogs tested	8	6	19	4	6	22
No. of seropositive dogs	0	0	0	1	2	6
Antibody titers ^b				512	232, 524	280-2242

^a The year of serum sample collection from racing greyhounds in Florida.

^b Microneutralization assay antibody titers for seropositive dogs, including the range for the six 2003 seropositive dogs.

5

Table 6. Antibody response to A/canine/Florida/43/2004 (H3N8) in racing greyhounds at 4 Florida tracks in June 2004.				
Response	Track A	Track B	Track C	Track D
Number of dogs tested ^a	37	10	22	25
Seroconversion (%) ^b	46	90	100	64
Seropositive (%) ^c	100	100	100	100
Geometric mean titer ^d	401	512	290	446

^a Number of clinically diseased dogs tested by HI using A/canine/Florida/43/2004 (H3N8).

^b Percentage of dogs with ≥ 4 -fold increase in antibody titer between acute and convalescent sera.

^c Percentage of dogs with a positive antibody titer (HI titer > 16) in the convalescent sera.

^d Geometric mean antibody titer for the convalescent sera.

10

MATERIALS AND METHODS FOR EXAMPLES 7-11

Canine tissues

15 Postmortem examinations were performed by the Anatomic Pathology Service at the University of Florida College of Veterinary Medicine on 6 mixed breed dogs that died in April/May 2005 during an influenza outbreak in a shelter facility in northeast Florida, and on a pet Yorkshire Terrier dog that died in May 2005 during an influenza outbreak in a

veterinary clinic in southeast Florida. Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5- μ m sections were stained with hematoxylin and eosin for histopathologic diagnosis. Unfixed tissues were stored at -80°C pending virological analyses.

5 RNA extraction from canine tissue samples

Frozen lung tissues from each of the 7 dogs were thawed and homogenized in minimum essential medium (MEM) supplemented with 0.5% bovine serum albumin (BSA) and antibiotics (gentamycin and ciprofloxacin) using a disposable tissue grinder (Kendall, Lifeline Medical Inc., Danbury, CT). Total RNA was extracted using a commercial kit
10 (RNeasy[®] Mini Kit, QIAGEN Inc., Valencia, CA) according to manufacturer's instructions and eluted in a final volume of 60 μ L of buffer. Total RNA was also extracted from lung tissue collected from dogs without respiratory disease.

Real-time RT-PCR

15 A single-step quantitative real-time RT-PCR was performed on total RNA extracted from the canine tissue samples using the QuantiTect[®] Probe RT-PCR Kit containing ROX as a passive reference dye (QIAGEN Inc., Valencia, CA). Briefly, 2 primer-probe sets were used for detection of influenza A sequences in each sample (**Table 7**). One primer-probe set was selective for canine hemagglutinin (H3) gene sequences. The other primer-probe set
20 targeted a highly conserved region of the matrix (M) gene of type A influenza virus. For each real-time RT-PCR reaction, 5 μ L of extracted total RNA were added to a reaction mixture containing 12.5 μ L of 2X QuantiTect[®] Probe RT-PCR Master Mix, 0.25 μ L of QuantiTect[®] RT Mix, forward and reverse primers (0.4 μ M final concentration for each), probe (0.1 μ M final concentration) and RNase-free water in a final volume of 25 μ L. The
25 TaqMan[®] Ribosomal RNA Control Reagents (Applied Biosystems, Foster City, CA) were used according to manufacturer's instructions for detection of 18S rRNA as an endogenous internal control for the presence of RNA extracted from the canine tissue samples.

Quantitative one-step real-time RT-PCR was performed on the reaction mixtures in a Mx3000P[®] QPCR System (Stratagene, La Jolla, CA). Cycling conditions included a reverse
30 transcription step at 50°C for 30 minutes, an initial denaturation step at 95°C for 15 minutes to activate the HotStarTaq[®] DNA polymerase, and amplification for 40 cycles. Each amplification cycle included denaturation at 94°C for 15 seconds followed by

annealing/extension at 60°C for 1 minute. The FAM (emission wavelength 518 nm) and VIC (emission wavelength 554 nm) fluorescent signals were recorded at the end of each cycle. The threshold cycle (Ct) was determined by setting the threshold fluorescence (dR) at 1000 in each individual experiment. The Mx3000P[®] version 2.0 software program (Stratagene, La Jolla, CA) was used for data acquisition and analysis. Samples were considered positive for influenza A virus when the threshold cycle (Ct) for the H3 or M gene was 3 units smaller than the Ct for lung tissues from dogs without respiratory disease. The positive control consisted of amplification of RNA extracted from A/canine/FL/242/03 (H3N8) virus.

10 Virus isolation in MDCK cells

Frozen lung tissues from each of the 7 dogs were thawed and homogenized in 10 volumes of Dulbecco's Modified Eagle Medium (DMEM) supplemented with 0.5% (BSA) and antibiotics (gentamycin and ciprofloxacin). Solid debris was removed by centrifugation and supernatants were inoculated onto Madin-Darby canine kidney (MDCK) cells cultured in DMEM supplemented with 1 µg/mL TPCK-treated trypsin (Sigma-Aldrich Corp., St. Louis, MO) and antibiotics (gentamycin and ciprofloxacin). Cells were grown in 25 cm² flasks at 37°C in a humidified atmosphere containing 5% CO₂. The cultures were observed daily for morphologic changes and harvested at 5 days post inoculation. The harvested cultures were clarified by centrifugation and the supernatants inoculated onto fresh MDCK cells as described for the initial inoculation; two additional passages were performed for samples that did not show evidence of influenza virus by hemagglutination or RT-PCR. Hemagglutination activity in the clarified supernatants was determined using 0.5% turkey red blood cells as previously described (Burleson, F. *et al.*, 1992; Kendal, P. *et al.*, 1982). RT-PCR was performed as described below.

25

Virus isolation in embryonated chicken eggs

Homogenates were prepared from frozen lung tissues as described above for inoculation of MDCK cells. The homogenates (0.2 mL) were inoculated into the allantoic sac of 10-day old embryonated chicken eggs. After 48 hours of incubation at 35°C, the eggs were chilled at 4°C overnight before harvesting the allantoic fluid. Hemagglutination activity in the clarified supernatants was determined using 0.5% turkey red blood cells as previously described (Burleson, F. *et al.*, 1992; Kendal, P. *et al.*, 1982). RT-PCR was performed as

30

described below. Two additional passages in embryonated eggs were performed for samples that did not show evidence of influenza virus after the initial inoculation.

RT-PCR, nucleotide sequencing, and phylogenetic analyses

5 Viral RNA was extracted from MDCK supernatant or allantoic fluid using the QIAamp[®] Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions. The viral RNA was reverse transcribed to cDNA using the QIAGEN[®] OneStep RT-PCR Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions. PCR amplification of the coding region of the 8 influenza viral genes in the cDNA was performed
10 as previously described (Klimov, A. *et al.*, 1992b), using universal gene-specific primer sets (primer sequences available on request). The resulting DNA amplicons were used as templates for automated sequencing in the ABI PRISM[®] 3100 automated DNA sequencer using cycle sequencing dye terminator chemistry (Applied Biosystems, Foster City, CA). Nucleotide sequences were analyzed using the Lasergene 6 Package[®] (DNASTAR, Inc.,
15 Madison, WI). The PHYLIP Version 3.5[®] software program was used to estimate phylogenies and calculate bootstrap values from the nucleotide sequences (Felsenstein, J., 1989). Phylogenetic trees were compared to those generated by neighbor-joining analysis with the Tamura-Nei model implemented in the MEGA[®] program (Kumar, S. *et al.*, 2004) and confirmed by the PAUP[®] 4.0 Beta program (Sinauer Associates, Inc., Sunderland, MA).

20

Hemagglutination inhibition (HI) assay

 Serum samples were incubated with receptor destroying enzyme (RDE, DENKA SEIKEN Co., Ltd., Tokyo, Japan) (1 part serum: 3 parts RDE) for 16 hours at 37°C prior to heat inactivation for 30 minutes at 56°C. Influenza A/Canine/Jacksonville/05 (H3N8) virus
25 was grown in MDCK cells for 72 hrs at 37°C in 5% CO₂. Virus culture supernatants were harvested, clarified by centrifugation, and stored at -80°C. All other viruses used in the HI assay were grown in 10-day old embryonated chicken eggs from which allantoic fluid was collected and stored at -80°C. The HI assay was performed as described previously (Kendal, P. *et al.*, 1982). Briefly, 4 hemagglutinating units of virus in 25µl were added to an equal
30 volume of serially diluted serum in 96-well plastic plates and incubated at room temperature for 30 minutes. An equal volume of 0.5% turkey erythrocytes was added and the hemagglutination titers were estimated visually after 30 minutes. The endpoint HI titer was defined as the last dilution of serum that completely inhibited hemagglutination.

EXAMPLE 7—CLINICAL CASES

In April and May 2005, a previously described (Crawford, P.C. *et al.*, 2005) respiratory disease outbreak occurred in dogs housed in a shelter facility in northeast Florida. The outbreak involved at least 58 dogs ranging in age from 3 months to 9 years, and included purebred dogs as well as mixed breeds. The most common clinical signs were purulent nasal discharge and a cough for 7 to 21 days. Of the 43 dogs that had clinical disease for ≥ 7 days, 41 had HI antibody titers to canine/FL/04 (H3N8) ranging from 32 to >1024 . At least 10 dogs progressed to pneumonia, of which 6 were euthanized. These 6 mixed breed dogs included 3 males and 3 females ranging in age from 4 months to 3 years. The duration of clinical signs ranged from 2 to 10 days at the time of euthanasia. On postmortem examination, these dogs had pulmonary congestion and edema. Histological examination of the respiratory tract revealed rhinitis, tracheitis, bronchitis, bronchiolitis, and suppurative bronchopneumonia. There was epithelial cell necrosis and erosion in the trachea, bronchi, bronchioles, and bronchial glands. The respiratory tissues were infiltrated by neutrophils and macrophages.

In May 2005, a respiratory disease outbreak occurred in 40 pet dogs at a veterinary clinic in southeast Florida. The most common clinical signs were purulent nasal discharge and a cough for 10 to 30 days. Of the 40 dogs, 17 were seropositive for canine/FL/04 (H3N8) with HI antibody tiers ranging from 32 to >1024 . Seroconversion occurred in 10 dogs for which paired acute and convalescent sera were available. Three dogs progressed to pneumonia. One of these dogs, a 9-year old male Yorkshire Terrier, died 3 days after onset of clinical signs. This dog had tracheobronchitis, pulmonary edema and congestion, and severe bronchopneumonia. Similar to the 6 shelter dogs, there was epithelial cell necrosis and erosion of the airways and neutrophilic infiltrates in the tissues.

EXAMPLE 8—REAL-TIME RT-PCR AND VIRAL ISOLATION

Lung tissues from the 7 dogs were analyzed by quantitative real-time RT-PCR assays that detect the M gene of influenza type A and the H3 gene of canine H3N8 influenza A virus. The lungs from all 7 dogs were positive for both the influenza A M gene and the canine influenza H3 gene (Table 8). After 3 passages in MDCK cells, influenza A subtype H3N8 virus was isolated from the lungs of a shelter dog that died after 3 days of pneumonia. This virus was named A/canine/Jacksonville/05 (H3N8) (canine/Jax/05). After 2 passages in

embryonated chicken eggs, influenza A subtype H3N8 virus was recovered from the lungs of the pet dog that also died after 3 days of pneumonia. This virus was named A/canine/Miami//05 (H3N8) (canine/Miami/05).

5 EXAMPLE 9—GENETIC ANALYSES OF THE CANINE INFLUENZA A H3N8 ISOLATES

Sequence analyses of canine/Jax/05 and canine/Miami/05 revealed that their hemagglutinin (HA) genes were 98% identical to the canine/FL/04, canine/TX/04, and canine/Iowa/05 isolates recovered from the lungs of racing greyhounds that died of
10 pneumonia during influenza outbreaks at tracks in 2004 and 2005 (Crawford, P.C. *et al.*, 2005; Yoon K-Y. *et al.*, 2005). In addition, the HA genes of canine/Jax/05 and canine/Miami/05 were 98% identical to contemporary equine influenza viruses isolated after the year 2000. Phylogenetic comparisons of the HA genes showed that the canine/Jax/05 and canine/Miami/05 viruses were clustered with the canine/FL/04, canine/TX/04, and
15 canine/Iowa/05 greyhound isolates and contemporary equine isolates, forming a distinct group from the older equine viruses isolated in the early 1990's (Figure 4). Furthermore, the canine/Jax/05, canine/Miami/05, and canine/Iowa/05 isolates were more closely related to canine/Tx/04 than to either canine/FL/04 or canine/FL/03. The 2005 isolates formed a subgroup that appears to branch off from the earlier 2003 and 2004 canine viruses with
20 differences at approximately 10 parsimony-informative sites. These differences support the hypothesis that canine influenza virus is being transmitted horizontally from dog-to-dog as opposed to being reintroduced periodically from an outside source. The accumulation of mutations from 2003 to 2005 illustrates the ongoing process of adaptation that the virus must undergo after being transmitted to a new host, as is expected to have happened for the canine
25 influenza viruses.

EXAMPLE 10—AMINO ACID ANALYSES OF THE CANINE INFLUENZA A H3N8 ISOLATES.

There were conserved amino acid substitutions in all 6 canine isolates that
30 differentiated them from contemporary equine influenza viruses (Table 9). These conserved substitutions were I15M, N83S, W222L, I328T, and N483T. Phylogenetic comparisons of the mature HA protein showed that the canine/Jax/05, canine/Miami/05, and canine/Iowa/05 viruses formed a subgroup with the canine/TX/04 isolate (Figure 4). There were 3 amino

acid changes (L118V, K261N, and G479E) that differentiated this subgroup from the other canine viruses (Table 9). There were 2 amino acid changes (F79L and G218E) that differentiated the 2005 isolates from their canine/TX/04 root. Furthermore, the 2005 isolates from non-greyhound dogs, canine/Jax/05 and canine/Miami/05, differed from the
5 canine/Iowa/05 greyhound isolate by one amino acid change, R492K. Finally, canine/Jax/05 differed from canine/Miami/05 at a single amino acid, S107P. In all other H3N8 equine and canine viruses, S is conserved at position 107 except for A/Equine/Jilin/1/89 which has a T (Guo Y. *et al.*, 1992).

10 EXAMPLE 11—ANTIGENIC ANALYSES OF THE CANINE INFLUENZA A H3N8 ISOLATES

Hemagglutination inhibition (HI) tests were performed using an antigen panel of older and contemporary equine influenza viruses and the canine influenza viruses, and serum collected in 2005 from horses and dogs that had been infected with influenza virus (Table
15 10). Serum from ferrets immunized against canine/FL/04 was also included in the analyses. The HI antibody titers in equine serum were 8 to 16-fold higher when tested with contemporary equine viruses compared to older isolates, but decreased by at least 4-fold when tested with the canine viruses. The canine serum was nonreactive with the older equine viruses, but the antibody titers increased 4-fold when tested with contemporary equine
20 isolates and canine isolates. This was also observed for the serum from ferrets immunized against canine influenza virus. These seroreactivity patterns demonstrated the antigenic similarity between the canine influenza viruses and contemporary equine influenza viruses and were consistent with the phylogenetic analyses. The antibody titers in equine, canine, and ferret sera to the canine/Miami/05 isolate were similar to those for the 2003 and 2004
25 canine isolates. However, the titers were 2 to 4-fold lower for the canine/Jax/05 isolate. This suggests that canine/Jax/05 is antigenically distinct from the other canine isolates, which may in part be related to the single amino acid change at position 107 in the mature HA.

Table 7. Primers and probes for quantitative real-time RT-PCR analysis for the matrix gene of influenza A virus and the H3 gene of canine influenza A (H3N8).			
Primer	Target	Sequence	Application
Ca-H3-F387	H3 (nt 387-406)	5'-tatgcatacgtccgatccat-3' (SEQ ID NO: 79)	Forward primer for H3
Ca-H3-R487	H3 (nt 487-467)	5'-gctccacttcttcggtttga-3' (SEQ ID NO: 80)	Reverse primer for H3
Ca-H3-P430	H3 (nt 430-459)	FAM-aattcacagcagaggatcattcacatggacag-BHQ1 (SEQ ID NO: 81)	TaqMan® probe
FluA-M-F151	M (nt 151-174)	5'-catggatggctaaagacaagacc-3' ^a (SEQ ID NO: 82)	Forward primer for M
FluA-M-R276	M (nt 276-253)	5'-agggcatttggacaaakogtcta-3' (SEQ ID NO: 83)	Reverse primer for M
FluA-M-P218	M (nt 218-235)	FAM-acgcTcacogTgccAgt-BHQ1 ^b (SEQ ID NO: 84)	TaqMan® probe

^a Underlined letter r represents nucleotide a or g and underlined letter k represents nucleotide g or t.

^b Uppercase letters represent locked nucleic acid residues.

Table 8. Quantitative real-time RT-PCR and viral isolation performed on lung tissues from dogs that died from pneumonia during respiratory disease outbreaks in a shelter and veterinary clinic in Florida.

Dog ID	Location	Duration of clinical disease	Real-time RT-PCR		Virus Isolation
			M (Ct)	HA (Ct)	
A/canine/FL/242/03 positive control			28.15	27.36	
1079	Shelter (NE FL)	2 days	29.81	28.84	none
1078	Shelter (NE FL)	3 days	30.37	29.71	MDCK 3 rd passage
318	Shelter (NE FL)	9 days	33.89	32.97	none
320	Shelter (NE FL)	10 days	39.44	37.09	none
319	Shelter (NE FL)	6 days	33.87	32.23	none
1080	Shelter (NE FL)	6 days	38.87	38.23	none
374	Veterinary clinic (SE FL)	3 days	24.05	22.65	Egg 2 nd passage

Table 9. Amino acid comparison of the mature HA for canine influenza viruses and contemporary equine influenza viruses.

	Amino Acid																	
	7	15	54	78	79	83	92	107	118	159	218	222	261	328	479	483	492	541
A/equine/KY/5/02	G	I	N	V	F	N	S	S	L	N	G	W	K	I	G	N	R	K
A/equine/MA/213/03	.	.	.	A	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/equine/OH/1/03	D	.	K	A	.	.	.	.	.	S	.	.	.	.	.	.	.	.
A/canine/FL/242/03	.	M	K	A	.	S	.	.	.	S	.	L	.	T	.	T	.	.
A/canine/FL/43/04	.	M	K	A	.	S	N	.	.	S	.	L	.	T	.	T	.	R
A/canine/TX/1/04	.	M	K	A	.	S	.	.	V	S	.	L	N	T	E	T	.	.
A/canine/Iowa/05	.	M	K	A	L	S	.	.	V	S	E	L	N	T	E	T	.	.
A/canine/Miami/05	.	M	K	A	L	S	.	.	V	S	E	L	N	T	E	T	K	.
A/canine/Jacksonville/05	.	M	K	A	L	S	.	P	V	S	E	L	N	T	E	T	K	.

Table 10. Antibody titers in equine, canine, and ferret serum to older and contemporary equine influenza viruses and canine influenza viruses.

	Serum	antibody	titers ^a
Antigens	Equine	Canine	Ferret ^b
equine/Miami/63	40	<10	16
equine/Ky/86	40	40	32
equine/KY/92	40	<10	32
equine/NY/99	320	40	128
equine/KY/05/02	320	160	256
equine/MA/213/03	640	160	512
equine/OH/01/03	640	160	512
canine/FL/03	160	160	512
canine/FL/04	160	80	512
canine/Tx/04	160	160	512
canine/Miami/05	160	80	256
canine/Jax/05	40	40	128

^a Antibody titers were determined in a hemagglutination inhibition assay performed with serial dilutions of equine, canine, or ferret serum and the viruses listed in the antigen column.

^b Serum from ferrets immunized with canine/FL/04 virus.

MATERIALS AND EXAMPLES METHODS FOR EXAMPLES 12-15

Canine influenza virus inoculum.

The virus inoculum was prepared by inoculation of Madin-Darby canine kidney (MDCK) epithelial cells with a stock of A/canine/FL/43/04 (H3N8) representing passage 3 of

the original isolate previously described (Crawford *et al.*, 2005). The inoculated MDCK cells in Dulbecco's Minimal Essential Media (DMEM) supplemented with 1 µg/mL TPCK-treated trypsin (Sigma-Aldrich Corp., St. Louis, MO) and antibiotics (gentamycin and ciprofloxacin) were grown in 250 cm² flasks at 37°C in a humidified atmosphere containing 5% CO₂. The cultures were observed daily for morphologic changes and harvested at 5 days post inoculation. The harvested cultures were clarified by centrifugation and the supernatants were stored at -80°C pending inoculation of dogs. An aliquot of supernatant was used for determination of virus titer by the Reed and Muench method. The titer was 10⁷ median tissue culture infectious doses (TCID₅₀) of A/canine/Florida/43/2004 (canine/FL/04) per mL.

Experimental inoculation.

Eight 4-month old colony bred mongrel dogs (Marshall BioResources, North Rose, NY) (4 males and 4 females) were used for the experimental inoculation study approved by the University of Florida Institutional Animal Care and Use Committee. The dogs' body weights ranged from 13 to 17 kg. The dogs were healthy based on physical examinations, baseline blood tests, and recording of body temperatures for 2 weeks prior to inoculation. All dogs were free from prior exposure to canine influenza virus based on serology tests performed on paired serum samples collected at the time of arrival into the facility and 2 weeks later. The dogs were anesthetized by intravenous injection of propofol (Diprivan®, Zeneca Pharmaceuticals, 0.4 mg/kg body weight to effect) for intubation with endotracheal tubes. Six dogs (3 males and 3 females) were each inoculated with 10⁷ TCID₅₀ of canine/FL/04 virus in 5 mL of sterile saline administered into the distal trachea through a small diameter rubber catheter inserted into the endotracheal tube. Two dogs (1 male and 1 female) were sham-inoculated with an equal volume of sterile saline. The sham-inoculated control dogs were housed in a different room from the virus-inoculated dogs and cared for by different personnel. Physical examinations and rectal temperature recordings were performed twice daily for 6 days post inoculation (p.i.).

Pharyngeal and rectal swab collection.

To monitor for virus shedding, oropharyngeal specimens were collected twice daily from each dog on days 0 to 6 p.i. using polyester swabs (Fisher Scientific International Inc., Pittsburgh, PA). The swabs were placed in 1 mL of sterile phosphate-buffered saline (PBS) containing 0.5% bovine serum albumin (BSA). Rectal swabs were collected from each dog

daily from days 0 to 6. Swab extracts were prepared by clarification of the swab transport media by centrifugation. An aliquot of swab extract was tested immediately for influenza A virus nucleoprotein using the Directigen™ commercial immunoassay kit (BD, Franklin Lakes, NJ) according to the manufacturer's instructions. The remaining extract was stored at
5 -80°C pending other virological assays.

Postmortem examinations.

On day 1 p.i., one sham-inoculated dog and one virus-inoculated dog were euthanatized by intravenous inoculation of Beuthanasia-D® solution (1 mL/5 kg body weight;
10 Schering-Plough Animal Health Corp). One virus-inoculated dog was similarly euthanatized each day from days 2 to 5 p.i. On day 6 p.i., the remaining sham-inoculated and virus-inoculated dog were euthanatized. Complete postmortem examinations were performed by one of the investigators (WLC). Tissues were fixed in 10% neutral buffered formalin, embedded in paraffin, and 5-µm sections were either stained with hematoxylin and eosin for
15 histopathologic diagnosis or processed for immunohistochemistry as described below. Unfixed lung tissues were submitted to the Diagnostic Clinical Microbiology/Parasitology/Serology Service at the University of Florida College of Veterinary Medicine for bacterial isolation and identification. The samples were cultured on nonselective media as well as media selective for *Bordetella* species (Regan-Lowe; Remel, Lenexa, KS) and *Mycoplasma*
20 species (Remel). All cultures were held for 21 days before reporting no growth. Unfixed tissues were also stored at -80°C pending virological analyses.

Immunohistochemistry.

Deparaffinized and rehydrated 5-µm trachea and lung tissue sections were mounted
25 on Bond-Rite™ slides (Richard-Allan Scientific, Kalamazoo, MI) and subsequently treated with proteinase K (DAKO Cytomation Inc., Carpinteria, CA) followed by peroxidase blocking reagent (DAKO® EnVision™ Peroxidase Kit, DAKO Corp., Carpinteria, CA). The sections were incubated with a 1:500 dilution of monoclonal antibody to influenza A H3 (Chemicon International, Inc., Temecula, CA) for 2 hours at room temperature. Controls
30 included incubation of the same sections with mouse IgG (1 mg/mL, Serotec, Inc. Raleigh, NC), and incubation of the monoclonal antibody with normal canine lung sections. Following treatment with the primary antibody, the sections were incubated with secondary immunoperoxidase and peroxidase substrate reagents (Dako® EnVision™ Peroxidase Kit,

Dako Corp.) according to the manufacturer's instructions. The sections were counterstained with hematoxylin, treated with Clarifier #2 and Bluing Reagent (Richard-Allan Scientific, Kalamazoo, MI), dehydrated, and coverslips applied with Permount (ProSciTech, Queensland, Australia).

5

RNA extraction from swabs and tissues.

Lung and tracheal tissues from each dog were thawed and homogenized in minimum essential medium (MEM) supplemented with 0.5% bovine serum albumin (BSA) and antibiotics (gentamycin and ciprofloxacin) using a disposable tissue grinder (Kendall, Lifeline Medical Inc., Danbury, CT). Total RNA was extracted from the tissue homogenates as well as oropharyngeal and rectal swab extracts using a commercial kit (RNeasy[®] Mini Kit, QIAGEN Inc., Valencia, CA) according to manufacturer's instructions and eluted in a final volume of 60 µL of buffer.

Real-time RT-PCR.

A single-step quantitative real-time RT-PCR was performed on the total RNA using the QuantiTect[®] Probe RT-PCR Kit containing ROX as a passive reference dye (QIAGEN Inc., Valencia, CA) and a primer-probe set that targeted a highly conserved region of the matrix (M) gene of type A influenza virus (Payungporn S. *et al.*, 2006a; Payungporn S. *et al.*, 2006b). For each real-time RT-PCR reaction, 5 µL of extracted total RNA were added to a reaction mixture containing 12.5 µL of 2X QuantiTect[®] Probe RT-PCR Master Mix, 0.25 µL of QuantiTect[®] RT Mix, forward and reverse primers (0.4 µM final concentration for each), probe (0.1 µM final concentration) and RNase-free water in a final volume of 25 µL. The TaqMan[®] GAPDH Control Reagents (Applied Biosystems, Foster City, CA) were used according to manufacturer's instructions for detection of GAPDH as an endogenous internal control for the presence of RNA extracted from the swab and tissue samples and as a normalization control.

Quantitative one-step real-time RT-PCR was performed on the reaction mixtures in a Mx3000P[®] QPCR System (Stratagene, La Jolla, CA). Cycling conditions included a reverse transcription step at 50°C for 30 minutes, an initial denaturation step at 95°C for 15 minutes to activate the HotStarTaq[®] DNA polymerase, and amplification for 40 cycles. Each amplification cycle included denaturation at 94°C for 15 seconds followed by

annealing/extension at 60°C for 1 minute. The FAM (emission wavelength 518 nm) and VIC (emission wavelength 554 nm) fluorescent signals were recorded at the end of each cycle. The threshold cycle (Ct) was determined by setting the threshold fluorescence (dR) at 1000 in each individual experiment. The Mx3000P[®] version 2.0 software program (Stratagene, La Jolla, CA) was used for data acquisition and analysis. The positive control consisted of amplification of RNA extracted from A/canine/FL/242/03 (H3N8) virus. The results were normalized by dividing the M Ct value by the corresponding GAPDH Ct value for each sample.

10 Virus re-isolation from tissues.

Frozen lung and trachea tissues from virus-inoculated dogs were thawed and homogenized in 10 volumes of DMEM supplemented with 0.5% BSA and antibiotics. Solid debris was removed by centrifugation and supernatants were inoculated onto MDCK cells cultured in DMEM supplemented with 1 µg/mL TPCK-treated trypsin (Sigma-Aldrich Corp., St. Louis, MO) and antibiotics as described above. Cells were grown in 25 cm² flasks at 37°C in a humidified atmosphere containing 5% CO₂. The cultures were observed daily for morphologic changes and harvested at 5 days post inoculation. The harvested cultures were clarified by centrifugation and the supernatants inoculated onto fresh MDCK cells as described for the initial inoculation; two additional passages were performed for samples that did not show evidence of influenza virus by hemagglutination or RT-PCR. Hemagglutination activity in the clarified supernatants was determined using 0.5% turkey red blood cells as previously described (Crawford *et al.*, 2005). RT-PCR was performed as described below.

RT-PCR, nucleotide sequencing, and phylogenetic analyses.

25 Viral RNA was extracted from MDCK supernatant using the QIAamp[®] Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions. The viral RNA was reverse transcribed to cDNA using the QIAGEN[®] OneStep RT-PCR Kit (QIAGEN Inc., Valencia, CA) according to manufacturer's instructions. PCR amplification of the coding region of the 8 influenza viral genes in the cDNA was performed as previously described (Crawford *et al.*, 2005), using universal gene-specific primer sets (primer sequences available on request). The resulting DNA amplicons were used as templates for automated sequencing in the ABI PRISM[®] 3100 automated DNA sequencer using cycle

sequencing dye terminator chemistry (Applied Biosystems, Foster City, CA). Nucleotide sequences were analyzed using the Lasergene 6 Package[®] (DNASTAR, Inc., Madison, WI). The nucleotide sequences for viruses recovered from infected dogs were compared to the sequences of the virus in the inoculum to determine if any changes had occurred during replication in the respiratory tract.

EXAMPLE 12—CLINICAL DISEASE

All 6 virus-inoculated dogs developed a transient fever (rectal temperature $\geq 39^{\circ}\text{C}$) for the first 2 days p.i., but none exhibited respiratory signs such as cough or nasal discharge over the 6-day observation period. The sham-inoculated dogs remained clinically healthy.

EXAMPLE 13—VIRUS SHEDDING

Influenza A nucleoprotein was detected in the oropharyngeal swab collected from one of the virus-inoculated dogs at 24 hours p.i. The oropharyngeal swabs collected from one dog at 72, 84, and 120 hours p.i., and another dog at 108, 120, and 132 hours p.i., were positive for virus by quantitative real-time RT-PCR (Table 11). The absolute number of influenza M gene copies per μL of swab extract increased with time from 3 to 6 days p.i. No virus was detected in the rectal swabs.

EXAMPLE 14—POSTMORTEM EXAMINATIONS

In contrast to the previous experimental infection using specific pathogen-free Beagles (Crawford *et al.*, 2005), the virus-inoculated mongrel dogs had pneumonia as evidenced by gross and histological analyses of the lungs from days 1 to 6 p.i. In addition to pneumonia, the dogs had rhinitis, tracheitis, bronchitis, and bronchiolitis similar to that described in naturally infected dogs (Crawford *et al.*, 2005). There was epithelial necrosis and erosion of the lining of the airways and bronchial glands with neutrophil and macrophage infiltration of the submucosal tissues (Figure 5, upper panels). Immunohistochemistry detected viral H3 antigen in the epithelial cells of bronchi, bronchioles, and bronchial glands (Figure 5, lower panels). No bacterial superinfection was present. The respiratory tissues from the 2 sham-inoculated dogs were normal.

EXAMPLE 15—VIRUS REPLICATION IN TRACHEA AND LUNGS

The trachea and lungs were positive for virus by quantitative real-time RT-PCR in all dogs from 1 to 6 days p.i. (Table 12). The absolute number of influenza M gene copies per μL of trachea homogenate increased from 1 to 5 days p.i., then decreased on day 6. The absolute number of M gene copies per μL of lung homogenate decreased from 1 to 6 days p.i. In general, the trachea contained $\geq$ one $\log_{10}$ more virus than the lung on each of the 6 days p.i.

Table 11. Detection of virus shedding in the oropharynx of mongrel dogs inoculated with canine influenza virus by quantitative real-time RT-PCR.			
Dog ID	Time p.i. (hours)^a	M/GAPDH ratio^b	Matrix gene (copies / μL)^c
860	72	1.20	1.57E+02
	84	1.30	8.25E+02
	120	1.23	1.47E+03
894	108	1.17	1.17E+02
	120	1.41	1.37E+02
	132	1.27	3.74E+02

^a Time that oropharyngeal swabs were collected from the dogs following inoculation with A/canine/FL/43/04 (H3N8) virus.

^b Normalization ratios were calculated by dividing the M (Ct) by the GAPDH (Ct) for each swab extract.

^c The absolute number of matrix gene copies per μL of swab extract.

Table 12. Detection of virus replication in the trachea and lung of mongrel dogs inoculated with canine influenza virus by quantitative real-time RT-PCR.					
Dog ID	Time p.i. (hours)^a	M/GAPDH ratio^b		Matrix gene (copies / uL)^c	
		Lung	Trachea	Lung	Trachea
797	24	1.20	1.43	8.22E+05	3.11E+04
801	48	1.33	0.99	1.15E+05	6.52E+06
789	72	1.44	1.12	2.39E+04	1.56E+05
819	96	1.40	1.27	3.19E+04	1.43E+05
860	120	1.59	1.04	3.48E+03	1.17E+06
894	144	1.70	1.15	4.78E+02	1.50E+03

^a Time that tissues were collected from the dogs following inoculation with A/canine/FL/43/04 (H3N8) virus.

^b Normalization ratios were calculated by dividing the M (Ct) by the GAPDH (Ct) for each tissue homogenate.

^c The absolute number of matrix gene copies per uL of tissue homogenate.

MATERIALS AND EXAMPLES METHODS FOR EXAMPLE 16

Virus strains

Canine influenza virus strains as well as those of avian, equine and human origin (listed in **Table 15**) were propagated in embryonated eggs or MDCK cells and their infectivity was titrated by endpoint dilution in chicken embryos, or plaque assay. Rapid virus quantification was performed by hemagglutination assay using turkey red blood cell erythrocytes.

Diagnostic specimens

A Total of 60 canine's lung tissues collected from suspect cases of viral respiratory disease during the year of 2005 were tested for the presence of canine influenza virus.

RNA extraction from canine tissue samples

Blocks of lung tissue weighing between 20 and 30 mg were homogenized in a disposable tissue grinder (Kendal). Total RNA was extracted using a commercial kit (RNeasy Mini Kit, Qiagen, Valencia, CA) and eluted in a final volume of 60 μ L, following the manufacturer's recommendations.

Primers and probes design

Multiple sequence alignments of the H3 and M genes from various subtypes and from diverse species were performed using the CLUSTAL X program (Version 1.8). Matrix (M) primers and probe were selected from the conserved regions of over the known sequences corresponding to different subtypes of influenza A virus, whereas the H3 hemagglutinin gene-specific primers and probe set were selected to specifically match equine and canine influenza A virus genes and mismatch the homologous avian and human genes (Table 13). Primer design software (OLIGOS Version 9.1) and the web based analysis tools provided by EXIQON (<http://lnatools.com>) was used for T_m calculation and prediction of secondary structure as well as self hybridization. A conserved region of an 18S rRNA gene was used as endogenous internal control for the presence of RNA extracted from canine tissue sample. The Pre-Developed TaqMan® Assay Reagents for Eukaryotic 18S rRNA (VIC/TAMRA) (Applied Biosystems) was used for the real-time detection of 18S rRNA in tissue samples.

Real-time RT-PCR condition

A single-step real-time RT-PCR was performed by using the Quantitect Probe RT-PCR Kit containing ROX as a passive reference dye (Qiagen, Valencia, CA). In each real-time RT-PCR reaction, 5 μ L of RNA sample were used as a template to combine with a reaction mixture containing 10 μ L of 2X QuantiTech Probe RT-PCR Master Mix, 0.2 μ L of QuantiTech RT Mix, primers (0.4 μ M final conc. for H3 gene or 0.6 μ M final conc. for M gene), probe (0.1 μ M final conc. for H3 gene or 0.2 μ M final conc. for M gene) and RNase-free water in a final volume of 20 μ L. One-step real-time RT-PCR was performed in the Mx3005P Real-Time QPCR System (Stratagene). Cycling conditions included a reverse transcription step at 50°C for 30 minutes. After an initial denaturation step at 95°C for 15 minutes in order to activate the HotStarTaq DNA polymerase, amplification was performed during 40 cycles including denaturation (94°C for 15 seconds) and annealing/extension (60°C

for 30 seconds). The FAM (emission wavelength 516 nm for H3 and M detection) and VIC (emission wavelength 555 nm for 18S rRNA detection) fluorescent signals were obtained once per cycle at the end of the extension step. Data acquisition and analysis of the real-time PCR assay were performed using the Mx3005P software version 2.02 (Stratagene).

Specificity of H3 primers/ probe set for canine influenza (H3N8) and universality of M primers/probe set for type A influenza virus

In order to test the specificity of each primers/probe set, RNA extracted from several known subtypes of influenza A viruses were used as a template in the real-time RT-PCR assay (Table 15).

RNA standard for determination of the real-time RT-PCR performance

The genes of canine influenza A virus (A/canine/Florida/242/2003(H3N8)) were used to generate the PCR amplicons for H3 (nt 1-487) and M (nt 1-276) by using primers linked with T7 promoter (Table 13). Then the purified PCR amplicons of H3 and M genes were used as templates for *in vitro* transcription by using Riboprobe *in vitro* Transcription System-T7 (Promega) following the manufacturer's recommendations. The concentration of the transcribed RNAs was calculated by measuring absorbance at 260 nm. The RNAs were then serially diluted 10-fold, ranging from 10^8 to 10 copies/ μL to perform sensitivity tests. Moreover, a standard curve was generated by plotting the log of initial RNA template concentrations (copies/ μL) against the threshold cycle (Ct) obtained from each dilution in order to determine the overall performance of real-time RT-PCR.

Comparative sensitivity tests between real-time RT-PCR and Directigen Flu A test kit

Stock viruses of two viral strains including A/Wyoming/3/2003 (H3N2) at $10^{6.67}$ EID₅₀/mL (HA=64) and A/canine/Florida/242/2003(H3N8) at $10^{7.17}$ EID₅₀/mL (HA=16) were used for the detection threshold assay. Logarithmic dilution of specimens in phosphate-buffered saline (PBS) (125 μL) were used in a rapid influenza A antigen detection kit, Directigen Flu A, (Becton, Dickinson and Company) following the manufacturer's instructions. Each Directigen Flu A test device has an H1N1 influenza antigen spot in the center of the membrane which develops as a purple dot and indicates the integrity of the test, which is based on a monoclonal antibody to the nucleoprotein (NP). The development of a

purple triangle surrounding the dot is indicative of the presence of influenza NP in the tested specimen. The intensity of the purple signal from the triangle was scored as + (outline of triangle), ++ (lightly colored triangle), +++ (dark-purple triangle) and ++++ (very dark-purple triangle). Viral RNA was extracted 125 μ L aliquots of each virus dilution by using QIAamp Viral RNA Mini Kit (Qiagen, Valencia, CA) and eluting in a final volume of 50 μ L. A volume of 5 μ L of the extracted viral RNAs were tested by real-time RT-PCR for comparative sensitivity test with Directigen Flu A kit.

EXAMPLE 16

The real-time RT-PCR assay for canine influenza relies on information from three molecular probes which target 18S rRNA from host cell as well as M and H3 from the influenza A virus genome (Table 14). Amplification of the host gene is a reporter of specimen quality and integrity. Clinical, necropsy or laboratory samples containing canine influenza (H3N8) virus are expected to yield amplification signal with the three probes. Specimens yielding amplification signal with M and 18S rRNA probes but negative for H3 would be indicative of an influenza virus subtype H3 from human, swine or avian origin or from non-H3 subtypes. These rare cases could be resolved by RT-PCR using HA universal primers to generate amplicon cDNA that can be analyzed by sequencing. Properly collected and handled specimens lacking influenza A virus yield 18S rRNA amplification signal only. Situations in which only the 18S rRNA probe and the H3 probes yield amplification signal are indicative of faulty technique, unless proven otherwise; either a false negative with M probes or false positive for H3 need to be demonstrated. Finally, specimens failing to yield amplification signals with the three probes are indicative of defective sample collection, degradation, faulty RNA extraction or the presence of inhibitors the polymerases used in PCR.

In order to test the specificity of the H3 primers/probe set for canine influenza A virus (H3N8) and the universality of M primers/probe set for type A influenza, several subtypes of influenza A viruses were tested by real-time RT-PCR. The results show that H3 primers/probe set yielded a positive amplification signal only with canine influenza (H3N8). No significant false positive or non-specific amplification signals were observed in other subtypes or human H3 strains. The M primers/probe set yielded positive amplification signal with all of the strains tested (Table 15). These results indicated that H3 primers/probe

specifically detects canine influenza A virus (H3N8) whereas M primers/probe detect multiple subtypes of type A influenza viruses.

The performance of real-time RT-PCR assays was evaluated by endpoint dilution of M and H3 *in vitro* transcribed RNAs. As expected, the threshold cycle (Ct) increased in
5 direct correlation with the dilution of the RNA standards. The fluorescent signals can be detected at RNA standard dilutions of M and H3 as low as 10^3 and 10^2 copies/ μ L, respectively (Figure 6A and 6B). The standard curves of M and H3 genes were constructed by plotting the log of starting RNA concentrations against the threshold cycle (Ct) obtained from each dilution (Figure 6C and 6D). The slope of the standard curve is used to determine
10 the PCR reaction efficiency, which is theoretically exponential; 100% amplification efficiency would imply doubling of amplicon concentration each cycle. The standard curves with a slope between approximately -3.1 and -3.6 are typically acceptable for most applications requiring accurate quantification (90-110 % reaction efficiency). An Rsq value is the fit of all data to the standard curve plot. If all the data lie perfectly on the line, the Rsq
15 will be 1.00. As the data fall further from the line, the Rsq decreases. An Rsq value ≥ 0.985 is acceptable for most assays. The M standard curve revealed a slope of -3.576 (efficiency= 90.4 %) and Rsq= 1.00 whereas H3 standard curve yielded a slope of -3.423 (efficiency= 95.9%) and Rsq= 0.999. These values indicate satisfactory amplification efficiency and overall performance of the real-time RT-PCR assays. We attribute the lower efficiency and
20 sensitivity of M primers/probe set as compared to H3 primers/probe set to the N-fold degeneracy of M primer sequences required to ensure broad coverage of M gene sequences variability across viruses of multiple subtypes, hosts and lineages.

The sensitivity of real-time RT-PCR assay was also compared with the commercial rapid antigen detection assay (Directigen Flu A). Logarithmic dilutions of
25 A/Wyoming/3/2003 (H3N2) and A/canine/Florida/242/2003(H3N8) were analyzed with Directigen Flu A and by real-time RT-PCR. The results of Directigen Flu A showed that the sensitivities against both viral strains are approximately 100-fold dilution from the stock viruses used in these experiments (Figure 7). The signals (purple color) generated by the canine virus (A/canine/Florida/242/2003: $10^{6 \times}$ PFU/ml) samples were much weaker than
30 those found in human virus (A/Wyoming/3/2003: $10^{7 \times}$ PFU/ml), in agreement with the lower virus concentration in these samples. Alternatively, lower signal for canine influenza could

be attributed to the molecular specificity of monoclonal antibodies against the NP; *i.e.* poor conservation of the amino acids within the NP epitope of canine influenza A viruses.

Real-time RT-PCR of the M gene yielded Ct values above threshold with virus 10 and 30 PFU equivalents per reaction of A/canine/Florida/242/2003 and A/Wyoming/3/2003, respectively (Table 16). The differences between the sensitivity value of 2 viral strains because the differences in the original viral titers. The H3 gene detection comparison between canine and human influenza viruses was not performed because the H3 primers/probe in our realtime RT-PCR assay amplifies exclusively canine influenza A virus. RT-PCR was 10^5 times more sensitive than the rapid antigen detection kit.

To evaluate the performance of the RT-PCR test in necropsy specimens from dogs with acute respiratory disease, sixty canine lung tissue samples submitted during the year of 2005 were tested for the presence of canine influenza A virus by real-time RT-PCR. A total of 12 out of 60 samples (20%) were positive with both M and H3 genes whereas the remaining 48 samples yielded negative result for both M and H3 gene. Virus isolation attempts were conducted by egg and MDCK cell inoculation, to evaluate the specificity of the realtime assay; 2 out 12 samples that were positive for canine influenza by RT-PCR yielded canine influenza virus (data not shown, manuscript in preparation). Although all of the tissues were collected from dogs with a history of severe respiratory disease, most of the samples yielded no canine influenza virus by either realtime PCR or conventional isolation, suggesting a high incidence of other respiratory pathogens such as *Bordetella bronchiseptica*, canine distemper or parainfluenza virus. The single step real-time RT-PCR assay herein provides a rapid, sensitive and cost-effective approach for canine influenza A virus (H3N8) detection. Rapid laboratory diagnosis of canine influenza A virus (H3N8) infections in the early stage of the disease can yield information relevant to clinical patient and facility management.

Table 13: Primers and probes used for real-time RT-PCR detection and in vitro transcription				
Oligo name	Type	Target	Sequence *	Application
Ca-H3-F387	Forward primer	H3 (nt 387-406)	5'-tatgcatcgctccgatccat-3' (SEQ ID NO: 79)	Real-time PCR
Ca-H3-R487	Reverse primer	H3 (nt 487-467)	5'-gctccacttcttccgttttga-3' (SEQ ID NO: 80)	
Ca-H3-P430	TaqMan probe	H3 (nt 430-459)	FAM-aattcacagcagagaggattcacatggacag-BHQ1 (SEQ ID NO: 81)	
FluA-M-F151	Forward primer	M (nt 151-174)	5'-catggartggctaagacaagacc-3' (SEQ ID NO: 82)	Real-time PCR
FluA-M-R276	Reverse primer	M (nt 276-253)	5'-agggcattttggacaaakcgtcta-3' (SEQ ID NO: 83)	
FluA-M-P218	LNA TaqMan probe	M (nt 218-235)	FAM-acgcTcaccgTgccAgt-BHQ1 (SEQ ID NO: 84)	
H3-F1	Forward primer	H3 (nt 1-14)	5'-tattcgtctcaggagcaaaagcagggg-3' (SEQ ID NO: 85)	<i>In vitro</i> transcription
T7/H3-R490	Reverse primer	T7 / H3 (nt 487-467)	5'-tgtaatacgaactcactataggggtccacttcttccgttttga-3' (SEQ ID NO: 86)	
M-F1	Forward primer	M (nt 1-15)	5'-gatcgcgtcttcaggagcaaaagcaggtag-3' (SEQ ID NO: 87)	<i>In vitro</i> transcription
T7/M-R276	Reverse primer	M (nt 276-253)	5'-tgtaatacgaactcactataggggcattttggacaaagcgtc-3' (SEQ ID NO: 88)	

* Note: Uppercases = LNA (Locked Nucleic Acid) residues, r = a or g, k = g or t, underline = T7 promoter sequence

Table 14: Interpretation of the real-time RT-PCR assay			
Interpretation	Results		
	M	H3	18S rRNA
Positive for canine influenza A virus (H3N8)	+	+	+
Positive for influenza A virus (unknown subtype)	+	-	+
Negative for influenza A virus	-	-	+
Error in RNA extraction or presence of PCR inhibitor	-	-	-

Table 15: Specificity test of canine H3 primers/probe set and universality test of M primers/probe set with several subtypes of influenza A viruses				
Subtypes	Strain Name	Host	Real-time RT-PCR detection	
			H3 gene (Ct)	M gene (Ct)
H1	A/Ohio/1983	Human	No Ct	15.40
	A/WSN/1933	Human	No Ct	20.09
H3	A/Wyoming/3/2003	Human	No Ct	28.85
	A/Victoria/3/1975	Human	No Ct	16.62
	A/canine/FL/242/2003	Canine	28.43	29.25
H4	Turkey/MN/1066/1980	Avian	No Ct	17.49
	Clinical sample*	Avian	No Ct	20.87
H5	AChicken/Thailand/CUK2/2004	Avian	No Ct	20.13
	A/Pheasant/NJ/1335/1998	Avian	No Ct	16.64
H6	Clinical sample*	Avian	No Ct	19.52
H10	Clinical sample*	Avian	No Ct	25.64
	Clinical sample*	Avian	No Ct	19.59
H11	Clinical sample*	Avian	No Ct	15.72
	Clinical sample*	Avian	No Ct	24.55

* Note that subtypes of clinical samples were confirmed by nucleotide sequencing.

Table 16: Comparative sensitivity tests for influenza A virus detection between real-time RT-PCR and Directigen Flu A

Virus dilutions	Directigen Flu A		Real-time RT-PCR of M (Ct)	
	A/canine/242/03	A/Wyoming/3/03	A/canine/242/03	A/Wyoming/3/2003
10^{-1}	++	++++	22.42	19.48
10^{-2}	+	+++	25.85	22.66
10^{-3}	-	-	29.27	25.76
10^{-4}	Not done	Not done	32.66	28.66
10^{-5}	Not done	Not done	35.48	33.14
10^{-6}	Not done	Not done	37.51	35.06
10^{-7}	Not done	Not done	39.09	36.44
10^{-8}	Not done	Not done	No Ct	38.93

Table 17.

Class of Amino Acid	Examples of Amino Acids
Nonpolar	Ala, Val, Leu, Ile, Pro, Met, Phe, Trp
Uncharged Polar	Gly, Ser, Thr, Cys, Tyr, Asn, Gln
Acidic	Asp, Glu
Basic	Lys, Arg, His

Table 18.			
Letter Symbol	Amino Acid	Letter Symbol	Amino Acid
A	Alanine	M	Methionine
B	Asparagine or aspartic acid	N	Asparagine
C	Cysteine	P	Proline
D	Aspartic Acid	Q	Glutamine
E	Glutamic Acid	R	Arginine
F	Phenylalanine	S	Serine
G	Glycine	T	Threonine
H	Histidine	V	Valine
I	Isoleucine	W	Tryptophan
K	Lysine	Y	Tyrosine
L	Leucine	Z	Glutamine or glutamic acid

Table 19. Amino acid differences between PB2 proteins of H3N8 equine and canine influenza viruses

Position	Equine Consensus *	Canine/FL/03	Canine/FL/04
5	K	K	E
12	S	L	L
37	G	G	E
175	R	R	I
374	L	I	I
375	R	R	K
447	Q	Q	H

Table 20. Amino acid differences between PB1 proteins of H3N8 equine and canine influenza viruses

Position	Equine Consensus *	Canine/FL/03	Canine/FL/04
114	V	I	I
154	D	G	G
221	A	T	T
317	M	I	I
459	I	I	V
682	I	I	V

Table 21. Amino acid differences between PA proteins of H3N8 equine and canine influenza viruses

Position	Equine Consensus *	Canine/FL/03	Canine/FL/04
27	D	N	N
62	I	V	V
213	R	K	K
337	A	T	T
343	A	E	E
345	L	I	I
353	K	R	R
400	T	T	A
450	V	I	I
460	M	M	I
673	R	R	K
675	N	D	D

*Based on available genes of viruses isolated between 1963 and 1998.

Table 22. Amino acid differences between NP proteins of H3N8 equine and canine influenza viruses

Position	Equine Consensus *	Canine/FL/03	Canine/FL/04
16	G	D	D
157	A	T	T
214	R	R	K
285	V	V	I
286	A	T	T
359	A	T	T
375	D	D	N
384	R	K	K
452	R	K	K

Table 23. Amino acid differences between NA proteins of H3N8 equine and canine influenza viruses			
Position	Equine Consensus *	Canine/FL/03	Canine/FL/04
9	A/T	T	A
12	S	F	F
20	L	I	I
40	G	R	R
42	G	D	D
46	N	K	K
52	E	E	K
61	R	K	K
69	N	S	S
72	E	K	K
201	V	I	I
261	I	V	V
301	I	I	V
396	N	D	D
397	L	P	P

Table 24. Amino acid differences between M1 proteins of H3N8 equine and canine influenza viruses			
Position	Equine Consensus *	Canine/FL/03	Canine/FL/04
M1 161	S	S	A
M1 208	K/Q	R	R

*Based on available genes of viruses isolated between 1963 and 1998.

5

Table 25. Amino acid differences between NS1 proteins of H3N8 equine and canine influenza viruses			
Position	Equine Consensus *	Canine/FL/03	Canine/FL/04
44	K	R	R
59	R	H	H
71	E	K	K
86	A	T	T
88	R	R	L
140	R	G	G
216	P	S	S

* Based on available genes of viruses isolated between 1963 and 1998.

10

EXAMPLE 17 - CANINE INFLUENZA CHALLENGE MODEL DEVELOPMENT.

The canine influenza (canine flu) virus, which was isolated from flu outbreaks in Florida, was observed to be a H3N8 type influenza virus, and closely related to equine flu virus strain, A/equine/Ohio/03 (Crawford *et al.*, SCIENCE Vol. 309, September 2005, incorporated by reference in its entirety into this patent). The potential of using the equine flu virus strain A/equine/Ohio/03 to induce influenza-like disease in dogs was investigated in this study.

Procedure:

Ten 13-week-old beagles of mixed sex were obtained from a commercial supplier, and housed in individual cages in a BSL-2 facility. The dogs were randomly assigned to two groups of 5 dogs each. As shown in Table 26, one group was subjected to a intratracheal challenge, and the other group was subjected to an oronasal challenge. The dogs were challenged at 14 weeks-of-age.

Table 26: Experimental Design

Group	Number of Dogs	Challenge Route
1	5	Intratracheal
2	5	Oronasal

A cell culture grown equine flu virus A/equine/Ohio/03 was used as the challenge virus. For intratracheal challenge, the challenge virus was administered via a delivery tube, which consisted of a cuffed tracheal tube (Size 4.0/4.5, Sheridan, USA) and feeding tube (size 5Fr, 1.7 mm, /16 inches in length, Kendall, USA) in 0.5 to 1.0 ml volume. For oronasal challenge, the challenge virus (10x7.0 to 10x8.0 TCID₅₀ per dog) was administered as a mist using a nebulizer (DeVilbiss Ultra-Neb®99 ultrasonic nebulizer, Sunrise Medical, USA) in a 2 to 3 ml volume.

The dogs were observed for flu related clinical signs for 14 days post-challenge. Serum samples were collected from each dog on day zero (before challenge), and days 7 and 14 post-challenge for determining the HI titer using a H3N8 equine influenza virus with a standard protocol (SAM 124, CVB, USDA, Ames, IA). All dogs were humanely euthanized and lung tissues were collected in 10% buffered formalin for histopathological evaluation.

Results:

The results of this experiment are summarized in Table 27. Influenza related clinical signs were observed in a few dogs after challenge. These signs included fever ($>103^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$) and cough. Two of 5 dogs (*i.e.*, 40%) had fevers ($>103^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$) in Group 1, compared to 1 of 5 (*i.e.*, 20%) dogs in Group 2. One dog from the oronasal challenge group had sneezing, and another had cough following the challenge. An HI titer range from 10 to 80, with a geometric mean titer (GMT) of 20, was observed for Group 1. A titer range of 40 to 160, with a GMT of 86, was observed for Group 2. One dog from each group had histopathological lesions compatible with or pathognomic for influenza.

Table 27. Canine flu challenge – clinical signs, virus isolation, histopathology results and serology results									
Dog* ID	Challenge method*	Clinical signs	Virus isolation			Microscopic lesion (histopathology)	Serology (HI titer)		
			Nasal/oral swab	Tracheal scraping	Lung tissues		Pre-challenge	7-days post challenge	14-days post challenge
AAH	Intratracheal	none	negative	negative	negative	negative	10	10	20
ADB	Intratracheal	none	negative	negative	negative	negative	10	80	20
ADC	Intratracheal	Fever*	negative	negative	negative	negative	10	20	20
AEB	Intratracheal	Fever	negative	negative	negative	positive	10	40	20
AEE	Intratracheal	none	negative	negative	negative	inconclusive	10	20	10
AAE	Oronasal	none	negative	negative	negative	negative	10	80	80
AAG	Oronasal	none	negative	negative	negative	negative	10	40	80
ABY	Oronasal	Occasional sneeze, occasional cough	negative	negative	negative	positive	10	80	160
ADY	Oronasal	Fever, occasional sneeze	negative	negative	negative	negative	10	80	80
ADZ	Oronasal	none	negative	negative	negative	negative	10	80	160

* The animals were challenged with an Equine flu isolate Ohio 03.

** Rectal temperature $\geq 103^{\circ}\text{F}$; $\geq 39.4^{\circ}\text{C}$

EXAMPLE 18 - EFFICACY OF AN EQUINE INFLUENZA VIRUS VACCINE FOR DOGS.

The canine influenza (canine flu) virus isolated from flu outbreaks in Florida was observed to be a H3N8 type influenza virus, and was closely related to equine flu virus, A/equine/Ohio/03 based on the sequence similarity. The following study was conducted to determine the efficacy of an experimental inactivated equine influenza virus vaccine.

Procedure:

Nine 7-week-old beagles of mixed sex were obtained from a commercial supplier, and housed in individual cages in a BSL-2 facility. These dogs were randomly assigned to two groups, as summarized in Table 28:

Table 28: Experimental Design		
Group	Number of Dogs	Treatment
1	5	Vaccine
2	4	Control

The first group consisted of 5 dogs, which were vaccinated with an inactivated, CARBIGENTM adjuvanted, equine flu virus A/equine/Ohio/03 vaccine at 8 and 12 weeks-of-age via subcutaneous (SQ) route. The A/equine/Ohio/03 was inactivated by binary ethylenimine ("BEI") using a standard method. Each dose of the vaccine contained 5% by mass CARBIGENTM, 4096 HA units of the inactivated virus, sufficient PBS to bring the total volume of the dose to 1 ml, and sufficient NaOH to adjust the pH to between 7.2 and 7.4. Serum samples were collected from all dogs on the day of first and second vaccination and day 7 and 14, post-first and -second vaccination, and at pre-challenge for determining the HI titer using a H3N8 equine influenza virus a standard protocol (SAM 124, CVB, USDA, Ames, IA). At 3 weeks post-second vaccination, the 5 vaccinated dogs and the second group (*i.e.*, the control group) consisting of 4 age-matched dogs were challenged oronasally with a cell-culture-grown equine influenza virus A/equine/Ohio/03 (107.0 to 108.0 TCID50 per dog) in a 1-2 ml volume per dose. The challenge virus was administered to the dogs as a mist using a nebulizer (DeVilbiss Ultra-Neb®99 ultrasonic nebulizer, Sunrise

Medical, USA). The dogs were observed for flu-related clinical signs for 14 days post-challenge. Five dogs (3 vaccinates and 2 controls) 7 days post-challenge and 4 dogs (2 controls and 2 vaccinates) 14 days post-challenge were humanely euthanized for collection of lung tissues in 10% buffered formalin for histopathological evaluation.

5

Results:

The results of this experiment are summarized in Tables 29 and 30. All vaccinated dogs seroconverted following the vaccination. An HI titer range from 40 to 640, with the GMT of 129, was observed during the post-vaccination period with equine influenza virus A/equine/Ohio/03, and a HI titer of 160 to 320, with a geometric mean titer of 211, was
10 observed with canine flu isolate, A/canine/Florida/242/03. Two of 6 vaccinates had a fever of $>103^{\circ}\text{F}$ ($>39.4^{\circ}\text{C}$) for one day and no other clinical signs were observed in any of the dogs following challenge.

15 Conclusion:

All the vaccinated dogs responded to the inactivated, CARBIGENTM adjuvanted equine influenza vaccine. The HI titer results with a canine influenza virus isolate suggest that the inactivated equine influenza vaccine did induce a detectable level of cross reactive antibody to canine influenza virus. Even though the challenge virus used in this did not
20 induce any noticeable clinical disease in beagle dogs, based on the HI titer with a canine influenza virus isolate, it was concluded that inactivated equine vaccine could be used in dogs to induce cross reactive antibodies, which could potentially protect dogs against "canine flu" disease caused by H3N8 type canine influenza viruses.

Table 29. Serology – Pre- and post-vaccination and post-challenge HI titers											
Dog*	Group	Pre-vaccination	HI titers							Post-challenge*	
			Post-1st vaccination		Post-2nd vaccination			Post-challenge*			
			7-d	14-d	7-d	14-d	21-d	7-d	14-d	7-d	14-d
AKT	Vaccinate**	< 10	40	80	640	640	640	320	320	320	320
ALH	Vaccinate**	< 10	40	80	320	160	160	80	***	80	***
ALU	Vaccinate**	< 10	40	80	320	160	160	80	80	80	80
ANJ	Vaccinate**	< 10	40	80	320	160	80	320	***	320	***
ANU	Vaccinate**	< 10	40	80	320	160	80	160	***	160	***
AJW	Control	< 10	< 10	< 10	< 10	< 10	< 10	< 10	***	10	***
AKR	Control	< 10	< 10	< 10	< 10	< 10	< 10	< 10	***	10	***
ALZ	Control	< 10	< 10	< 10	< 10	< 10	< 10	< 10	20	20	20
ARC	Control	< 10	< 10	< 10	< 10	< 10	< 10	< 10	10	10	10

* The animals were challenged with an equine flu isolate Ohio 03

** CARBIGEN™ adjuvanted inactivated equine flu virus Ohio 03 vaccine was used for vaccination

*** Euthanized 7-days post-challenge

Table 30. Canine flu challenge* -- clinical signs, virus isolation, histopathology results						
Dog ID	Treatment group	Clinical signs	Virus isolation			Microscopic lesion (histopathology)
			Nasal swab	Tracheal scraping	Lung tissues	
AKT	Vaccinate**	none	negative	negative	negative	negative
ALH	Vaccinate**	none	negative	negative	negative	negative
ALU	Vaccinate**	none	negative	negative	negative	negative
ANJ	Vaccinate**	none	negative	negative	negative	negative
ANU	Vaccinate**	none	negative	negative	negative	negative
AJW	Control	none	negative	negative	negative	negative
AKR	Control	none	negative	negative	negative	negative
ALZ	Control	none	negative	negative	negative	negative
ARC	Control	none	negative	negative	negative	negative

* The animals were challenged with an Equine flu isolate Ohio 03

** CARBIGEN™ adjuvanted inactivated equine flu virus Ohio 03 vaccine was used for vaccination

EXAMPLE 19 – EFFICACY OF AN EQUINE INFLUENZA VIRUS VACCINE FOR DOGS.

The canine influenza virus isolated from flu outbreaks in Florida was characterized is closely related to a number of H3N8 type equine influenza virus isolates. By DNA and amino acid sequence similarity analysis it was demonstrated that the canine influenza virus is very similar to an equine influenza virus, A/equine/Ohio/03. The following study was conducted in dogs to determine the efficacy of commercially available equine influenza vaccines in dogs.

10 Procedure:

Approximately 16 month old, 20 mongrels and 20 beagles of mixed sex were used in the study. The dogs were randomly assigned to 6 groups (Table 31) of 6-7 dogs each. Dogs in groups 1 and 4 were vaccinated with a commercially available inactivated, adjuvanted equine influenza vaccine (EQUICINE IITM, Intervet Inc., Millsboro, DE) at 16 and 17 months of age via subcutaneous (SQ) route. The dogs in groups 2 and 5 were vaccinated with a modified live equine/Kentucky/91 influenza vaccine in a 1 ml volume via intranasal route (single nostril). Blood samples were collected on the day of vaccination, day 7 and 14 post first vaccination (groups 1, 2, 4, and 5) and post second vaccination (groups 1 and 4) for determining the HI titer using an H3N8 equine influenza virus and a canine influenza virus using per a standard protocol (SAM 124, CVB, USDA, Ames, IA).

Vaccinates (at 72 days post final vaccination) and the controls were challenged oronasally with a cell-culture grown equine influenza virus strain A/equine/Ohio/03 (10x7.0 to 10x8.0 TCID50 per dog) in a 1-2 ml volume. The challenge virus was administered to the dogs as mist using a nebulizer (DeVilbiss Ultra-Neb®99 ultrasonic nebulizer, Sunrise Medical, USA). The dogs were observed for influenza-related clinical signs for 12 days post-challenge. The nasal and oropharyngeal swabs were collected in Earl's MEM medium with antibiotics (neomycin and polymyxin B) daily from day -1 to day 12 post challenge for virus isolation. The presence of virus in the swabs indicates that the animal is excreting the virus in nasal/oral secretions. All dogs were humanely euthanized on day 12 post-challenge and lung tissues were collected in 10% buffered formalin for histopathological evaluation.

Table 31. Experimental design

Group	Number of dogs	Breed	Treatment	Number of doses	Route of vaccination
1	7	Beagle	EQUICINE II™**	2	Subcutaneous
2	7	Beagle	A/KY/91***	1	Intranasal
3	6	Beagle	Control	N/A*	N/A*
4	7	Mongrel	EQUICINE II™	2	Subcutaneous
5	7	Mongrel	A/KY/91	1	Intranasal
6	6	Mongrel	Control	N/A*	N/A*

* Not applicable

** EQUICINE II™ is marketed by Intervet Inc. as a liquid vaccine. EQUICINE II™ contains inactivated A/Pennsylvania/63 influenza (or "A/Pa/63") virus and A/equine/Kentucky/93 influenza (or "A/KY/93") virus with carbopol (*i.e.*, HAVLOGEN® (Intervet Inc.)). More specifically, a dose of EQUICINE II™ contains: inactivated A/Pa/63 at $10^{6.0}$ EID₅₀, inactivated A/KY/93 at $10^{6.7}$ EID₅₀, 0.25% by volume carbopol, and sufficient PBS to create a total volume of 1 ml.

*** A/KY/91 is a freeze-dried vaccine that was reconstituted with water. Such reconstitution was conducted using vaccine-grade water sufficient to bring the vaccine dosage to a total volume of 1 ml. The vaccine contained equine/Kentucky/91 influenza (or "A/KY/91") virus, and is discussed in, for example, U.S. Patent Nos. 6,436,408; 6,398,774; and 6,177,082, which are incorporated by reference in their entirety into this patent. When reconstituted, a dose of the vaccine contained A/KY/91 at $10^{7.2}$ TCID₅₀ per ml, 0.015 grams N-Z AMINE AS™ per ml, 0.0025 grams gelatin per ml, and 0.04 grams D lactose per ml. N-Z AMINE AS™ is a refined source of amino acids and peptides produced by enzymatic hydrolysis of casein. N-Z AMINE AS™ is marketed by Kerry Bio-Science (Norwich, NY, USA).

Results:

All vaccinated dogs seroconverted following the vaccination and the HI titers ranged from 10 to 80 for EQUICINE II™ vaccine group dogs compared to 10 to 40 for the A/KY/91 vaccine group dogs using an equine influenza virus (H3N8 type).

The samples collected at 2 weeks post vaccination (post second vaccination for EQUICINE II™ vaccine) were analyzed for HI titer determination with a canine influenza as well as with an equine influenza virus (H3N8 type). The HI results are shown in Table 32. The clinical signs include fever ($>103^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$), occasional cough, and mild nasal discharge observed following the challenge.

Table 32. Serology – HI titers at 2 weeks post vaccination

Group	Number of dogs	Breed	Treatment	HI titer with			
				Equine influenza virus		Canine influenza virus	
				Range	GMT	Range	GMT
1	7	Beagle	Equicine II™	10-80	36	10-80	33
2	7	Beagle	A/KY/91	10-20	12	20-160	54
3	6	Beagle	Control	N/A*	N/A*	N/A*	N/A*
4	7	Mongrel	Equicine II™	40-80	54	40-80	50
5	7	Mongrel	A/KY/91	10-40	24	40-80	49
6	6	Mongrel	Control	N/A*	N/A*	N/A*	N/A*

* Not applicable

Among beagles, 2 of 6 dogs in the EQUICINE II™ vaccine group (Group 1), 1 of 7 dogs in the A/KY/91 vaccine group (Group 2) and 2 of 6 dogs in the control group (Group 3) had fever. One of 6 dogs in Group 3 (control) was positive for virus in the cell culture supernatant of nasal swab material by hemagglutination assay with 0.25% chicken red blood cells (CRBC). One of 6 dogs in the control group (Group 3) and 1 of 7 dogs in the A/KY/91 vaccine group (Group 2) had mild nasal discharge during the post challenge observation period. There was no statistical significant difference ($P > 0.05$) between control and vaccine groups for beagle dogs.

Among mongrels, 5 of 7 dogs in the EQUICINE II™ vaccine group (Group 4), 1 of 7 dogs in the A/KY/91 vaccine group (Group 5) and 5 of 6 dogs in the control group (Group 6) had fever. One dog from each of Group 4 and 6 had a mild nasal discharge, and one dog from Group 5 had an occasional cough. Two of 7 dogs in the EQUICINE II™ vaccine group (Group 4) and 3 of 6 dogs in the control group (Group 6) were positive for influenza virus in the nasal swab by HA assay. None of the dogs from the A/KY/91 group (Group 5) were positive for influenza virus in the nasal swab materials.

20 Conclusion:

By serology, it was demonstrated that vaccination of dogs with commercially available equine influenza vaccines stimulated a moderate level influenza antibody response. There may be some breed difference in development of influenza-related clinical signs in dogs following a challenge with H3N8 type influenza virus. The live attenuated equine influenza vaccine (A/KY/91) provided a significant ($P < 0.05$) protection

from clinical disease development in rectal temperature in mongrels. Also, the live attenuated viral vaccine prevented the shedding of influenza virus in the nasal secretions.

EXAMPLE 20 – CANINE INFLUENZA CHALLENGE MODEL DEVELOPMENT

5 In view of reports that inducing disease in canines for purposes of study had not proven successful, the potential for using a canine influenza virus, H3N8, to develop a canine influenza challenge model in dogs was investigated in the following study.

Procedure:

10 Ten mongrels of mixed sex were obtained from a commercial supplier, and housed in cages in a BSL-2 facility. The dogs were randomly assigned to two groups of 5 dogs each. As shown in Table 33, one group was subjected to an intratracheal/intranasal challenge, and the other group was subjected.

Table 33. Experimental design

Group	Number of dogs	Challenge route
1	5	Intratracheal/intranasal
2	5	Oronasal

15 The dogs were challenged at approximately 12 weeks-of-age. Embryonated-chicken-egg grown canine influenza virus (A/canine/Florida/242/03) virus was used as challenge virus. Each dog received a total of approximately 107.2 TCID₅₀ of virus in either 2 ml (for oronasal route) or 4 ml (intratracheal/intranasal route) volume.

20 For intratracheal/intranasal challenge, 3 ml of the challenge virus was administered into the trachea first, followed by 5 ml of PBS using a delivery tube, which consisted of a cuffed tracheal tube (Size 4.5/5.0, Sheridan, USA) and feeding tube (size 5Fr, 1.7 mm; 16 inches (41 cm) in length, Kendall, USA), and a 1 ml challenge virus, followed by 3 ml of atmospheric air was administered into nostrils using a syringe.

25 For oronasal challenge, the challenge virus was administered as a mist using a nebulizer (Nebulair™, DVM Pharmaceuticals, Inc., Miami, FL) in approximately 2 ml volume. The dogs were observed for flu-related clinical signs for 14 days post-challenge. The dogs were euthanized at day 14 post challenge, and tissue (lung and trachea) samples were collected in 10% buffered formalin for histopathological examination.

Results:

All dogs in groups 1 and 2 developed canine influenza clinical signs within 24 to 48 hours. Each dog had 2 or more of the following clinical signs: fever ($>103.0^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$), cough, serous or mucopurulent ocular discharge, serous or mucopurulent nasal discharge, vomiting, diarrhea, depression, weight loss, gagging, hemoptysis, and audible rales. Lung tissues from 5 of 5 dogs from group 1 and 4 of 5 dogs from group 2 had histopathological lesions which included one or more of the following: diffuse suppurative bronchopneumonia, bronchitis/bronchiolitis with plugs of neutrophilic exudate in the lumina and marked mononuclear cell aggregation in mucosa and peribronchiolar tissue, mixed exudate within alveoli with large numbers of foamy macrophages, lymphocellular and plasma cellular as well as granulocytic cell infiltration, and thickening of alveolar septa with proliferation of type II pneumocytes compatible with or pathognomic to an influenza virus infection. The trachea tissue samples were normal.

Conclusion:

An H3N8 canine influenza isolate such as the one used in this study may be used for inducing canine influenza disease in dogs using one of the methods described in this study or a similar method.

EXAMPLE 21 - CANINE INFLUENZA CHALLENGE MODEL DEVELOPMENT.

The potential for using a canine influenza virus, H3N8, to develop a canine influenza challenge model in dogs was further investigated in the following study.

Procedure:

Fifteen 17- to 18-week-old mongrels and five 15-week-old beagles were obtained from commercial suppliers, and were housed in cages in a BSL-2 facility. The mongrels were randomly assigned to 3 groups (Groups 1 to 3) of 5 dogs each. All beagles were assigned to one group (Group 4) as shown in Table 34:

Table 34. Experimental design

Group	Breed	Number of dogs	Challenge virus dose
1	Mongrels	5	$10^{6.8}$ TCID ₅₀
2	Mongrels	5	$10^{5.8}$ TCID ₅₀
3	Mongrels	5	$10^{4.8}$ TCID ₅₀
4	Beagles	5	$10^{6.8}$ TCID ₅₀

The dogs were challenged oronasally with a virulent canine influenza virus, A/Canine/Florida/242/2003 (isolated from lung of a greyhound dog with canine influenza disease (provided by Dr. Cynda Crawford at the University of Florida)). The challenge virus was administered as a mist using a nebulizer (Nebulair™) in approximately 2 ml volume. The dogs were observed for flu-related clinical signs for 14 days post-challenge.

Results:

Eighty percent (4 of 5) of the dogs in Group 1 and 4, 100% of the dogs in Group 2 and 3, developed canine influenza clinical signs within 48 hours. Each dog had one or more of the following clinical signs: fever ($>103.0^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$), cough, serous or mucopurulent ocular discharge, serous or mucopurulent nasal discharge, vomiting, diarrhea, depression, weight loss, gagging, and rales. The clinical signs observed in beagles were generally milder and short-course compared to mongrels.

Conclusion:

An H3N8 canine influenza isolate such as the one used in this study may be used for inducing canine-influenza-like or kennel-cough-like disease in dogs using method described in this study or a similar method with a challenge dose range from 104.8 to 106.8 TCID₅₀. There were some differences in clinical signs observed in mongrels and beagles. In general, beagles tend to have milder flu-related clinical signs compared to mongrels.

EXAMPLE 22 – CANINE INFLUENZA VACCINE EFFICACY STUDY.

The following study was conducted to assess the efficacy of an H3N8 equine influenza vaccine in dogs against canine influenza virus.

Procedure:

Seventeen 14-week-old mongrels and ten 8-week-old beagles were obtained from commercial suppliers. The dogs were randomly assigned to 5 groups as shown in Table 35, and housed in a research facility.

Table 35. Experimental design

<u>Group</u>	<u>Age</u>	<u>Number of dogs</u>	<u>Treatment</u>	<u>Number of doses</u>	<u>Age at Vaccination (weeks)</u>
1	14 weeks	7	Vaccinate	2	14 & 18
2	14 weeks	5	Vaccinate	1	18
3	14 weeks	5	Control	--	--
4	8 weeks	5	Vaccinate	2	8 & 12
5	8 weeks	5	Control	--	--

The vaccine used in this study was a HAVLOGEN®-adjuvanted, inactivated equine influenza virus (A/equine/KY/02) vaccine. To prepare this vaccine, the virus was inactivated by binary ethylenimine (BEI) using a standard method. Each vaccine dose contained HAVLOGEN® (10% v/v), 6144 HA units of the inactivated virus, 0.1% (v/v) of 10% thimerosal, 0.1 % (v/v) of phenol red, sufficient NaOH to adjust the pH to from 6.8 to 7.2, and sufficient PBS to bring the total dose volume to 1 ml.

The dogs in Groups 1 and 4 were vaccinated with 2 doses of the vaccine. The second dose (*i.e.*, the booster) was administered 4 weeks after the first. The dogs in Group 2 were vaccinated with 1 dose at 18 weeks-of-age. Blood samples were collected to assess HI titer using a standard protocol (*e.g.*, SAM 124, CVB, USDA, Ames, IA) with an H3N8 canine influenza isolate on days zero (before vaccination), 7, and 14 post first and second vaccinations. Approximately 5 days before challenge, the dogs were moved to a BSL-2 facility and housed in individual cages.

All vaccinates and age-matched control dogs were challenged oronasally with a virulent canine influenza virus (107.7 TCID₅₀ of A/Canine/Florida/242/2003 per dog) at 2 weeks post second vaccination of Groups 1 and 4 and first vaccination of Group 2. The challenge virus was administered as a mist using a nebulizer (Nebulair™) at 2 ml per dog. The dogs were observed for influenza-related clinical signs for 17 days post-challenge. Nasal and oropharyngeal swabs were collected in tubes containing 2 ml of virus transport medium for virus isolation from day -1 (*i.e.*, one day before challenge) to day 17 days post-challenge. All dogs were euthanized at day 17 post-challenge and lung and tracheal samples were collected in 10% buffered formalin for histopathology. Blood samples were collected on days 7 and 14 post challenge for HI titer determination. The clinical sign score assignments used for the post challenge observation are shown in Table 36.

Results:

All dogs in 2-dose vaccination groups (Group 1 and 4) developed HI antibody titer responses to the canine influenza virus isolate (Table 37). Following the challenge, approximately a 4-fold increase in titer on day 14 post challenge in all groups indirectly indicated that all dogs were exposed to the challenge virus. All dogs exhibited one or more of the following signs of canine influenza: fever ($>103.0^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$), cough, serous or mucopurulent ocular discharge, serous or mucopurulent nasal discharge, vomiting, diarrhea, depression, weight loss, and dyspnea. Vaccinates had less severe clinical signs, compared to age-matched controls (Table 38). There was a significant reduction in clinical signs due to the 2-dose vaccination in both 8-week-old ($P = 0.040$) and 14-week-old ($P = 0.003$) dogs (Groups 4 and 1 respectively). In this experiment, one-dose vaccination did not provide a significant ($P = 0.294$) reduction in clinical signs (Group 2)

Virus isolation results are shown in Table 39. Among 14-week-old dogs, canine influenza virus was isolated from swab samples collected from 2 of 7 dogs (29%) from the 2-dose vaccine group (Group 1), 3 of 5 dogs (60%) from the 1-dose vaccine group (Group 2), and 5 of 5 dogs (100%) from the control group (Group 3). Among 8-week-old dogs, the virus was isolated from 1 of 5 dogs (20%) from the 2-dose vaccine group (Group 4), and 4 of 5 dogs (80%) from the control group (Group 5). There was a significant reduction ($P = 0.003$) in the number of dogs positive for canine influenza virus in swab samples due to 2-dose vaccination (Groups 1 and 4) compared to unvaccinated controls (Groups 3 and 5). Although there was a reduction in the number of dogs (60% vs. 100%) positive for canine influenza virus in swab samples between 1-dose vaccine group (Group 2) and the control group (Group 3), the difference was not statistically significant ($P = 0.222$).

Histopathological evaluation of lung and tracheal tissue samples for lesions was conducted to identify lesions compatible with or pathognomic to canine influenza disease. This includes, for example, determination of whether one or more of the following exist: areas with suppurative bronchopneumonia; peribronchitis/peribronchiolitis with mononuclear cell aggregation (lymphocytes, plasma cells); presence of plugs of granulocytic cellular debris in the lumina; hyperplasia of respiratory epithelium; mixed exudate in the alveoli with large amount of granulocytic cells and cell debris; aggregates of (foamy) macrophages, plasma cells, and lymphocytes; and thickening of alveolar septa with proliferation of type II pneumocytes.

Table 40 provides a summary of the extent of lesions in this experiment for the dogs. Among 14-week-old dogs, the lung lesions were less extensive and less severe in 5 of 7 dogs in the 2-dose vaccination group (Group 2), and 4 of 5 dogs in the 1-dose vaccination group (Group 1). All controls dogs (Group 3) had severe and extensive lesions suggestive of no protection. There was no difference in tracheal lesions due to 1- or 2-dose vaccination among 14- week-old dogs. Among 8-week-old dogs, there was no difference in lung lesions between 2-dose vaccinates and control dogs. None of the dogs had any tracheal lesions.

Conclusion:

The results from this study demonstrate that: (1) inactivated H3N8 equine influenza virus can induce canine influenza virus cross reactive HI antibody responses in vaccinated dogs, (2) use of an H3N8 equine influenza virus vaccine can reduce the severity of canine influenza virus disease in dogs, and (3) use of an H3N8 equine influenza virus vaccine can reduce virus excretion in nasal and/or oral secretions.

Table 36. Clinical signs and scoring system

<u>Clinical signs</u>	<u>Score per day</u>
Temp	
<103.0°F (<39.4°C)	0
103.0 – 103.9°F (39.4-	2
104.0-104.9°F (40.0-40.5°C)	3
>105.0°F (>40.6°C)	4
Coughing	
No coughing	0
Occasional	2
Paroxysmal	4
Sneezing	
No sneezing	0
Occasional	1
Paroxysmal	2
Nasal discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3

Table 36. Clinical signs and scoring system

Clinical signs	Score per day
Ocular discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3
Hemoptysis	
No	0
Yes	5
Depression	
No	0
Yes	1
Anorexia	
No	0
Yes	1
Respiratory signs	
None	0
Rales	3
Dyspnea	4
Gasping	5
Mucous expectorate	
No	0
Yes	2
Vomiting	
No	0
Yes	1
Fecal abnormalities	
No	0
Yes	1

Table 37. Serology – Hemagglutination inhibition titers

Group No	Dog ID	Age (week)	Treatment	Number of doses	HI titer									
					Days post first vaccination of Groups 1 and 4					Days post challenge				
					0*	7	14	28**	35	42***	7	14	20	320
1	921	14	Vaccinate	2	<10	<10	10	20	40	20	<10	<10	160	320
1	926	14	Vaccinate	2	<10	<10	<10	40	40	80	<10	<10	80	>640
1	931	14	Vaccinate	2	<10	<10	<10	10	20	20	<10	<10	80	>640
1	955	14	Vaccinate	2	<10	<10	<10	10	40	40	<10	<10	160	320
1	011	14	Vaccinate	2	<10	<10	<10	10	20	40	<10	<10	160	320
1	013	14	Vaccinate	2	<10	<10	<10	20	40	40	<10	<10	160	320
1	019	14	Vaccinate	2	<10	<10	<10	10	20	40	<10	<10	160	320
2	922	14	Vaccinate	1	<10	<10	<10	<10	20	40	<10	<10	80	>640
2	953	14	Vaccinate	1	<10	<10	<10	<10	<10	<10	<10	<10	>640	>640
2	015	14	Vaccinate	1	<10	<10	<10	<10	<10	<10	<10	<10	320	>640
2	016	14	Vaccinate	1	<10	<10	<10	<10	<10	<10	<10	<10	320	>640
2	017	14	Vaccinate	1	<10	<10	<10	<10	<10	<10	<10	<10	160	320
3	923	14	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	320	>640
3	012	14	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	40	160
3	014	14	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	40	320
3	018	14	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	40	160
3	01A	14	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	40	160
4	406	8	Vaccinate	2	<10	<10	<10	<10	<10	<10	<10	<10	40	160
4	407	8	Vaccinate	2	<10	<10	10	40	80	80	<10	<10	160	>640
4	504	8	Vaccinate	2	<10	<10	20	40	40	40	<10	<10	320	>640
4	704	8	Vaccinate	2	<10	<10	10	20	20	80	<10	<10	160	>640
4	705	8	Vaccinate	2	<10	<10	10	40	80	160	<10	<10	160	>640
5	404	8	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	80	160
5	405	8	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	80	80
5	610	8	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	20	40
5	702	8	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	80	160
5	703	8	Control	N/A	<10	<10	<10	<10	<10	<10	<10	<10	40	160

*First vaccination – Groups 1 and 4

**Second vaccination – Groups 1 and 4; First vaccination – Group 2

***Day of challenge

Table 38. Analysis of total canine influenza disease clinical scores

<u>Group</u>	<u>Treatment</u>	<u>Number of doses of vaccine</u>	<u>Age at first vaccination of Groups 1 and 4</u>	<u>Average total Score per dog</u>	<u>P-value*</u>
1	Vaccinate	2	14 weeks	8.7	0.003 (Group 1 vs. 3)
2	Vaccinate	1	14 weeks (these dogs were vaccinated once, when they were 18 weeks old)	21.8	0.294 (Group 2 vs. 3)
3	Control	--	14 weeks (these dogs were not vaccinated)	25.4	--
4	Vaccinate	2	8 weeks	2.0	0.040 (Group 4 vs. 5)
5	Control	--	8 weeks (these dogs were not vaccinated)	5.4	--

* Analyzed using a NPARIWAY procedure of SAS® Version 8.2 (the vaccine groups were compared using the Wilcoxon rank sum test)

Table 40. Histopathological evaluation of tissue samples						
Group No	Dog ID	Age (week)	Treatment	Number of doses	Microscopic lesion (Histopathology)	
					Lungs	Trachea
1	921	14	Vaccinate	2	+/-	-
1	926	14	Vaccinate	2	-	+/-
1	931	14	Vaccinate	2	-	-
1	955	14	Vaccinate	2	+/-	-
1	011	14	Vaccinate	2	+/-	-
1	013	14	Vaccinate	2	+/-	+/-
1	019	14	Vaccinate	2	+/-	+/-
2	922	14	Vaccinate	1	+/-	-
2	953	14	Vaccinate	1	+/-	+/-
2	015	14	Vaccinate	1	+/-	+
2	016	14	Vaccinate	1	-	-
2	017	14	Vaccinate	1	+/-	+/-
3	923	14	Control	N/A	+	+/-
3	012	14	Control	N/A	+	-
3	014	14	Control	N/A	+	-
3	018	14	Control	N/A	+	-
3	01A	14	Control	N/A	+	+/-
4	406	8	Vaccinate	2	+/-	-
4	407	8	Vaccinate	2	-	-
4	504	8	Vaccinate	2	+/-	-
4	704	8	Vaccinate	2	-	-
4	705	8	Vaccinate	2	-	-
5	404	8	Control	N/A	-	-
5	405	8	Control	N/A	-	-
5	610	8	Control	N/A	+/-	-
5	702	8	Control	N/A	+/-	-
5	703	8	Control	N/A	-	-

“+” Severe lesion consistent or pathognomic to an influenza infection

5 “+/-” Mild lesion (inconclusive)

“-” Normal

EXAMPLE 23 - CANINE INFLUENZA VACCINE EFFICACY STUDY

The following study was conducted to determine the efficacy of a multivalent
 10 H3N8 equine influenza vaccine against canine influenza virus in dogs.

Procedure:

Seventeen 15-week-old beagles were obtained from a commercial supplier. The dogs were randomly assigned to 3 groups as shown in Table 41, and housed in a research facility.

Table 41. Experimental design				
<u>Group</u>	<u>Number of dogs</u>	<u>Treatment</u>	<u>Number of doses</u>	<u>Age at Vaccination (weeks)</u>
1	7	Vaccinate	2	15 & 19
2	5	Vaccinate	1	19
3	5	Control	--	--

The vaccine used in this study was a HAVLOGEN® adjuvanted, inactivated equine influenza (A/equine/KY/02, A/equine/KY/93, and A/equine/NM/2/93) vaccine. To prepare this vaccine, the viruses were inactivated by binary ethylenimine (BEI) using a standard method. Each vaccine dose contained HAVLOGEN® (10% v/v), 2048 HA units of each of the inactivated virus, 0.1% (v/v) of 10% thimerosal, 0.1 % (v/v) of phenol red, sufficient NaOH to adjust the pH to 6.8 to 7.2, and sufficient PBS to bring the total dose volume to 1 ml.

The dogs in Group 1 were vaccinated with 2 doses of the vaccine. The second (*i.e.*, booster) dose was administered 4 weeks after the first dose. The dogs in Group 2 were vaccinated with 1 dose of vaccine at 19 weeks-of-age. Blood samples were collected to assess HI titer using a standard protocol with an H3N8 canine influenza isolate on days zero (before vaccination), 7, and 14 post first and second vaccinations. Seven days before challenge, the dogs were moved to a BSL-2 facility and housed in individual cages.

All vaccinates and age-matched control dogs were challenged oronasally with a virulent canine influenza virus (107.3 TCID₅₀ of A/Canine/Florida/242/2003 per dog) at 2 weeks post second vaccination of Group 1 and first vaccination of Group 2. The challenge virus was administered as a mist using a nebulizer (Nebulair™) at 2 ml per dog. The dogs were observed for influenza-related clinical signs for 14 days post challenge. All dogs were euthanized at day 14 post-challenge, and lung and trachea samples were collected in 10% buffered formalin for histopathology. Blood samples were collected on days 7 and 14 post challenge for HI titer determination. The clinical sign score assignments used for the post challenge observation are shown in Table 42.

Results:

All vaccinated dogs developed HI antibody titer responses to the canine influenza virus isolate (Table 43). Following the challenge, approximately a 4 fold increase in HI titer on day 14 post challenge compared to the pre-challenge HI titer in all groups indirectly indicate that all dogs were exposed to the challenge virus. All dogs exhibited signs canine influenza disease with each dog demonstrating one or more of the following clinical signs: fever ($>103.0^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$), cough, serous or mucopurulent ocular discharge, serous or mucopurulent nasal discharge, vomiting, diarrhea, depression, weight loss, and dyspnea. Vaccinates had less severe clinical signs, compared to age-matched controls (Table 44). There was a significant ($P = 0.028$) reduction in clinical signs due to the 2-dose vaccination in dogs (Group 1). One dose vaccination did not provide a significant ($P = 0.068$) reduction in clinical signs (Group 2).

As in Example 22, histopathological evaluation of lung and tracheal tissue samples for lesions was conducted to identify lesions compatible with or pathognomic to canine influenza disease. Table 45 provides a summary of the extent of lesions in this experiment for the dogs. Among 15-week-old dogs, vaccination of dogs with either 1 dose or 2 doses prevented the lung lesions in all dogs. Four of 5 control dogs (80%) had severe suppurative bronchopneumonia consistent with an influenza disease. One of 7 dogs from the 2-dose vaccine group (Group 1) and 1 of 5 dogs from the control group (Group 3) had mild trachea lesions suggestive of tracheitis which could be attributed to influenza disease.

Conclusion:

The results from this study demonstrate that 1) inactivated H3N8 equine influenza virus can induce canine influenza virus cross reactive HI antibody responses in vaccinated dogs, and 2) Use of a H3N8 equine influenza virus vaccine can reduce the severity of canine influenza virus disease in dogs.

Table 42. Clinical signs and scoring system	
<u>Clinical signs</u>	<u>Score per day</u>
Temp	
$<103.0^{\circ}\text{F}$ ($<39.4^{\circ}\text{C}$)	0
$103.0 - 103.9^{\circ}\text{F}$ ($39.4 -$	2
$104.0 - 104.9^{\circ}\text{F}$ ($40.0 -$	3
$>105.0^{\circ}\text{F}$ ($>40.6^{\circ}\text{C}$)	4

Table 42. Clinical signs and scoring system	
<u>Clinical signs</u>	<u>Score per day</u>
Coughing	
No coughing	0
Occasional	2
Paroxysmal	4
Sneezing	
No sneezing	0
Occasional	1
Paroxysmal	2
Nasal discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3
Ocular discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3
Hemoptysis	
No	0
Yes	5
Depression	
No	0
Yes	1
Anorexia	
No	0
Yes	1
Respiratory signs	
None	0
Rales	3
Dyspnea	4
Gasping	5
Mucous expectorate	
No	0
Yes	2
Vomiting	
No	0
Yes	1
Fecal abnormalities	
No	0
Yes	1

Table 43. Serology – Hemagglutination inhibition titers

Group No	Dog ID	Treatment	Number of doses	HI titer							
				Days post first vaccination of Group 1						Days post challenge	
				0*	7	14	28**	35	42***	7	14
1	ALK	Vaccinate	2	<10	<10	20	20	80	40	160	320
1	AMF	Vaccinate	2	<10	<10	10	20	20	40	160	320
1	AKY	Vaccinate	2	<10	20	20	20	40	40	160	80
1	ALC	Vaccinate	2	<10	10	10	10	40	40	160	160
1	ALL	Vaccinate	2	<10	<10	10	10	40	20	160	320
1	ALM	Vaccinate	2	<10	<10	10	20	40	40	80	160
1	AMU	Vaccinate	2	<10	20	40	40	40	40	40	160
2	ALA	Vaccinate	1	<10	<10	<10	<10	<10	10	320	160
2	AMA	Vaccinate	1	<10	<10	<10	<10	<10	20	> 640	80
2	APD	Vaccinate	1	<10	<10	<10	<10	<10	10	> 640	320
2	APG	Vaccinate	1	<10	<10	<10	<10	<10	10	320	80
2	APT	Vaccinate	1	<10	<10	<10	<10	<10	10	320	320
3	ALT	Control	N/A	<10	<10	<10	<10	<10	<10	40	160
3	AMS	Control	N/A	<10	<10	<10	<10	<10	<10	80	160
3	AKX	Control	N/A	<10	<10	<10	<10	<10	<10	20	80
3	ALX	Control	N/A	<10	<10	<10	<10	<10	<10	80	80
3	AMI	Control	N/A	<10	<10	<10	<10	<10	<10	40	80

*First vaccination - Group 1

**Second vaccination – Group 1; First vaccination – Group 2

***Day of challenge

Table 44. Analysis of total canine influenza disease clinical scores					
Group	Treatment	Number of doses	Age at first vaccination of Group 1	Average total Score per dog	P-value*
1	Vaccinate	2	15 weeks	6.3	0.028 (Group 1 vs. 3)
2	Vaccinate	1	15 weeks (these dogs were vaccinated once, when they were 19 weeks old)	14.2	0.068 (Group 2 vs. 3)
3	Control	--	15 weeks (these dogs were not vaccinated)	24.4	--

* Analyzed using a NPARIWAY procedure of SAS® Version 8.2 (the vaccine groups were compared using the Wilcoxon rank sum test)

Table 45. Histopathological evaluation of tissue samples					
Group No	Dog ID	Treatment	Number of doses	Microscopic lesion (Histopathology)	
				Lung	Trachea
1	ALK	Vaccinate	2	-	+/-
1	AMF	Vaccinate	2	-	-
1	AKY	Vaccinate	2	-	-
1	ALC	Vaccinate	2	-	-
1	ALL	Vaccinate	2	-	-
1	ALM	Vaccinate	2	-	-
1	AMU	Vaccinate	2	-	-
2	ALA	Vaccinate	1	-	-
2	AMA	Vaccinate	1	-	-
2	APD	Vaccinate	1	-	-
2	APG	Vaccinate	1	-	-
2	APT	Vaccinate	1	-	-
3	ALT	Control	N/A	+/-	-
3	AMS	Control	N/A	+	-
3	AKX	Control	N/A	+	-
3	ALX	Control	N/A	+	+/-
3	AMI	Control	N/A	-	-

“+” Severe lesion consistent or pathognomic to an influenza infection
 “+/-” Mild lesions (inconclusive)
 “-” Normal

EXAMPLE 24 - CANINE INFLUENZA VACCINE EFFICACY STUDY

The following study was conducted to determine: (1) the efficacy of monovalent versus multivalent H3N8 equine influenza vaccines against canine influenza virus in dogs, and (2) the effect of route of administration on vaccine efficacy.

Procedure:

Thirty 10-week old mongrels were obtained from a commercial supplier. The dogs were randomly assigned to 6 groups as shown in Table 46, and housed in a research facility.

Table 46. Experimental design

Group	Number of dogs	Treatment	Route of vaccination	Number of doses	Age at Vaccination (weeks)
1	5	VAX-1	IN	2	10 & 14
2	5	VAX-2	SQ	2	10 & 14
3	5	VAX-2	IN	2	10 & 14
4	5	VAX-3	SQ	2	10 & 14
5	5	VAX-3	IN	2	10 & 14
6	5	Control	--	--	--

Three types of vaccines (VAX-1, VAX-2, and VAX-3) were used. The VAX-1 was a HAVLOGEN®-adjuvanted, inactivated equine influenza virus (A/equine/KY/02) monovalent vaccine, and each dose contained HAVLOGEN® (10% v/v), 6144 HA units of the inactivated virus, 0.1% (v/v) of 10% thimerosal, 0.1 % (v/v) of phenol red, sufficient NaOH to adjust the pH to 6.8 to 7.2, and sufficient PBS to bring the total dose volume to 1 ml. The VAX-2 was a HAVLOGEN®-adjuvanted, inactivated equine influenza virus (A/equine/KY/02) monovalent vaccine, and each dose of vaccine contained HAVLOGEN® (10% v/v), 4096 HA units of the inactivated virus, 0.1% (v/v) of 10% thimerosal, 0.1 % (v/v) of phenol red, sufficient NaOH to adjust the pH to 6.8 to 7.2, and sufficient PBS to bring the total dose volume to 1 ml. The VAX-3 was a HAVLOGEN®-adjuvanted, inactivated equine influenza (A/equine/KY/02, A/equine/KY/93, and A/equine/NM/2/93) multivalent vaccine, and contained HAVLOGEN® (10% v/v), 2048 HA units of inactivated virus per strain, 0.1% (v/v) of 10% thimerosal, 0.1 % (v/v) of phenol red, sufficient NaOH to adjust the pH to 6.8 to 7.2, and sufficient PBS to bring the total dose volume to 1 ml. All influenza viruses used for

the vaccine formulation were inactivated by binary ethylenimine (BEI) using a standard method.

The vaccines and routes of administration for each group are described in Table 46. All dogs in the vaccinated groups were vaccinated either via the intranasal (IN) or the subcutaneous (SQ) route, and each dog received 2 doses. The second (*i.e.*, booster) dose was administered 4 weeks after the first dose. Blood samples were collected to assess HI titer using a standard protocol with an H3N8 canine influenza isolate on days zero (before vaccination), 7, and 14 post first and second vaccinations. Seven days before challenge, the dogs were moved to a BSL-2 facility and housed in individual cages.

All vaccinates and age-matched control dogs were challenged oronasally with a virulent canine influenza virus (107.4 TCID₅₀ of A/Canine/Florida/242/2003 per dog) at 2 weeks post second vaccination. The challenge virus was administered as a mist using a nebulizer (Nebulair™) in a 2 ml volume per day. The dogs were observed for influenza-related clinical signs for 14 days post-challenge. Blood samples were collected on days 7 and 14 post challenge for HI titer determination. All dogs were euthanized at day 14 post-challenge, and lung and trachea samples were collected in 10% buffered formalin for histopathology. The clinical sign score assignments used for the post challenge observation are shown in Table 47.

Results:

All dogs vaccinated via the SQ route developed HI antibody titer responses to the canine influenza virus isolate, regardless of the vaccine type (Table 48). None of the dogs from the IN vaccination groups (*i.e.*, Groups 1, 3, and 5) developed HI antibody titer responses to the canine influenza virus isolate, regardless of the vaccine type, during the post vaccination period. There was, however, a 4-fold increase in titer by day 14 post challenge in all dogs indirectly, indicating that all dogs were exposed to the challenge virus (Table 47).

All dogs exhibited one or more of the following clinical signs of canine influenza: fever (>103.0°F; >39.4°C), cough, serous or mucopurulent ocular discharge, serous or mucopurulent nasal discharge, vomiting, diarrhea, depression, weight loss, and dyspnea. Vaccinates had less severe clinical signs, compared to age-matched controls (Table 49). There was a significant reduction in clinical signs in dogs vaccinated with VAX-3 via the SQ route (Group 4). In this experiment, IN administration of either VAX-1, VAX-2, or VAX-3 did not provide a significant reduction in clinical signs of canine influenza virus.

As in Examples 22 and 23, histopathological evaluation of lung and tracheal tissue samples for lesions was conducted to identify lesions compatible with or pathognomic to canine influenza disease. Table 50 provides a summary of the extent of lesions in this experiment for the dogs. Five of 5 control dogs (Group 6) had lung lesions consistence
5 with an influenza infection. Two of 5 dogs vaccinated with VAX-2 via the SC route (Group 2) and 3 of 5 dogs vaccinated with VAX-3 via the SC route (Group 4) were free of any influenza-related lung lesions. All the dogs that received the vaccine via the intranasal route, irrespective of the vaccine type, had severe lung lesions consistent with an influenza infection. The trachea lesions observed in this study were very mild.

Conclusion:

The results from this study demonstrate that: (1) inactivated H3N8 equine influenza virus can induce canine influenza virus cross reactive HI antibody responses in dogs vaccinated via the SQ route, (2) intranasal administration of either monovalent
15 (VAX-1 and VAX-2) or multivalent vaccine (VAX-3) was not efficacious in dogs, and (3) subcutaneous administration of multivalent vaccine (VAX-3) provided a significant ($P=0.016$) reduction in severity of canine influenza virus disease in dogs.

Table 47. Clinical signs and scoring system	
<u>Clinical signs</u>	<u>Score per day</u>
Temp	
<103.0°F (<39.4°C)	0
103.0 – 103.9°F (39.4–	2
104.0-104.9°F (40.0–	3
>105.0°F (>40.6°C)	4
Coughing	
No coughing	0
Occasional	2
Paroxysmal	4
Sneezing	
No sneezing	0
Occasional	1
Paroxysmal	2
Nasal discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3
Ocular discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3
Hemoptysis	
No	0
Yes	5
Depression	
No	0
Yes	1
Anorexia	
No	0
Yes	1
Respiratory signs	
None	0
Rales	3
Dyspnea	4
Gasping	5
Mucous expectorate	
No	0
Yes	2

Table 47. Clinical signs and scoring system	
<u>Clinical signs</u>	<u>Score per day</u>
Vomiting	
No	0
Yes	1
Fecal abnormalities	
No	0
Yes	1

Table 48. Serology – Hemagglutination inhibition titers

[illegible]

Table 48. Serology – Hemagglutination inhibition titers												
Group No	Dog ID	Treatment	Route of vaccination	Number of doses	HI titer							
					Days post vaccination						Days post challenge	
					0*	7	14	28**	35	42***		
5	466	Vaccinate	IN	2	<10	<10	<10	<10	<10	<10	7	14
5	46B	Vaccinate	IN	2	<10	<10	<10	<10	<10	<10	80	80
6	236	Control	--	2	<10	<10	<10	<10	<10	<10	80	160
6	504	Control	--	2	<10	<10	<10	<10	<10	<10	160	160
6	507	Control	--	2	<10	<10	<10	<10	<10	<10	80	160
6	515	Control	--	2	<10	<10	<10	<10	<10	<10	80	160
6	468	Control	--	2	<10	<10	<10	<10	<10	<10	80	160

* First vaccination

** Second vaccination

*** Day of challenge

Table 49. Analysis of total canine influenza disease clinical scores

Group	Treatment	Route of vaccination	Average total Score per dog	P-value*
1	VAX-1	IN	35.2	0.500 (Group 1 vs. 6)
2	VAX-2	SQ	31.0	0.345 (Group 2 vs. 6)
3	VAX-2	IN	39.4	0.631 (Group 3 vs. 6)
4	VAX-3	SQ	13.0	0.016 (Group 4 vs. 6)
5	VAX-3	IN	42.6	0.790 (Group 4 vs. 6)
6	Control	--	36.8	--

* Analyzed using a NPARIWAY procedure of SAS® Version 8.2 (the vaccine groups were compared using the Wilcoxon rank sum test)

Table 50. Histopathological evaluation of tissue samples

Group No	Dog ID	Treatment	Route of vaccination	Number of doses	Microscopic lesion (Histopathology)	
					Lung	Trachea
1	248	Vaccinate	IN	2	+	-
1	501	Vaccinate	IN	2	+	-
1	502	Vaccinate	IN	2	+	-
1	469	Vaccinate	IN	2	+	+
1	46A	Vaccinate	IN	2	+	+
2	232	Vaccinate	SQ	2	+	-
2	511	Vaccinate	SQ	2	+	-
2	514	Vaccinate	SQ	2	-	-
2	461	Vaccinate	SQ	2	+	-
2	463	Vaccinate	SQ	2	-	-
3	246	Vaccinate	IN	2	+	-
3	505	Vaccinate	IN	2	+	-
3	506	Vaccinate	IN	2	+	+
3	464	Vaccinate	IN	2	+	-
3	465	Vaccinate	IN	2	+	+
4	23B	Vaccinate	SQ	2	-	-
4	247	Vaccinate	SQ	2	+/-	-
4	508	Vaccinate	SQ	2	-	-
4	512	Vaccinate	SQ	2	-	+/-
4	516	Vaccinate	SQ	2	+	+
5	503	Vaccinate	IN	2	+	+/-
5	513	Vaccinate	IN	2	+	+
5	462	Vaccinate	IN	2	+	+/-
5	466	Vaccinate	IN	2	+	+
5	46B	Vaccinate	IN	2	+	-
6	236	Control	--	2	+	-
6	504	Control	--	2	+	+
6	507	Control	--	2	+	+
6	515	Control	--	2	+	+/-
6	468	Control	--	2	+	+

“+” Severe lesion consistent or pathognomic to an influenza infection

“+/-” Mild lesion (inconclusive)

“-” Normal

EXAMPLE 25 – CANINE INFLUENZA VACCINE EFFICACY STUDY

Canine influenza disease is caused by an H3N8 influenza virus (CIV). CIV is very closely related to equine H3N8 viruses (Crawford *et al.*, 2005) and infects all exposed dogs.

Approximately 80% of exposed dogs develop clinical signs. In the following study the efficacy of an inactivated H3N8 equine influenza virus vaccine and a canine influenza virus vaccine were determined.

Procedure:

Thirty-five beagles and five mongrels were used in this study. Beagles were randomly assigned to three groups (Table 51). All mongrels were assigned to control group (Group 3). All dogs were fed with a standard growth diet and water was available as libitum.

Table 51. Experimental design					
Group	Treatment	Vaccination route	Number of dogs	Age at vaccination (weeks)	Challenge
1	VAX-1	IM	15	8 & 12	Yes
2	VAX-2	SC	5	8 & 12	Yes
3	Control	N/A	20	N/A	Yes

The dogs in Groups 1 and 2 were vaccinated with either VAX-1 or VAX-2 (Table 51). VAX-1 was a HAVLOGEN® adjuvanted, inactivated equine influenza virus (A/equine/KY/02) vaccine. For vaccine preparation, the vaccine virus was inactivated by binary ethylenimine (BEI) using a standard method. Each dose of vaccine contained HAVLOGEN® (10% v/v), 6144 HA units of the inactivated virus, 0.1% (v/v) of 10% thimerosal, 0.1 % (v/v) of phenol red and sufficient PBS to bring the total dose volume to 1 ml and sufficient NaOH to adjust the pH to 6.8 to 7.2.

VAX-2 was an inactivated, CARBIGENTM adjuvanted, canine influenza antigen vaccine (A/canine/FI/43/2004). The A/canine/FI/43/2004 was inactivated by binary ethylenimine ("BEI") using a standard method. Each dose of the vaccine contained 5% by mass CARBIGENTM, approximately 1280 HA units of the inactivated virus, sufficient PBS to bring the total volume of the dose to 1 ml, and sufficient NaOH to adjust the pH to between 7.2 and 7.4. Serum samples were collected from all dogs on the day of first and second vaccination, days 7 and 14 post first and second vaccinations, and at pre-challenge to determine the HI titers using an H3N8 equine influenza virus standard protocol (SAM 124, CVB, USDA, Ames, IA). Seven days before challenge, the dogs were moved to a ABSL-2 facility and housed in individual cages.

All vaccinates and age-matched control dogs were challenged oronasally with virulent canine influenza virus (107.2 TCID₅₀ of A/Canine/Florida/242/2003 per dog) at 2 weeks post second vaccination. The challenge virus was administered as a mist (2ml/dog) using a nebulizer (Nebulair™). The dogs were observed for influenza-related clinical signs for 14 days post-challenge. Nasal and oropharyngeal swabs were collected daily in tubes containing 2 ml of virus transport medium for virus isolation from day -1 (*i.e.*, one day before challenge) through day 14 post-challenge. Blood samples were collected on days 7 and 14 post challenge for HI titer determination. The clinical sign score assignments used for post challenge observation are shown in Table 52.

Results:

All vaccinated dogs (Groups 1 and 2) developed HI antibody titer responses to the canine influenza virus isolate (Table 53). All dogs exhibited one or more of the following signs of canine influenza: fever ($>103.0^{\circ}\text{F}$; $>39.4^{\circ}\text{C}$), cough, serous or mucopurulent ocular discharge, serous or mucopurulent nasal discharge, vomiting, diarrhea, depression, and anorexia. Vaccinates had less severe clinical signs, compared to age-matched controls (Table 54). There was a significant ($P < 0.001$) reduction in clinical signs in dogs vaccinated with either VAX-1 (Group 1) or VAX-2 (Group 2).

Virus isolation results are shown in Tables 55 and 56. Following a virulent canine influenza virus challenge, the canine influenza virus was isolated from 5 of 15 (33%) dogs from Group 1 (VAX-1), 0 of 5 (0%) dogs from Group 2 (VAX-2) and 17 of 20 (85%) controls (Group 3). Both inactivated equine influenza vaccine (VAX-1) and canine influenza virus (VAX-2) vaccinates demonstrated a significant ($P = 0.004$) reduction in virus shedding in nasal or oral secretions or both (Table 55) compared to controls.

Conclusion:

The results from this study demonstrate that: (1) inactivated H3N8 equine influenza virus and canine influenza virus vaccines can induce canine influenza virus reactive HI antibody responses in vaccinated dogs, (2) use of an H3N8 equine influenza virus or canine influenza virus vaccine can reduce the severity of canine influenza virus disease in dogs, and (3) use of an H3N8 equine influenza virus or canine influenza virus vaccine can reduce virus excretion in nasal and/or oral secretions.

Table 52. Clinical signs and scoring system	
Clinical signs	Score per day
Temp	
<103.0°F (<39.4°C)	0
103.0 – 103.9°F (39.4–	2
104.0-104.9°F (40.0-40.5°C)	3
>105.0°F (>40.6°C)	4
Coughing	
No coughing	0
Occasional	2
Paroxysmal	4
Sneezing	
No sneezing	0
Occasional	1
Paroxysmal	2
Nasal discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3
Ocular discharge	
No discharge	0
Serous -slight	1
Serous -copious	1
Mucopurulent-slight	2
Mucopurulent-copious	3
Hemoptysis	
No	0
Yes	5
Depression	
No	0
Yes	1
Anorexia	
No	0
Yes	1
Respiratory signs	
None	0
Rales	3
Dyspnea	4
Gaspings	5
Mucous expectorate	
No	0
Yes	2
Vomiting	
No	0
Yes	1

Table 52. Clinical signs and scoring system	
<u>Clinical signs</u>	<u>Score per day</u>
Fecal abnormalities	
No	0
Yes	1

Table 53. Serology – Hemagglutination inhibition titers

Group No	Dog ID	Treatment	Vaccination route	HI titer											
				Days post vaccination				Days post challenge							
				0*	7	14	28**	35	42***	7	14				
1	AYS	Vaccinate	IM	<10	<10	<10	20	40	40	80	> 640				
1	AZV	Vaccinate	IM	<10	<10	<10	20	40	40	160	> 640				
1	BAD	Vaccinate	IM	<10	<10	<10	40	40	80	80	320				
1	BAE	Vaccinate	IM	<10	<10	10	20	20	20	40	320				
1	BAH	Vaccinate	IM	<10	<10	10	10	40	40	160	> 640				
1	BAJ	Vaccinate	IM	<10	<10	10	20	80	80	40	320				
1	BAN	Vaccinate	IM	<10	10	10	20	40	40	40	320				
1	BBN	Vaccinate	IM	<10	10	10	20	80	80	40	320				
1	BBT	Vaccinate	IM	<10	<10	<10	20	40	40	40	160				
1	BBY	Vaccinate	IM	<10	<10	<10	20	80	80	160	> 640				
1	BCS	Vaccinate	IM	<10	10	40	40	160	160	160	160				
1	BCZ	Vaccinate	IM	<10	10	10	20	80	40	160	160				
1	BDP	Vaccinate	IM	<10	<10	<10	20	40	40	80	> 640				
1	BEE	Vaccinate	IM	<10	10	20	40	80	80	160	320				
1	BEY	Vaccinate	IM	<10	<10	10	10	40	40	160	160				
2	AZH	Vaccinate	SC	<10	<10	10	20	80	80	160	160				
2	AZT	Vaccinate	SC	<10	<10	10	10	40	80	320	> 640				
2	BBC	Vaccinate	SC	<10	<10	20	40	160	160	80	160				
2	BCM	Vaccinate	SC	<10	<10	10	20	80	40	80	160				
2	BEB	Vaccinate	SC	<10	<10	<10	10	20	40	80	160				
3	AYT	Control	N/A	<10	<10	<10	<10	<10	<10	40	320				
3	AZJ	Control	N/A	<10	<10	<10	<10	<10	<10	20	160				
3	AZL	Control	N/A	<10	<10	<10	<10	<10	<10	40	160				
3	AZN	Control	N/A	<10	<10	<10	<10	<10	<10	160	160				
3	BAB	Control	N/A	<10	<10	<10	<10	<10	<10	40	320				

Table 53. Serology – Hemagglutination inhibition titers

Group No	Dog ID	Treatment	Vaccination route	HI titer									
				Days post vaccination				Days post challenge					
				0*	7	14	28**	35	42***	7	14		
3	BBD	Control	N/A	<10	<10	<10	<10	<10	<10	<10	>640		
3	BBU	Control	N/A	<10	<10	<10	<10	<10	<10	<10	160		
3	BBZ	Control	N/A	<10	<10	<10	<10	<10	<10	<10	160		
3	BCC	Control	N/A	<10	<10	<10	<10	<10	<10	<10	320		
3	BCD	Control	N/A	<10	<10	<10	<10	<10	<10	<10	>640		
3	BCG	Control	N/A	<10	<10	<10	<10	<10	<10	<10	>640		
3	BCI	Control	N/A	<10	<10	<10	<10	<10	<10	<10	320		
3	BCL	Control	N/A	<10	<10	<10	<10	<10	<10	<10	>640		
3	BCV	Control	N/A	<10	<10	<10	<10	<10	<10	<10	320		
3	BDU	Control	N/A	<10	<10	<10	<10	<10	<10	<10	>640		
3	MFI	Control	N/A	NT	NT	NT	NT	<10	<10	<10	320		
3	MFJ	Control	N/A	NT	NT	NT	NT	<10	<10	<10	320		
3	MFK	Control	N/A	NT	NT	NT	NT	<10	<10	<10	320		
3	MFR	Control	N/A	NT	NT	NT	NT	<10	<10	<10	320		
3	MFS	Control	N/A	NT	NT	NT	NT	<10	<10	<10	>640		

* First vaccination

** Second vaccination

*** Day of challenge

Table 54. Analysis of total canine influenza disease clinical scores

Group	Treatment	Average total Score per dog	P-value*
1	VAX-1	9.1	< 0.001 (Group 1 vs. 3)
2	VAX-2	5.4	< 0.001 (Group 2 vs. 3)
3	Control	24.1	--

* Analyzed using a NPARIWAY procedure of SAS® Version 9.1 (the vaccine groups were compared using the GLM procedure)

Table 55. Post-challenge virus shedding

Group	Treatment	Percent dogs excreted the virus	P-value*
1	VAX-1	33% (5/15)	0.004 (Group 1 vs. 3)
2	VAX-2	0% (0/5)	0.004 (Group 2 vs. 3)
3	Control	85% (17/20)	--

* Analyzed using a FREQ procedure of SAS® (Version 9.1) and P-value associated with Fisher's exact test

Table 56. Serology -- Hemagglutination inhibition titers																		
Group No	Dog ID	Treatment	Vaccination route	Days post-challenge														
				-1	0	1	2	3	4	5	6	7	8	9	10	11	12	13
3	BBD	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BBU	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BBZ	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BCC	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BCD	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BCG	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BCI	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BCL	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BCV	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	BDU	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	MFI	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	MFJ	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	MFK	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	MFR	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N
3	MFS	Control	N/A	N	N	N	N	N	N	N	N	N	N	N	N	N	N	N

N- No virus isolated from oral or nasal swabs

P – Virus isolated from nasal or oral or nasal and oral swabs.

Table 57. Hemagglutinin, neuraminidase and nucleoprotein gene amino acid sequence similarities among influenza viruses

Gene (Canine /Florida/43/2004)	Amino acid sequence similarity	Gene of influenza virus used for comparison
Hemagglutinin	88	equine/Algiers/72
HA	90	equine/Sao paulo/6/69
HA	91	equine/Miami/1/63
HA	93	equine/Newmarket/79
HA	94	equine/Kentucky/1/81
HA	95	Equi-2/Ludhiana/87
HA	96	Equine/Alaska/1/91
HA	97	equine/Tennessee/5/86
HA	98	equine/Kentucky/5/02
HA	99	equine/Ohio/1/2003
HA	99	canine/Florida/242/2003
Neuraminidase	88	Eq/Algiers/72
NA	90	equine/Sao Paulo/6/69
NA	91	equine/Miami/1/63
NA	93	equine/Newmarket/79
NA	94	equine/Kentucky/1/81
NA	95	Equi-2/Ludhiana/87
NA	96	equine/Santiago/85
NA	97	equine/Tennessee/5/86
NA	98	equine/Kentucky/5/2002
NA	99	equine/Ohio/1/2003
NA	99	canine/Florida/242/2003
Nucleoprotein ("NP")	94	equi/Miami/1/63
NP	97	equine/Kentucky/1/81
NP	99	equine/Kentucky/5/02
NP	99	equine/Ohio/1/2003
NP	99	canine/Florida/242/2003

The words "comprise," "comprises," and "comprising" in this patent (including the
5 claims) are to be interpreted inclusively rather than exclusively. This interpretation is intended to be the same as the interpretation that these words are given under United States patent law.

The above detailed description of preferred embodiments is intended only to acquaint others skilled in the art with the invention, its principles, and its practical application so that

others skilled in the art may adapt and apply the invention in its numerous forms, as they may be best suited to the requirements of a particular use. This invention, therefore, is not limited to the above embodiments, and may be variously modified.

5

It should be understood that the examples and embodiments described herein are for illustrative purposes only and that various modifications or changes in light thereof will be suggested to persons skilled in the art and are to be included within the spirit and purview of this application.

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1. An isolated canine influenza virus wherein said influenza virus comprises a polynucleotide which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:33, or a polynucleotide encodes a polypeptide having 99% or greater sequence identity with the amino acid sequence of SEQ ID NO:33.

2. The canine influenza virus according to claim 1, wherein said influenza virus is inactivated or attenuated.

3. The canine influenza virus according to claim 1, wherein said influenza virus is provided in a pharmaceutically acceptable carrier or diluent.

4. An isolated cell comprising a canine influenza virus of claim 1.

5. The cell according to claim 4, wherein said cell is a canid animal cell.

6. A vaccine comprising a canine influenza virus of claim 2.

7. The canine influenza vaccine according to claim 6, wherein said influenza virus is inactivated.

8. The canine influenza vaccine according to claim 6, wherein said influenza virus is attenuated.

9. A composition comprising a canine influenza virus wherein said influenza virus comprises a polynucleotide which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:33, or a polynucleotide encodes a polypeptide having 99% or greater sequence identity with the amino acid sequence of SEQ ID NO:33.

10. The composition according to claim 9, wherein said composition further comprises cell-free whole virus, or a portion thereof; a viral polynucleotide; a viral protein; a viral polypeptide or peptide; a virus infected cell; a recombinant viral vector based construct; a reassortant virus; or naked nucleic acid of said virus.

11. The canine influenza virus according to claim 9, wherein said influenza virus is inactivated or attenuated.

12. The composition according to claim 11, wherein said composition is formulated in a pharmaceutically acceptable carrier or diluent.

13. A reassortant virus comprising a polynucleotide which encodes a polypeptide comprising the amino acid sequence of SEQ ID NO:33, or said polynucleotide encodes a polypeptide having 99% or greater sequence identity with the amino acid sequence of SEQ ID NO:33.

14. The reassortant virus according to claim 13, wherein said reassortant virus is formulated in a pharmaceutically acceptable carrier or diluent.

15. An isolated virus that comprises one or more polynucleotides wherein said one or more polynucleotides encode a canine influenza virus polypeptide comprising the amino acid sequence of SEQ ID NO:33, or said polynucleotide encodes a polypeptide having 99% or greater sequence identity with the amino acid sequence of SEQ ID NO:33.

16. The virus according to claim 15, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.

17. An isolated canine influenza virus, wherein said influenza virus comprises a polynucleotide encoding a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ

ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence.

18. An isolated canine influenza virus, wherein said influenza virus comprises a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence.

19. A composition comprising a canine influenza virus, wherein said canine influenza virus comprises a polynucleotide encoding a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence, wherein said canine influenza virus is capable of inducing an immune response against an influenza virus that is capable of infecting a canid animal.

20. A composition comprising a canine influenza virus, wherein said canine influenza virus comprises a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence, wherein said canine influenza virus is capable of inducing an immune response against an influenza virus that is capable of infecting a canid animal.

21. A reassortant virus comprising a polynucleotide encoding a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence.

22. A reassortant virus comprising a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence.

23. An isolated virus comprising a polynucleotide encoding a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence.

24. An isolated virus comprising a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:33, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:33, wherein said HA protein comprises a serine at position 82, a leucine at position 221, a threonine at position 327, and a threonine at position 482 of the amino acid sequence.

25. The canine influenza virus according to claim 17, wherein said polynucleotide encodes a hemagglutinin (HA) that comprises the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.

26. The canine influenza virus according to claim 18, wherein said hemagglutinin (HA) comprises the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.

27. The reassortant virus according to claim 21, wherein said reassortant virus comprises a polynucleotide encoding a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID

NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.

28. The reassortant virus according to claim 22, wherein said reassortant virus comprises a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.

29. The virus according to claim 23, wherein said virus comprises a polynucleotide encoding a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.

30. The virus according to claim 24, wherein said virus comprises a hemagglutinin (HA) comprising the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.

31. The canine influenza virus according to claim 17, wherein said influenza virus is inactivated.

32. The canine influenza virus according to claim 17, wherein said influenza virus is provided in a pharmaceutically acceptable carrier or diluent.

33. The canine influenza virus according to claim 18, wherein said influenza virus is inactivated.

34. The canine influenza virus according to claim 18, wherein said influenza virus is provided in a pharmaceutically acceptable carrier or diluent.

35. The reassortant virus according to claim 21, wherein said virus is inactivated.

36. The reassortant virus according to claim 21, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.

37. The reassortant virus according to claim 22, wherein said virus is inactivated.

38. The reassortant virus according to claim 22, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.

39. The virus according to claim 23, wherein said virus is inactivated.

40. The virus according to claim 23, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.

41. The virus according to claim 24, wherein said virus is inactivated.

42. The virus according to claim 24, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.

43. The canine influenza virus according to claim 25, wherein said influenza virus is inactivated.

44. The canine influenza virus according to claim 25, wherein said influenza virus is provided in a pharmaceutically acceptable carrier or diluent.

45. The canine influenza virus according to claim 26, wherein said influenza virus is inactivated.

46. The canine influenza virus according to claim 26, wherein said influenza virus is provided in a pharmaceutically acceptable carrier or diluent.

47. The reassortant virus according to claim 27, wherein said virus is inactivated.
48. The reassortant virus according to claim 27, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.
49. The reassortant virus according to claim 28, wherein said virus is inactivated.
50. The reassortant virus according to claim 28, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.
51. The virus according to claim 29, wherein said virus is inactivated.
52. The virus according to claim 29, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.
53. The virus according to claim 30, wherein said virus is inactivated.
54. The virus according to claim 30, wherein said virus is provided in a pharmaceutically acceptable carrier or diluent.
55. The canine influenza virus according to claim 17, wherein said influenza virus is attenuated.
56. The canine influenza virus according to claim 18, wherein said influenza virus is attenuated.
57. The reassortant virus according to claim 21, wherein said virus is attenuated.
58. The reassortant virus according to claim 22, wherein said virus is attenuated.

59. The virus according to claim 23, wherein said virus is attenuated.
60. The virus according to claim 24, wherein said virus is attenuated.
61. The canine influenza virus according to claim 25, wherein said influenza virus is attenuated.
62. The canine influenza virus according to claim 26, wherein said influenza virus is attenuated.
63. The reassortant virus according to claim 27, wherein said virus is attenuated.
64. The reassortant virus according to claim 28, wherein said virus is attenuated.
65. The virus according to claim 29, wherein said virus is attenuated.
66. The virus according to claim 30, wherein said virus is attenuated.
67. A vaccine comprising a canine influenza virus of claims 31 and 55.
68. A vaccine comprising a canine influenza virus of claims 33 and 56.
69. The canine influenza vaccine according to claim 67, wherein said polynucleotide encodes a hemagglutinin (HA) that comprises the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.
70. The canine influenza vaccine according to claim 68, wherein said hemagglutinin (H-A) comprises the amino acid sequence of SEQ ID NO:16, or an amino acid sequence having greater than 95% amino acid sequence identity to SEQ ID NO:16.

71. The canine influenza vaccine according to claim 67, wherein said influenza virus is inactivated.

72. The canine influenza vaccine according to claim 67, wherein said influenza virus is attenuated.

73. The canine influenza vaccine according to claim 68, wherein said influenza virus is inactivated.

74. The canine influenza vaccine according to claim 68, wherein said influenza virus is attenuated.

75. The canine influenza vaccine according to claim 69, wherein said influenza virus is inactivated.

76. The canine influenza vaccine according to claim 69, wherein said influenza virus is attenuated.

77. The canine influenza vaccine according to claim 70, wherein said influenza virus is inactivated.

78. The canine influenza vaccine according to claim 70, wherein said influenza virus is attenuated.

79. A canine influenza virus wherein said influenza virus is the viral isolate designated as A/canine/Florida/43/2004 (ATCC Accession No. PTA-7914).

80. A canine influenza virus wherein said influenza virus is the viral isolate designated as A/canine/Florida/242/2003 (ATCC Accession No. PTA-7915).

81. A canine influenza virus wherein said influenza virus is the viral isolate designated as canine/Jax/05 (ATCC Accession No. PTA-7941).

82. A canine influenza virus wherein said influenza virus is the viral isolate designated as canine/Miami/05 (ATCC Accession No. PTA-7940).