DISSERTATION

EPIDEMIOLOGY, ECOLOGY, AND EVOLUTION OF CANINE INFLUENZA VIRUS H3N8 IN UNITED STATES DOGS

Submitted by

Heidi Lee Pecoraro

Department of Microbiology, Immunology, and Pathology

In partial fulfillment of the requirements

For the Degree of Doctor of Philosophy

Colorado State University

Fort Collins, Colorado

Fall 2012

Doctoral Committee:

Advisor: Gabriele Landolt

Carol Blair
Richard Bowen
Kathryn Huyvaert

Copyright by Heidi Lee Pecoraro 2012

All Rights Reserved

ABSTRACT

EPIDEMIOLOGY, ECOLOGY, AND EVOLUTION OF CANINE INFLUENZA VIRUS H3N8 IN UNITED STATES DOGS

Canine influenza virus (CIV) first emerged in dogs at a Florida racing track in 2004, although serological evidence suggests the virus has been circulating in the Unites States since as early as 1999. Phylogenetic analysis shows that CIV isolates are related to equine influenza virus of the Florida Clade 1 sublineage. However, sustained transmission of CIV among dogs and further genetic evolution of the virus has established CIV as a canine-specific influenza A virus (IAV). During the early years after emergence, studies determining the impact of CIV on dog populations were scarce. The few published findings were also alarming, with case fatality rates as high as 36% and seropositivity as high as 97% in certain dog populations. Despite these reports, the prevalence of CIV infection in dogs, the transmission dynamics among dog populations, risk factors for CIV infection, and how the virus was evolving within the canine host had yet to be examined. The research described here sought to fill these knowledge gaps by accomplishing four main objectives: (1) to evaluate diagnostic tests for detecting CIV infection in nasal swab samples and to determine which test(s) would be most sensitive for rapid and accurate CIV diagnosis, (2) to estimate the seroprevalence of CIV antibodies in several dog populations, including household pet, racing sled, and shelter dogs, and to evaluate any risk factors associated with CIV seropositivity, (3) to identify epidemiological and ecological determinants of CIV infection within U.S. shelter dogs, and (4) to determine the degree of genomic evolution and antigenic variability of CIV in U.S. dogs.

For the first objective, nasal and pharyngeal swab samples were collected from 124 shelter and household dogs seen at Colorado State University Veterinary Teaching Hospital (CSU VTH) for canine infectious respiratory disease between April 2006 and March 2007 and from 1372 dogs housed in six shelters from December 2009 to November 2010. Several methods – virus isolation, real time reverse transcriptase polymerase chain reaction (rRT-PCR), and rapid influenza detection tests (RIDT) – were used to detect presence of CIV in these samples and compared to determine which test was most sensitive. Using a stochastic latent class modeling approach, the median sensitivities of virus isolation,

Directigen Flu A+B®, and rRT-PCR were estimated at 72%, 65%, and 96%, respectively. Furthermore, a different RIDT, the Flu Detect™, performed poorly for detecting CIV nasal shedding compared to rRT-PCR.

Objective two was addressed using data from three different studies. In the first study, to determine seroprevalence and risk factors for CIV in Colorado household dogs, serum samples from 140 dogs presenting to the CSU VTH Community Practice service, 110 dogs seen at other clinical services in 2009, and samples from 75 dogs seen prior to 2004 were tested with hemagglutination inhibition (HI) assay, using three CIV isolates. To identify risk factors for CIV infection, 140 owners completed questionnaires at the time of sampling. Results showed CIV seroprevalence was 2.8% (4/140) for dogs seen by the Community Practice service and 4.5% (5/110) for dogs seen by other hospital services. All sera collected prior to 2004 tested negative for CIV. No differences were seen in antibody titers to the three CIV isolates tested, suggesting that measurable antigenic drift had not yet occurred as of 2009. Data from questionnaires indicated an association between CIV seropositivity and canine day-care visits.

A similar study was conducted in sled dogs from the United States and Canada racing in the 2010 Iditarod to determine the seroprevalence of CIV in the sled dog population. Questionnaires were completed detailing medical and CIV vaccination history, kennel size and location, travel history, and social interactions for each team. Three hundred ninety-nine dogs were tested for CIV antibodies by HI assay. Of these, including 39 samples from dogs reported as CIV vaccinated, none was seropositive for CIV antibodies. Furthermore, all of the vaccinated dogs were also negative on virus microneutralization assay. Although risk factors for CIV seropositivity could not be determined due to lack of positive samples, this was the first published study investigating the prevalence of CIV in sled dogs.

For the third study, which also sought to address the third objective, risk factors for both CIV-shedding and CIV-seroprevalence in shelter dogs were examined, as well as transmission dynamics between shelter and community dogs. Nasal swab and serum samples were collected from over 5160 dogs upon admission or discharge from six U.S. humane shelters from December 2009 through January 2012. HI and rRT-PCR assays were performed for each nasal and serum sample, respectively. True prevalence was estimated by stochastic latent class analysis. Results showed that the New York and Colorado shelters had the highest numbers of both CIV nasal shedding and CIV seropositivity.

Differences in CIV rates upon intake and discharge depended on the humane shelter sampled. An information theoretic approach to model selection and multi-model inference were used to identify risk factors associated with CIV infection and seropositivity. These risk factor analyses suggest that CIV shedding and CIV seropositivity are associated with region, month, year and co-mingling/co-housing. Additionally, the number of days within a shelter was associated with CIV seroprevalence.

Finally, the last objective was addressed by a study focused on the evolution of CIV to determine if antigenic drift is occurring, especially since the widespread use of a recently developed killed CIV vaccine. To this end, the full-length hemagglutinin gene of 19 CIV isolates from dogs sampled from Colorado, New York, and South Carolina humane shelters were sequenced. Both the nucleotide and amino acid sequences were compared to all those published for CIV strains isolated since 2003. Phylogenetic analysis suggests that CIV is diverging into two genetically distinct clades. Using a mixed-effects model for evolution (MEME), five amino acid sites were found to be undergoing episodic selection pressure: 107, 169, 216, 453, and 464. Additionally, a total of five amino acid changes were observed in two antigenic sites for CIVs isolated from the New York and South Carolina humane shelters between 2009 and 2011 (H75Q, K172E, N216H, V223I, and P262T). Although preliminary data suggest that the New York clade is evolving into a distinct antigenic cluster, controlled experiments are required to determine true extent of antigenic drift occurring within circulating CIV.

In conclusion, the studies conducted here report several important findings that deepen our understanding of CIV. (1) Real-time RT-PCR assays should be used as a rapid test for detecting CIV and can minimize false negatives, allowing a more accurate depiction of CIV infection in dogs. (2) Both CIV shedding and seroprevalence in the dog populations sampled are not as high as reported in racing greyhounds during the first CIV outbreak in Florida, suggesting that there may be other genetic or environmental factors contributing to fatal CIV infection or that perhaps there are host-immune response mechanisms restricting severe CIV infection. (3) Despite low prevalence of disease, however, control measures should still be implemented for at-risk dogs, such as dogs in boarding facilities, attending canine day-care, and residing in humane shelters. (4) Additionally, CIV, like other influenza A viruses, appears to be a disease of space and time. Therefore, modeling could be helpful for predicting future CIV outbreaks. (5) Finally, CIV is evolving into two genetically distinct clades, one of which is antigenically

different from previous CIV isolates. Controlled experiments are required to determine true extent of antigenic drift occurring within circulating CIV and, if necessary, CIV vaccines should be updated accordingly.

ACKNOWLEDGMENTS

The work presented here was funded in part by the American College of Veterinary Internal Medicine Foundation, the International Sled Dog Veterinary Medical Association, and the Morris Animal Foundation. These studies could not have been performed without the help of many individuals, including the veterinarians, staff, and students from the Colorado State University Veterinary Teaching Hospital, the 2010 Iditarod Veterinary team, veterinarians and staff from participating shelters, and the laboratory of Dr. Christopher Olsen at the University of Wisconsin-Madison. A special thanks is given to Dr. Katharine Benedict who taught me how to perform the stochastic latent class analysis, Dr. Paul Morley who reviewed the CIV risk assessment in Colorado household dogs, Dr. Kathryn Huyvaert for helping with the information theoretic approach to model selection and multi-model inference, and (soon to be) Dr. Justin Lee who provided advice and guidance for the CIV genomic analysis. Finally, it was kindness in the form of food, childcare, and moral support from many friends and family (especially Kali Saxton-Shaw, Daniel Saxton, Carl Davis, Justin Lee, Patti Kiser, Kathy Benedict, Heather Pilarski, and Shannon Pecoraro), which enabled this dissertation to be finally completed.

DEDICATION

This dissertation is dedicated to my mother, Mary Pilarski. All she ever wanted was for me to go to college and she sacrificed everything to get me there.

Also, for Shannon Pecoraro, who has suffered through nearly 15 years of courses, papers, exams, night-school, day-school, part-time jobs, full-time jobs, caffeine addictions, lapsed gym memberships, and poor, very poor housekeeping. Thank you for the encouragement and for planting the idea in the first place. You have given me the best project of all time and I am happy we get to perform this latest experiment together.

TABLE OF CONTENTS

CHAPTER ONE: INTRODUCTION

Background for this study

Influenza A viruses (IAV) are considered commensal pathogens of their reservoir waterfowl hosts.[1] Occasionally, however, IAV is transmitted from aquatic birds to mammals and poultry, causing clinical disease and even death in infected individuals. While most cross-species IAV transmission events result in incidental infection, in which the virus is not further transmitted to naïve individuals, host-adaptation does indeed occur, allowing the new IAV to spread efficiently among susceptible hosts.[2] Moreover, once an IAV becomes maintained within a species, host immune selection pressures further influence IAV evolution along a host-specific lineage. Human, equine, and swine IAVs are noteworthy examples of particularly successful avian-to-mammal transmission events, resulting in established host-specific influenza lineages.[1] Interestingly, horses suffered two such occurrences in the latter half of the 20th century. In 1956, an H7N7 IAV was isolated from a horse in Prague.[3] H7N7 viruses circulated in equids until 1980, when the last known H7N7 equine influenza virus (EIV) was isolated from an Yugoslavian horse.[4] However, serological evidence suggests that H7N7 might still be circulating, as H7-specific antibodies have been detected in unvaccinated horses as recently as 2004.[5] The second transmission event was reported in 1963 after the isolation of an H3N8 IAV from a horse in Miami.[6] It is from this latter EIV subtype that canine influenza virus (CIV) emerged in 2004.[7]

The advent of a canine-adapted IAV provided the perfect opportunity to examine mechanisms of species-specificity and to identify host range determinants that hinder transmission of IAV among species. In fact, early research in our laboratory was focused on this particular area (see Appendix A). However, it soon became apparent that little was known about CIV in naturally-infected dogs – the who, what, where, how, and why questions of an epidemiological study were still under investigation. Early reports indicated CIV infection resulted in high mortality and was highly infectious (capable of being spread from infected to susceptible dogs), as the case fatality rate of CIV-infected Florida racing greyhounds was 36%[7] and shelters experiencing canine infectious respiratory disease (CIRD) had up to 97% seropositivity among dog sera tested for CIV antibodies.[7] Obviously, this new threat to canine health was cause for concern among veterinarians and dog owners, as well as to other health care practitioners

who were uncertain if CIV could be spread from dogs to their human companions.[8] Our study, therefore, expanded to include a more diagnostic and surveillance-oriented approach for identifying the epidemiological and ecological factors that help to maintain canine-adapted IAV in dogs after cross-species transmission. The overall goal of the research presented here was to examine the epidemiology, ecology, and evolution of CIV in U.S. dogs in order to advance our general understanding of how IAV jumps the species barrier.

Objectives of research

There were four main objectives of my research. The first was to evaluate diagnostic tests for detecting CIV infection in nasal swab samples and to determine which test(s) would be most sensitive for rapid and accurate CIV diagnosis. For the second objective, we sought to estimate the seroprevalence of CIV in several dog populations, including household pet, racing sled, and shelter dogs, to better understand the extent of CIV infection within U.S. dog populations. Another part of this objective was to evaluate any risk factors associated with CIV seropositivity. The third objective was to identify epidemiological or ecological determinants of CIV infection within U.S. shelter dogs. Finally, for the last objective, we wanted to determine the degree of genomic evolution and antigenic variability of CIV in U.S. dogs.

Main hypotheses of research

While there were several objectives for these studies, with individual aims (described in each subsequent section), there was one overarching hypothesis directing our research from the beginning: CIV is being transmitted and maintained within dog populations, resulting in the establishment of a canine-specific influenza A lineage. Although simple, it is important to note that EIV had been spread to dogs before[9,10] and the mechanism that allowed EIV to adapt to the canine host and be transmitted among dogs is still unknown. If this hypothesis is correct, there are many implications, but several that pertain specifically to the following dissertation are (1) CIV prevalence may be different among canine populations, (2) such differences likely depend on CIV-associated ecological and epidemiological factors, and (3) as an IAV, CIV is potentially evolving genomically and antigenically within dogs.

Organization of dissertation

As each research chapter includes an introduction to the study's relevant literature, Chapter 2 consists of a brief literature review on influenza A viruses, in general, and the emergence of CIV, in particular. Following the literature review, five chapters (3-7) describe studies conducted from 2006 to 2012. In Chapter 3, the specificities of tests use to detect CIV in swabs are estimated and compared using a stochastic latent class analysis (or Bayesian approach). Chapters 4 through 6 detail findings from surveillance studies to determine the prevalence of CIV in three different dog populations – Colorado household dogs, North American racing sled dogs, and dogs residing in shelters located throughout the U.S. – and to determine any risk factors associated with CIV in these populations. In Chapter 7, attention is re-directed to the evolution of CIV, with the characterization of genomic evolution and antigenic variation of CIV since emergence, and on how evolution might contribute to antigenic drift of the virus within dogs. Finally, in Chapter 8, conclusions and future directions are presented. One final research paper from early studies conducted in the Landolt laboratory describes a clinical study on EIV and CIV infection in dogs, as well as receptor-binding preferences for these viruses (included as Appendix A).

CHAPTER TWO: LITERATURE REVIEW

Introduction to influenza A viruses (IAV)

Influenza A viruses belong to the family *Orthomyxoviridae*, which are negative-sense, single-stranded RNA viruses. These segmented RNA viruses include five genera: influenza A, B, and C, Thogotovirus, and Isavirus.[11] Influenza A viruses, the causative agents of pandemic influenza outbreaks such as the 1918 "Spanish flu" and 2009 "swine flu," infect many mammalian and avian species.[1] Although other mammals have been infected with influenza B and C (namely, seals[12] and swine,[13] respectively), viruses from these two genera primarily infect humans. Thogotoviruses are typically transmitted from ticks to mammals,[14] while, isavirus (infectious salmon anemia) affects, as its name implies, salmonid species.[15]

The fact that influenza A viruses (IAVs) are segmented RNA viruses poses several problems for IAV outbreak prevention and control measures. Firstly, viral RNA-dependent RNA-polymerase necessary for transcription is inherently error-prone. For IAV, the rate of mutation is estimated to be 2×10^{-3} nucleotide substitutions per position per virus generation,[1] which may result in IAV immune escape if mutations are located in specific antigenic regions – a process termed antigenic drift.[16] Annual influenza A vaccination is one strategy to counterbalance mutating IAVs in human populations.[2] Secondly, whole gene segments may be exchanged among IAV isolates, known as antigenic shift,[16] as was most recently seen in the latest influenza A H1N1 pandemic of 2009. In this case, a quadruple gene reassortment occurred among avian, swine, and human IAVs.[17] With eight gene segments, two IAVs, theoretically, have the potential to reassort and generate 256 (2^8) new viruses.

Virion structure

The IAV virion, which can be either spherical, elongated, or filamentous in shape,[18] contains an outer lipid membrane (the envelope of the virus), two surface glycoproteins (trimeric hemagglutinin [HA] and tetrameric neuraminic [NA] proteins) which accumulate around lipid rafts, a tetrameric integral protein (matrix 2 [M2]) which functions as an hydrogen ion-channel, and the matrix 1 (M1) protein located on the underside of the lipid membrane.[11] The M1 protein contributes to the virion structure by adding rigidity to

the lipid membrane.[19] In addition, M1 is associated with the eight internal panhandle-shaped IAV ribonucleoproteins (vRNPs), which are formed by viral RNA (vRNA), nucleoprotein (NP), and the heterotrimeric polymerase complex (polymerase acidic [PA], polymerase basic 1 [PB1], and polymerase basic 2 [PB2] proteins).[20] The two viral surface proteins (HA and NA) have been shown to play essential roles in antigenicity of IAV.[21,22] To date, 17 HAs and 10 NAs have been identified (the latest HA and NA were recently isolated from Guatemalan fruit bats[23]). The HA and NA designate the IAV subtype. Although nearly all HA and NA subtypes have been found in waterfowl species, the reservoir for IAV, only specific HA and NA subtypes have been isolated from non-avian hosts. For example, recently circulating human IAV are subtype H1N1 or H3N2.[22] IAV isolates from equids since 1956 have been primarily H7N7 (1963-1980)[3,4] and H3N8.[6,24] Interestingly, IAVs from bats before the isolation of a distinct bat-lineage influenza virus (H17N10) were often contemporary human subtypes.[25,26]

Cell entry and membrane fusion

Binding of the virus to a host cell involves sialic acid receptors on the surface of the host cell (respiratory epithelial cells for mammals and birds, as well as gastrointestinal epithelial cells for birds) and a receptor binding pocket on the viral HA surface protein, which, in the HA subtype 3 (H3) numbering system, includes amino acid residues 226 through 228.[27] Viral attachment to cellular sialic acid (SA) receptors depends on the linkage of host cell oligosaccharides to SA,[28] as well as on the biochemical structure of the SA itself (e.g., *N*-acetylneuraminic [Neu5Ac] or *N*-glycolneuraminic [Neu5Gc] acids).[29] It has long been recognized that avian and equine influenza viruses preferentially bind to receptors that have a SA linked to a galactose (gal) residue by an α2,3 linkage, while human lineage viruses prefer α2,6-linked receptors.[27,30-32] Additionally, receptor specificity has been found to correlate with the availability of the SA receptors within the host animal respiratory tract.[9,33-37]

IAV enters cells via the apical surface of epithelium, where host cell SA receptors are located. Interestingly, human viruses preferentially attach to non-ciliated human epithelial cells while avian viruses prefer to bind to ciliated cells.[38] Viral entry is mediated by clathrin-coated endocytosis, although non-clathrin non-caveolae endocytosis has also been reported.[39,40] Fusion between the viral and endosomal membranes requires cleavage of the HA0 precursor by endogenous proteases[41-43] into the two subunits

HA1 and HA2. The low pH (5-6) within the endosome results in a conformational change in the newly formed HA2 N-terminal fusion peptide, promoting insertion of the hydrophobic N-terminus into the endosomal membrane to modulate fusion with the viral membrane.[44]

Several models have been proposed to describe the next steps during membrane fusion.[45] The stalk-pore hypothesis[46,47] involves the creation of a fusion pore by the refolding of the coiled α-helices of the HA while the two different HA subunits are attached to the two different membranes (i.e., HA1 to the viral membrane and HA2 to the target or endosomal membrane), leading to the formation of a stalk which eventually narrows into a breakable hemifused diaphragm. Another model[48,49] suggests that an oligomeric ring-like structure is formed by the HA trimers, resulting in a breakable proteinaceous bridge. Yet another alternative model[50] hypothesizes that the fusion peptide is not inserted into the endosomal membrane, but rather into the viral membrane, causing HA refolding and formation of a dimple that extends to the endosomal membrane. Whatever the mechanism, the end result is the same: fusion of the viral envelope and endosomal membranes. This fusion event triggers the uncoating of the virus, which results in dissociation of vRNPs from the M1 protein and subsequent release of vRNPs into the cytoplasm.

Viral replication and transcription

Because the virion requires host nuclear cell machinery for splicing the M and nonstructural (NS) genes into two transcripts each,[11] nuclear import of vRNPs is required. Active transport through nuclear pore complexes (NPCs) requires nuclear localization signals (NLSs).[51] Although NLSs have been found on the M1, polymerase, and the NS2 (also called the nuclear export protein or NEP) proteins, the NLSs on NPs (which bind ~24 viral RNA nucleotides per NP), are perhaps the most important.[11] In general, NLSs contain binding sites for importin (karyopherin) α, which in turns binds importin β. Interestingly, the subtype of cellular importin α has been found to contribute to species-specificity.[52] The NP-importin complex docks at the NPC and, in the presence of ATP and Ran-GAP, is translocated via cytoplasmic Ran-GTP.[51] Viral RNA then undergoes transcription to messenger (m)RNA or replication from complimentary (c)RNA.

Both vRNA transcription and replication involves a vRNA promoter, consisting of the vRNA 5' and 3' ends base-paired to one another, and the viral RNA-dependent RNA polymerase. Transcription of

vRNA requires a capped pre-mRNA fragment that functions as a primer. The cap is snatched by PB2[53] and cleaved by PA.[54] The addition of a guanosine to the 3' end of the capped primer pairs with the penultimate cystine residue on the 3' end of the vRNA, initiating vRNA transcription.[11] PB1 then catalyzes each nucleotide.[55] Termination occurs ~17 nucleotides from the 5' end of the vRNA and a poly(A) tail is added onto the mRNA transcript. The cap and poly(A) tail are essential for mRNA nuclear export to the cytoplasm and subsequent translation.[51] Unlike during transcription, vRNA replication requires the synthesis of encapsidated positive-sense cRNA in a primer-independent manner. The newly formed cRNA is a full-length complementary copy of the vRNA template.[11] vRNA is then synthesized from cRNA, using the complementary cRNA promoter, and eventually packaged with NP, PA, PB1, and PB2 proteins to make up the vRNP, which then associate with M1 and NEP proteins for nuclear export.

Nuclear export of vRNPs occurs after expression of late viral M1 and NEP genes and termination of vRNA replication.[11] M1 interacts with both NEP and vRNPs. As NEP contains a nuclear export signal, it is able to recruit the exportin chromosome maintenance gene 1 (Crm1) and is able to undergo caspase-dependent nucleo-cytoplasmic trafficking in the absence of M1 and NEP. The nuclear nucleotide exchange factor RCC1 triggers Ran-GDP to Ran-GTP exchange, which, in turn, associates with Crm1 to facilitate vRNP nuclear transport through the NPC. Additionally, phosphorylation of the M1, NEP, and NP is likely required for nuclear export.[51]

Virion assembly and budding

During the assembly process, viral proteins are trafficked to the apical domain of polarized infected cells[56] along cellular pathways to either lipid rafts (for HA and NA) or non-lipid raft domains (for M2).[57] NP has been shown to be associated with actin microfilaments (suggesting cytoskeleton trafficking) and lipid rafts.[57] M1 binds lipids, RNA, RNP, and NP, is associated with HA and NA tails, and may rely on piggybacking on other viral proteins for transportation to the apical domain. Bud initiation requires outward bending of the plasma membrane.[18] Lipid rafts, as well as viral proteins HA, NA, M2, and M1, play a role in the membrane curvature and bud initiation at the budding site.[18] Fusion and fission (a separation or splicing) are required for bud closure of new virions – fusion between the viral and cellular membranes and then fission of the viral bud from the cell membrane.[18] The IAV viral bud pinches-

off at the neck region, which is devoid of lipid rafts and, therefore, does not contain HA and NA or M1 on the outer and inner membrane leaflets, respectively (but does contain M2).[11] Finally, the NA functions as a sialidase, releasing progeny virion from the sialic acid receptor of infected cells.[58,59]

Cellular signaling pathways

There are several cellular pathways involved during IAV infection. The IκB kinase (IKK)/nuclear factor kappa-light-chain-enhancer of activated B cells (NF-κB) pathway has traditionally been considered a regulator of IAV infection due to its induction of interferon (IFN) type 1 expression (IFN-α and IFN-β).[60] In addition to IFN type-I up-regulation, however, the IKK/NF-κB pathway has also been shown to be required for influenza infection.[61] Briefly, IKK-2 is activated by vRNA accumulation and/or expression of viral proteins.[62] A cascade of phosphorylation events occurs where IκB-α is phosphorylated and releases p50/p65 NF-κB transcription factors for nuclear import and NF-κB activation.[63] NF-κB activation in turn leads to expression of IFN-β, proapoptotic factors (e.g., TRAIL, Fas, and FasL),[64] and suppressor of cytokine signaling (SOCS)-3.[65] While IFN-β primarily functions as an innate antiviral cytokine, the simultaneous expression of SOCS-3 limits this response.[65] Furthermore, TRAIL and FasL activate caspase-3 which allows release of vRNP from the nucleus due to caspase-mediated disruption of the NPC.[66,67]

Like the IKK/NF-κB pathway, phosphatidylinositol 3-kinase (PI3K)/Akt (a serine/threonine protein kinase) activation induces both antiviral and proviral responses.[68,69] The pathway is activated when the PI3K regulatory protein p85 binds to the viral protein NS1. PI3K phosphorylates interferon regulatory factor (IRF)-3, stimulating IFN-β expression. PI3K also phosphorylates phosphatidylinositol-4,5-biphosphate (PIP2), which then phosphorylates phosphatidylinositol-3,4,5-triphosphate (PIP3).[70] PIP3 then indirectly phosphorylates Akt, which activates NF-κB and inhibits proapoptotic factors such as caspase 3, caspase 9, and glycogen synthase kinase 3 (another serine/threonine protein kinase),[69] thus, preventing premature cell apoptosis.

Finally, the Raf/mitogen-activated protein kinase kinase (MAP2K or MEK)/extracellular signal-regulated kinase (ERK) pathway is stimulated by the clustering of HAs on lipid rafts during late infection and corresponds with vRNP nuclear export.[71] IAV replication triggers increased diffusion of the small GTPase H-Ras and activation of protein kinase Cα (PKCα). Both H-Ras and PKCα activate Raf, which

initiates a cascade of phosphorylation events, starting with the phosphorylation of MEK.[72] MEK in turn phosphorylates and activates ERK, which ultimately leads to cytokine up-regulation. ERK also regulates vRNP export via direct phosphorylation of either NP or NEP.[73]

Host-pathogen interactions

Virions that are able to enter the host cell are recognized by a variety of pathogen-associated molecular patterns that stimulate pattern-recognition receptors to begin down-stream cell induction of Type I IFN, interleukins (IL), and other cytokines. Toll-like receptor-7 (TLR-7) is activated by single stranded RNA (ssRNA),[74,75] and results in production of pro-inflammatory cytokines and chemokines, as well as expression of co-stimulatory molecules via activation of transcription factor NF-κB. The presence of ssRNA also activates retinoic acid inducible gene-1 (RIG-1),[76] which contains RNA helicase-like domains that bind double stranded RNA (a replication intermediate) and two caspase activation and recruitment domains (CARDs) that interact with adaptor proteins[77] (such as IFN-β promoter stimulator -1). Activation of CARDs up-regulates IRF-3 and IRF-7 by phosphorylation of I-κB kinase family members. IPS-3 activates NF-κB and Jun N-terminal kinase (JNK), which enhances binding of the ATF-2/c-jun transcription factor to the IFN-β promoter.[78] IFN-α and -β bind to interferon receptors coupled to Janus-family tyrosine kinase, which phosphorylates STAT proteins along the JAK-STAT pathway, activating transcription of oligoadenylate synthetase, PKR kinase, and the *Mx* gene.[79] IFN-α and -β also increase MHC class I expression and antigen expression in cells, thus, rendering IAV-infected cells more susceptible to cytotoxic T cells (CTLs) and activated dendritic cells (involved in neutralization and antigen presentation), macrophages (involved in phagocytosis and antigen presentation), and NK cells (mediates antibody-dependent cell cytotoxicity).[80] Interestingly, NS1 protein inhibits the activation and/or signaling of antiviral proteins such as RIG-I, PKR, 2',5' oligoadenylate synthetase, activators of mitogen-activated protein kinase, and Type I IFN transcription factors.[79,81]

Cellular signaling ultimately leads to activation of alveolar macrophages and dendritic cells.[80] Antigen presentation by these cells results in an adaptive immune response. There are both IAV HA-specific[82-84] and NA-specific[85] antibody binding regions, which, at least in the case of HA-bound antibodies, results in hindering the acid pH-induced conformational change of the HA and virus

neutralization.[84] Cellular responses include both CD4+ (T helper-1 or Th1) and CD8+ (CTL) cells. Upon activation, respiratory dendritic cells (rDC) migrate to lymph nodes where rDC pass on antigen to resident CD8α+DC and then both interact with naïve antigen-specific CD8 T cells.[80] CTLs undergo activation, proliferation, differentiation, and, finally, migration to the site of infection, while CD8 cells undergo a second direct interaction with MHC I-expressing antigen presenting cells.[80] Meanwhile, CD4 cells experience both antigen-dependent and -independent phases. During the antigen-dependent phases (days 0-2 of infection), naïve CD4 cells are activated by antigen presenting cells and begin secreting IL-2. Antigen drives further Th1 cell proliferation before eventual death of the antigen-presenting cell.[86] The antigen-independent phase (days 2-5 of infection) involves cytokine induction (especially by IL-2) of CD4 T cell expansion and differentiation, while activated Th1 cells differentiate into highly polarized cells.[86]

IAV species-specificity

Although every gene segment has been shown to contribute to IAV species-specificity,[87-91] the HA, which contains the receptor-binding site, has been found to be the major host range determinant maintaining the influenza species barrier. Matrosovich and others have published reports on the species-specificity of the alpha-linkage between terminal SA and adjacent galactose (gal) of glycans covalently attached to glycoproteins or glycolipids on epithelial cells.[31,32] SA receptors linked to gal by an α2,3gal linkage have been found to preferentially bind avian and equine IAVs, while α2,6gal linked SA receptors bind human IAVs.[27,30-32] Naturally, it follows that the receptors expressed in respiratory epithelium coincide with the binding preference of host-specific IAVs. For example, the human respiratory tract expresses mostly α2,6gal linked SA receptors, while the avian gastrointestinal and the equine respiratory tracts express mostly α2,3gal linked SA receptors.[9,33-37] Interestingly, lower respiratory tract tissues (i.e., lung) express receptors with both types of SA linkages in several species.[35,Appendix A] It is this mixture of SA receptors in the lower respiratory tract that has been postulated as the mechanism for H5N1 infection in humans.[35] Quail and swine express both SA-α2,3-gal and SA-α2,6-gal glycans throughout their respiratory epithelium, and, therefore, pose as potential mixing vessels for IAVs.[92,93] The biochemical structure of the SA (Neu5Ac or Neu5Gc acids) also contributes to species-specificity. For example, the

equine respiratory and duck gastrointestinal tracts consist of mostly Neu5Gc SA, while human and swine respiratory epithelium express mostly Neu5Ac SA.[29,37,94]

IAV infection in dogs and CIV emergence

Despite occasional accounts of IAV infection in dogs,[95-97] historically, dogs were not considered natural hosts for IAV. In past instances where dogs have been infected with EIV, there were no reports of sustained transmission of the virus among canine hosts.[9,10,98] However, in 2004, racing greyhounds at a Florida track were afflicted with an IAV and appeared to be spreading the virus to other dogs at the facility.[7] Early cases of influenza infection in these dogs resulted in ether mild clinical infections signaled by fever and 10-14 days of coughing (14/22 dogs) or severe hemorrhagic pneumonia and death (8/22).[7] Furthermore, it was difficult to decipher whether greyhounds were more susceptible to IAV because of genetic differences in the breed, immunosuppression and stress that might accompany the rigors of racing, or severe cytokine/chemokine induction caused by the virus itself. Chemokine storm, high viral load, and neutrophil migration have been associated with bronchointerstitial pneumonia in dogs experimentally infected with canine-adapted H3N2 IAV.[99] Indeed, CIV-inoculated alveolar macrophages have been shown to induce tumor necrosis factor-alpha and IL-10,[100] and viral antigen has been found in alveolar macrophages in experimentally infected dogs.[101] In fatal cases of H5N1-infected humans, both hypercytokinemia and high viral loads were noted.[102] However, regardless of the reason for severe clinical illness in greyhounds, the Florida IAV outbreak was the first documentation of sustained dog-to-dog IAV transmission and established CIV as a potential threat to canine health. By April 2005, CIV had spread to racing tracks in Iowa, and, though the case fatality rate (<5%) was much lower than in Florida, the attack rate was 100%, indicating all dogs at these facilities were susceptible to CIV.[103]

Generally, the onset of CIV symptoms in dogs occurs about three days after exposure to the virus. Clinical signs include an elevated temperature (>103.0°F), a dry, persistent, non-productive cough, serous or mucopurulent discharge, as well as weight loss, anorexia, and lethargy.[104] Viral shedding has been observed from day 2 through day 7 after exposure to the virus, with an antibody response detectable after day 7 (Appendix A). The timeline below (Figure 2-1) illustrates the phases of a non-complicated CIV infection in the canine host.

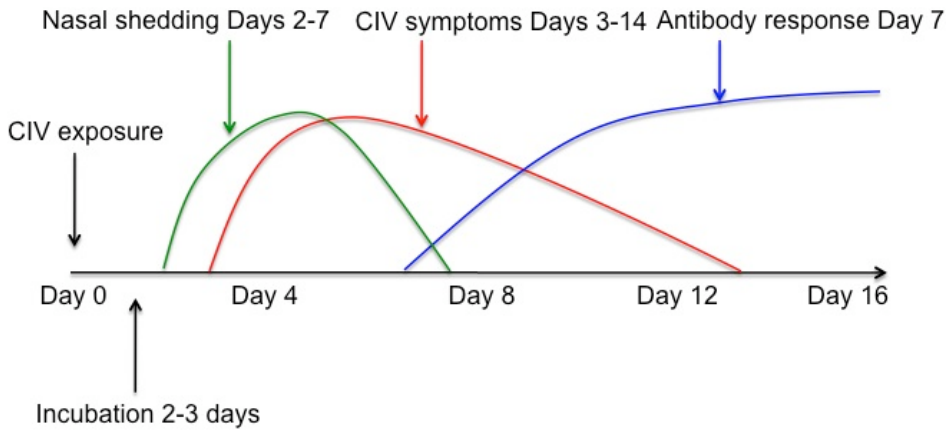


Figure 2-1. Timeline of CIV infection in dogs.

Genetic analysis of IAVs isolated from dogs revealed close relatedness with equine influenza virus (EIV) H3N8 isolates from the Florida Clade 1 sublineage.[8] Indeed, several studies have shown that EIV and CIV share the same receptor-binding preference (SA-α2,3-gal).[105,Appendix A] Initially, at least five changes from the HA EIV consensus amino acid sequence were identified for CIV,[7,8] although this number has been expanded to 19 conserved (or consistent) amino acid substitutions among six IAV proteins in CIV strains isolated since 2006.[106] These include a total of nine HA mutations (residues 29, 54, 83, 118, 222, 261, 328, 479, and 483), four PB2 mutations (residues 107, 221, 292, and 374), one PB1 mutation (residue 200), one NP mutation (residue 375), and two NA mutations (residues 62 and 147). Studies examining intrahost evolution of CIV in dogs pre- and post-vaccination showed that mean genetic diversities on the HA gene range from 0.1-0.2% in samples taken from the same individual on different days, suggesting high mutation rates in vaccinated dogs.[107] Furthermore, isolates from dogs taken in humane shelters, racing tracks, and veterinary hospitals had a mean nucleotide substitution rate of 2.1 x 10^{-3} per site per year for the HA gene.[108] Evolution of CIV into a canine-specific lineage might perhaps explain the reduced replication of recent CIVs seen within the CIV-infected equine host.[105,109] It is interesting to note that EIV is not similarly restricted in dogs, as seen recently in both experimentally and naturally EIV-infected dogs.[98,110,Appendix A]

Although it is clear that CIV is evolving within the canine host, the selection pressures for such evolution are less apparent. One major factor that might contribute to genetic selectivity is herd-immunity.

With the recent production of a CIV vaccine,[111] as well as evidence of high CIV seroprevalences (42-97%) reported in shelter dogs,[7,112] it is possible that mutations allowing viral immune escape are being positively selected. Genetic analyses of CIV isolates since 2006 have thus far found substitutions of two amino acid residues within HA antigenic regions.[108] Clearly, more research is needed to determine CIV prevalence in the general dog population to accurately determine whether selection might be due to a large number of immuno-protected dogs or to some other factor. In addition, there is a gap in knowledge of how CIV is spread among dogs and between dog populations. Several studies suggest direct contact to be the route of transmission for CIV in dogs.[113,Appendix A] However, the transmission dynamics between dog populations is virtually unknown, as are the epidemiological and ecological factors contributing to infections within these populations. Finally, frequent genomic analyses are required to monitor antigenic drift of CIV in dogs. The studies presented hereafter attempt to address these gaps, as well as increase our understanding of CIV and its threat to canine health.

CHAPTER THREE: SENSITIVITIES OF DIAGNOSTIC TESTS FOR DETECTING CANINE INFLUENZA VIRUS IN NASAL SWAB SAMPLES

INTRODUCTION

Canine influenza virus (CIV) was first detected in greyhounds at a Florida racetrack in 2004 and has since been isolated from dogs throughout the United States.[7,114] Phylogenetic analysis indicates that CIV is related to contemporary equine H3N8 influenza viruses (single-stranded negative-sense RNA viruses; family *Orthomyxoviridae*), though CIV is clearly evolving into its own distinct H3 lineage.[8,106] Symptoms of CIV infection include serous or mucopurulent nasal discharge, fever (>103.0°F), and a dry, persistent, non-productive cough, as well as weight loss, anorexia, and lethargy.[104] Because these symptoms mirror the clinical signs of infectious tracheobronchitis (referred to as "kennel cough" or "canine infectious respiratory disease" [CIRD]), which can be caused by *Bordetella bronchioseptica*, canine parainfluenza virus, *Mycoplasma* spp., canine adenovirus-2, and/or canine distemper virus, CIV should be considered in the differential diagnosis for dogs with acute respiratory disease. The ability to differentiate severe respiratory illness caused by CIV from other pathogens associated with CIRD is paramount to initiation of appropriate therapeutic and biosecurity interventions and to preventing further transmission of CIV.

Currently, serologic tests are a key tool in the diagnosis of canine influenza infection. As most serologic assays are fairly easy to perform and cost-effective and multiple samples can be collected and tested simultaneously, serologic testing is particularly useful for large-scale disease surveillance. While valuable, there are also several limitations to serologic testing. For instance, serum antibodies indicate exposure to the virus, but do not necessarily indicate active infection or disease. The most common reason for a negative result, despite clinical disease, is that insufficient time has passed between exposure and measurement of an antibody response. While specific influenza antibodies have been detected as early as seven days following experimental H3N8 influenza infection,[115] serologic testing often provides only retrospective information. However, decisions regarding outbreak control and treatment must often be made before serologic testing can confirm active infection.

Virus isolation (VI) in embryonated chicken eggs or cell culture has traditionally been considered the “gold standard” in influenza diagnostics;[116] however, due the fact that the procedure can be time consuming and cumbersome, other sensitive diagnostic methods have been developed for rapid influenza detection. To this end, several approaches, including reverse transcriptase (RT)-polymerase chain reaction (PCR) assays, enzyme-linked immunosorbent assays (ELISA), and antigen capture tests, are routinely performed to determine influenza infection in patients with acute respiratory illness. Nucleoprotein (NP) rapid influenza diagnostic tests (RIDTs) have particularly become popular in the hospital and field setting. These antigen-detecting tests, such as Directigen Flu A+B (BD Diagnostic Systems, Sparks, MD) and Flu Detect (Synbiotics, San Diego, CA), allow for rapid on-site diagnosis of influenza. However, low sensitivities of RIDTs for the detection of influenza virus shedding have been reported.[117-119] Alternatively, highly sensitive quantitative or real-time RT-PCR (rRT-PCR)-based assays have been developed and implemented in many diagnostic laboratories for detection and quantification of influenza viruses.[120-122] By employing a target-specific fluorescent probe, rRT-PCR-based assays eliminate the need to distinguish the target by PCR fragment size on electrophoresis or hybridization with probes after PCR. Additionally, by combining reverse transcriptase activity, a necessary first step in amplification of genomes of RNA viruses, and a traditional PCR reaction in one assay, the risk of cross-contamination decreases. The advantages of rRT-PCR over other diagnostic methods include its high sensitivity, high specificity, and rapid turnaround time,[120,121,123,124] and, as such, rRT-PCR assay meets the requirements for CIV detection in dogs with CIRD. Recently, members of the Landolt laboratory developed a one-step rRT-PCR assay method based on the amplification of the CIV matrix (M) gene for rapid diagnosis of influenza infection and quantification of nasal virus shedding in dogs. The objectives of the current studies were to (1) examine whether the rRT-PCR assay could serve as an effective method for influenza virus detection and quantification, and (2) evaluate the performance of the “patient side” lateral flow RIDT compared to the rRT-PCR assay in detecting CIV nasal shedding in high-risk shelter dogs.

MATERIAL AND METHODS

Viruses. Viral stocks of equine influenza H3N8 strains, A/equine/KY/1/1981 (kindly provided by Dr. D. P. Lunn, Colorado State University) and A/equine/WI/1/2003 (kindly provided by C. W. Olsen, University of Wisconsin-Madison), as well as and canine H3N8 influenza clinical isolates, A/canine/CO/224986/2006 (GenBank HQ917678, HQ993101) and A/canine/CO/148902/2006 (HQ917679, HQ993102), were grown in either 10-day old embryonated chicken eggs or Madin Darby canine kidney (MDCK) cells.

Clinical samples. There were two separate canine populations from which clinical samples were collected. A total of 124 shelter and household dogs demonstrating symptoms of CIRD (e.g., tachypnea, cough, nasal discharge) were swabbed between March 2006 and April 2007 by clinicians at Colorado State University Veterinary Teaching Hospital (CSU VTH) or on-site at shelters reporting CIRD. Briefly, a sterile polyester-tipped swab (Dacron Polyester, Hardwood Products Company, Guilford, ME) was inserted into the nostril of each dog and, if possible, a second swab was collected from the pharynx of each animal. The swab samples were immediately placed in different vials of 1 ml viral transport medium (phosphate buffered saline [PBS], 5mg/ml bovine serum albumin [BSA], 2,000 U/ml potassium penicillin G, 4 mg/ml streptomycin, 16 µg/ml gentamicin, and 100 U/ml nystatin) and stored at -80°C. Additionally, two nasal swabs were collected at the same time from 1372 dogs housed in one of six different humane shelters participating in a large-scale CIV surveillance program, which included shelters from California, Colorado, Florida, New York, South Carolina, and Texas between December 2009 and November 2010. The first nasal swab sample was collected and processed according to the recommendation of the manufacturer of the Flu Detect RIDT on site at the humane shelter. The second nasal swab sample was collected as described above and stored at 4°C before being shipped to the laboratory overnight on ice. All studies were reviewed and approved for conduct by the CSU Institutional Animal Care and Use Committee prior to initiation.

Virus Isolation. Influenza viruses were isolated by inoculating both MDCK cells and the allantoic cavity of 10-day old embryonated chicken eggs with 200 µl of thawed viral transport medium from the nasal and pharyngeal swab specimens collected at CSU VTH. Inoculated eggs were kept at 37°C in a humid incubator for 72 hours. MDCK cells were grown in 6-well cell culture plates in Eagle's minimum

essential medium (MEM; Gibco/Invitrogen, Carlsbad, CA) supplemented with 10% fetal bovine serum (FBS; Gibco/Invitrogen). Before inoculation, cells were washed twice with MEM. Each plate contained a negative (mock-inoculated) and positive (A/canine/CO/224986/2006) control well. After inoculation, 2 ml of MEM supplemented with 2.25mg/ml BSA, 50 mg/ml penicillin-streptomycin, 250 µg/ml amphotericin B, and 1 µg/ml tolylsulfonyl phenylalanyl chloromethyl ketone (TPCK)-treated trypsin (Worthington Biochemical Corp, Lakewood, NJ) was added to each well. Inoculated cells were incubated for 48 to 72 hours at 37°C and 5% CO_2 before cell culture supernatants were harvested. Cell culture supernatants were passaged twice and allantoic fluid was passaged up to three times in embryonated chicken eggs. Immunocytochemistry using the anti-influenza A NP antibody 68D2 (kindly provided by Drs. M. McGregor and Y. Kawaoka, University of Wisconsin-Madison) was used to detect influenza virus infection and replication in the MDCK cells. Presence of influenza virus was further confirmed in both embryonated chicken egg- and MDCK-grown viral stocks by RT-PCR amplification (described below) and gel electrophoresis, using a 10% polyacrylamide gel (10% acrylamide 1 × Tris-borate-EDTA [BioRad, Hercules, CA] gel), of the M, hemagglutinin (HA), and neuraminidase (NA) genes.

RT-PCR amplification and sequencing of HA and NA genes. The full-length protein coding regions of the HA and NA gene segments from each of the egg- and cell-isolated viruses were amplified by a two-step RT-PCR. Briefly, isolated viral RNA was reverse transcribed using SZAHA or SZANA primers[125] and SuperScript™ III reverse transcriptase (Gibco/Invitrogen). HA and NA cDNAs were further amplified by PCR using Platinum® *Taq* DNA polymerase high fidelity (Gibco/Invitrogen). PCR products were analyzed by direct cycle sequencing (ABI Big Dye; PE Applied Biosystems, Foster City, CA) at the Proteomics and Metabolomics Facility at CSU (http://www.dnatools.com). Nucleotide and amino acid sequences were determined using DNASTAR (Lasergene 7.0, Madison, WI) software.

Primers and probes. rRT-PCR primers and probes were designed based on influenza A virus M gene sequence information obtained from a GenBank BLAST search (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). To identify conserved regions in the M gene, nine sequences of equine and canine H3N8 influenza viruses isolated in North America since 1963 were aligned with ClustalW (http://www.genome.jp/tools/clustalw/). Primers and probes were selected using the Beacon Designer software (Premier Biosoft Inc., Palo Alto, CA). A 144-bp product from the M gene

between nucleotides 130 and 274 was amplified with the forward primer (5' GAA CAC CGA TCT TGA GGC ACT C 3') and reverse primer (5' GGC ATT TTG GAC AAA GCG TCT AC 3'). The 5' reporter dye (6-carboxyfluorescein [FAM]) labeled probe consisted of 23 nucleotides (5'-AGT CCT CGC TCA CTG GGC ACG GT-3').

One-step rRT-PCR assay. From each nasal and pharyngeal swab sample collected at CSU VTH and the six humane shelters, viral RNA was extracted from 140 μl of viral transport medium using the QIAamp Viral RNA Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions. The viral nucleic acid was eluted in a final volume of 60 μl RNase-free water before storage at -80°C. The one-step rRT-PCR assay was performed according to the manufacturers' recommendation with modifications. Briefly, a 5 μl-aliquot of RNA template was mixed with 20 μl of premixed reaction solution (iScript One-Step RT-PCR Kit [BioRad]) containing 200 nmol each of the forward and reverse primers, 80 nmol of the probe, as well as all the components necessary for the RT-PCR reaction. RNA from each swab sample was run in duplicate. Negative controls consisted of water and neat transport medium. The positive control consisted of 10 $TCID_{50}$ of A/canine/CO/224986/2006 (H3N8) in viral transport medium. To increase the throughput for sample processing, the epMotion Ep5070 (Eppendorf, Hamburg, Germany) automated system was used to load 96 well plates. Amplification and detection were performed using the Mastercycler Realplex (Eppendorf) under the following conditions: 10 min at 52°C, 5 min at 95°C, followed by 45 cycles of 10 sec at 95°C and 45 sec at 68.4°C. Optimization of the thermal-amplification profile and concentration of primers were performed using SYBER GREEN (BioRad). To confirm product size and PCR specificity, 25 μl of the PCR product were analyzed by gel electrophoresis, using a 10% polyacrylamide gel (10% acrylamide × Tris-borate-EDTA gel). Amplified products were visualized by UV transillumination following staining with 0.5 μg/ml ethidium bromide.

RNA synthesis by *in vitro* transcription. Purified full-length M gene RNA was used as the standard for calibration of influenza virus M gene copy number, which was *in vitro* transcribed from the corresponding full-length M gene template of A/equine/WI/1/2003 (which is homologous with the CIV M gene) cloned into a plasmid vector (pGEM-T Easy vector [Promega, Madison, WI]). The directionality of the insert was confirmed by direct cycle sequencing using T7 primers. M gene RNA was transcribed with the RiboMAX Large Scale RNA production systems kit (Promega) from the T7 promoter according to the

manufacturer's instructions, treated with RNase-free DNase I, suspended in RNase-free water, quantified by spectrophotometer, and stored at -80°C at a concentration of 0.12 ng/μl.

rRT-PCR standardization and determination of detection limits. The rRT-PCR assay was standardized using dilution series of four H3N8 isolates grown in MDCK cultures (A/equine/ KY/1/1981, A/equine/WI/1/2003, A/canine/CO/224986/2006, and A/canine/CO/148902/2006) ranging from 10^4 to 10^{-6} $TCID_{50}$/ml in viral transport medium and from nasal swab samples obtained from dogs showing no clinical signs of CIRD. To determine the minimum detection level of virus RNA by rRT-PCR, the *in vitro* transcribed RNA was serially diluted in RNase-free water to produce dilutions ranging from 10^7 to 10^0 genomic equivalents/μl of viral M gene RNA. A cycle threshold (Ct) cut-off value for the rRT-PCR assay was determined by a parametric receiver operating characteristic (ROC) curve analysis, using the raw Ct values of the nasal and pharyngeal swab samples. To account for assay variation, the median Ct values of over 50 RNA standard curves, run on different days, were determined.

Rapid influenza diagnostic tests. Two different RIDTs were used in these studies. Directigen Flu A +B (herein referred to as DFA; BD Diagnostic Systems, Sparks, MD), an enzyme immunoassay test, was performed on every nasal and pharyngeal swab that was also assayed by rRT-PCR from dogs sampled by a CSU VTH veterinarian. Briefly, 125 μL of viral transport medium containing the swab sample was added to the DFA test membrane. Influenza A virus NP antigen that bound to the membrane was subsequently detected using an enzyme-conjugated anti-NP monoclonal antibody. Each swab sample was evaluated and scored as positive (characterized by the development of a purple triangle) or negative. The second RIDT, Flu Detect (herein referred to as FD; Synbiotics, San Diego, CA), a lateral flow avian influenza A antigen test kit, also detects the NP antigen. To determine efficacy of the FD test, humane shelter personnel collected nasal swab samples from incoming dogs and immediately performed the FD test on-site. Briefly, the nasal swab was placed into a tube containing extraction buffer before rotating the swab 5-10 times. The swab was then pressed up against the sides of the tube to remove any remaining liquid. A test strip was submerged into the tube and allowed to incubate for 15 min at room temperature before being read as either positive (two pink/purple bands – the test line and control line) or negative (one pink/purple band – the control line only). All reagents, tubes, and swabs were provided with the test kit. For consistency, only one or two people from each shelter conducted all of the FD tests.

Statistical analyses. To determine significance between standardized Ct values among samples and between shelter and household dog populations, Student t-tests were calculated using GraphPad Prism version 5 for Mac OS X (GraphPad Software, La Jolla, CA). Diagnostic test sensitivity and specificity estimates were determined by the stochastic latent class analysis modeling approach described by Branscum et al.[126] using Gibbs sampling.[127] The dataset was divided into two populations based on whether the dog sampled at CSU VTH resided in a shelter (n=97) or household (n=27), as these two dog populations have been shown to have different seroprevalence rates of CIV.[7,112,128] Independence and dependence is determined by the type of biological sample tested.[126] Therefore, three models were used to evaluate detection of CIV in the samples obtained from CSU VTH: two conditional independent tests in two populations comparing VI to DFA and VI to rRT-PCR (VI is a measure of infectivity and replication, while DFA and rRT-PCR specifically detect genes or gene products), two conditional dependent tests in two populations comparing DFA to rRT-PCR, and three tests (two dependent [DFA, rRT-PCR], one independent [VI]) in two populations. "Conditional" refers to the tests being conditional on the infection status of the individuals sampled. As both the rRT-PCR and DFA test results were separately compared to VI in the two conditional independent tests assimilations, a total of four models were analyzed. Table 3-1 describes the sensitivity and specificity prior probability distributions for the three diagnostic tests evaluated. The priors for CIV prevalence were based on previous studies[7,112,128] to a mode of 0.04 and 0.40 for household and shelter dogs, respectively, both with a 95th percentile of 0.7. Inferences were based on 100,000 iterations (with a burn-in of 5,000 iterations) after six chains indicated no lack of convergence. All models were run with WinBUGS 1.4.3 freeware.[129]

Table 3-1. Estimates, modes, 95% probability intervals, and beta distributions used as the prior distributions in the stochastic latent class models.

	Estimate	Mode	95% Probability Interval		Beta distribution	
Viral isolation sensitivity	>0.5	0.90	0.436	0.982	a=5.3842	b=1.4871
Viral isolation specificity	>0.5	1.00	0.426	0.994	a=4.3219	b=1.0000
Directigen sensitivity	>0.5	0.70	0.464	0.861	a=13.3221	b=6.2809
Directigen specificity	>0.5	0.90	0.436	0.982	a=5.3842	b=1.4871
rRT-PCR sensitivity	>0.5	0.90	0.436	0.982	a=5.3842	b=1.4871
rRT-PCR specificity	>0.5	0.90	0.436	0.982	a=5.3842	b=1.4871

As there was only one population (shelter dogs) sampled for the RIDT FD analysis, sensitivity, specificity, positive predictive value, and negative predictive value were determined using traditional calculations from a 2 x 2 contingency table.

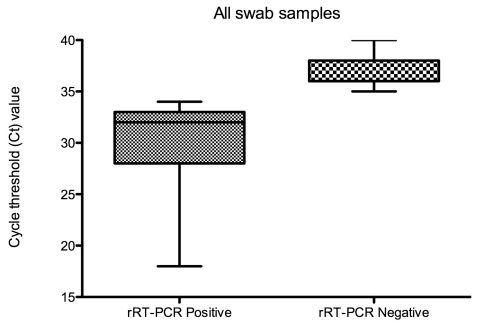
RESULTS

Development of rRT-PCR and sample analysis. The minimum detection level of influenza virus M gene RNA was determined to be 1000 copies per reaction, or a Ct value of 34, by rRT-PCR. Negative samples were considered to have Ct values above 34 up to 40 (which was considered undetectable). Amplification of dilutions of the M gene RNA transcripts showed linearity over a range of eight orders of magnitude, from 10^0 through 10^7 (Table 3-2). For the RNAs from the H3N8 influenza strains serially diluted from 10^4 to 10^{-6} $TCID_{50}$/ml, the minimal levels of detection for the isolates were as follows: A/canine/CO/224986/2006, 1 $TCID_{50}$; A/canine/CO/148902/2006, 1 $TCID_{50}$; A/equine/KY/1/1981, 10 $TCID_{50}$; and A/equine/WI/1/2003, 10 $TCID_{50}$. The threshold of the rRT-PCR assay on the basis of the mid-point of the linear region of the amplicon development curve was defined as Rn=0.1. Assay variability was calculated based on the Ct values. Coefficients of variation were between 1.5 and 4.7% for the Ct values and were comparable to those from previously published rRT-PCR studies.[120] Of the total 122 swab samples tested for influenza by rRT-PCR, the overall Ct mean difference between positive (Ct of 34 and under) and negative (Ct over 34) samples was statistically significant (P<0.0001; t=12.25 df=120), with a mean Ct value of 29.6 (±0.7) (median 32.0) for positive swabs and 36.9 (±0.2) (median 36.0) for negative swabs (Figure 3-1a). The negative and positive results from the 83 samples that were inoculated into eggs or MDCK cells for VI and the 119 samples tested by DFA were significantly different as well (P_{VI}<0.0001, t_{VI}=7.538 df_{VI}=81; P_{DFA}<0.0001, t_{DFA}=11.97 df_{DFA}=117), although there was no difference between the VI and DFA positive shelter and household dog samples (P=0.49, t=0.6899, df=120) (Figure 3-1b). The Ct values of positive VI and DFA ranged from 18 to 33 and from 18 to 36, respectively. Among the 13 dogs with both

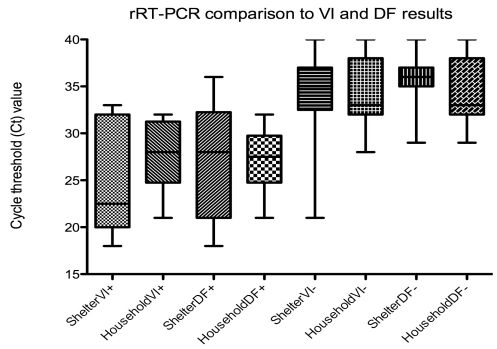
Table 3-2. Median cycle threshold values corresponding to each dilution of matrix gene RNA.

Matrix gene copy number	Median cycle threshold value (±SEM)
$<10^1$	>38.1 or undetectable
10^2	37.2 (0.15)
10^3	34.5 (0.20)
10^4	31.1 (0.24)
10^5	27.0 (0.19)
10^6	24.1 (0.16)
10^7	20.2 (0.16)

3-1a.



3-1b.



3-1c.

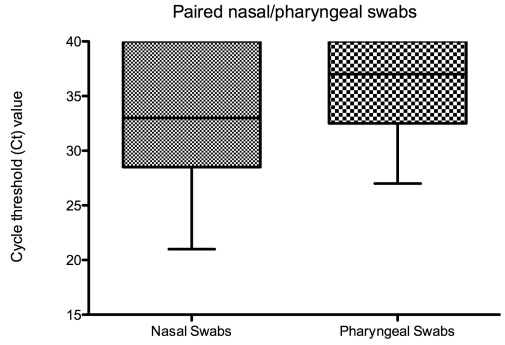


Figure 3-1. Comparison of cycle threshold values for (a) all nasal and pharyngeal swabs collected from dogs with canine infectious respiratory disease, (b) shelter and household dog samples testing positive and negative on virus isolation (VI) and/or Directigen (DFA), and (c) paired nasal and pharyngeal swabs from dogs with canine infectious respiratory disease. The bottom and top of the box represent the 25th and 75th percentiles, the horizontal bands are the medians, and the whiskers depict the maximums and minimums.

nasal and pharyngeal swab samples available for testing, seven dogs had positive nasal swabs on rRT-PCR (Ct_{mean}=29.1 ±1.7; median 29.0), but only four out of those seven animals also had a positive pharyngeal swab (Ct_{mean}=31.0 ±1.4; median 32.0). The difference in Ct values between the 13 paired nasal and pharyngeal swab samples (Figure 3-1c) was not statistically significant (*P*=0.06; t=2.076 df=12).

VI from nasal and pharyngeal swabs. Influenza virus was isolated in 34.1% (15/44) of the 10-day old embryonated chicken eggs and 7.1% (5/70) of the MDCK cell cultures inoculated with nasal and pharyngeal secretions. In total, 16 influenza viruses were isolated. There was agreement between 21 of the 31 (67.7%) swabs that were inoculated into both eggs and cell culture, with 4 positives and 17 negatives. Interestingly, there were 11 viruses isolated in eggs, but not in MDCK cells. Conversely, there was one isolate that replicated in MDCK cells only. Four viruses replicated in both. All CIV isolates fell into the canine H3N8 sub-lineage differing from EIV H3N8 at the five HA amino acid residues previously described for CIV (N54K, N83S, W222L, I328T, N483T).[8]

Comparison of VI, DFA, and rRT-PCR in detecting CIV. The three tests had varying sensitivities for detecting CIV from the swab samples collected (Table 3-3). While 19 (16.0%) of the 119 total nasal and pharyngeal swab samples tested by DFA were positive, only 10 of those were also positive by VI. In total, VI and DFA agreed on 68 of 81 (84.0%) samples. Using a Ct cut-off of 34, of the 83 swab samples tested by both rRT-PCR and VI, 16 (19.3%) were positive by both assays and 38 (45.8%) were negative by both assays. There were 29 (34.9%) samples that were positive by rRT-PCR, but virus was not isolated in either eggs or cell culture. Surprisingly, the percentages of positives, although different among the tests performed, were consistently higher for household dogs compared to the shelter dogs sampled (Table 3-4).

Table 3-3. Comparison of CIV detection by virus isolation (VI), Directigen (DFA), and rRT-PCR from nasal and pharyngeal swabs.

rRT-PCR (n=122)	DFA (n=119)	VI (n=85)	Total (n=124)
+	+	+	10
+	+	–	7
+	–	+	6
+	–	–	22
–	–	–	36
+	–	NT*	3
–	+	NT	2
–	–	NT	33
–	NT	–	2
–	NT	NT	1
NT	NT	–	2

* NT: Not Tested

Table 3-4. Comparison of CIV detection by virus isolation (VI), Directigen (DFA), and rRT-PCR from nasal and pharyngeal swabs from shelter (s) and household (h) CIRD dogs.

	VI (s=61, h=24)		DFA (s=95, h=24)		rRT-PCR (s=95, h=27)	
Swab samples	Positive	Negative	Positive	Negative	Positive	Negative
Shelter	16.4%	83.6%	13.7%	86.3%	33.7%	66.3%
Household	25.0%	75.0%	25.0%	75.0%	59.3%	40.7%
Total	18.8%	81.2%	16.0%	84.0%	39.3%	60.7%

Sensitivities of VI, DFA, and rRT-PCR assays. Three stochastic latent class models were used to estimate the diagnostic sensitivities and specificities of VI, DFA, and rRT-PCR in detecting CIV in dogs. However, as these studies concentrated on the ability of the various tests to detect positivity in swab samples and no known true negative samples were collected, only the sensitivities of the three tests are reported here (Table 3-5). These sensitivities have wide ranges among the three tests and among the models used. The model comparing all three three-tests in two different populations, the most appropriate model for these data, shows DFA to have a median sensitivity of 0.65, while VI median sensitivity was a little higher at 0.72. In this model, the rRT-PCR assay had the highest sensitivity at 0.95. The other models, with the one-on-one test assessments, showed similar results.

Table 3-5. Median sensitivity estimates and 95% probability intervals (PI) determined by stochastic latent class analyses

	VI		DFA		rRT-PCR	
Test comparison model	Estimate	95% PI	Estimate	95% PI	Estimate	95% PI
Three tests	0.72	0.47, 0.96	0.65	0.48, 0.80	0.95	0.83-0.99
Two tests (VI, DFA)	0.80	0.52, 0.98	0.696	0.52, 0.85	--	--
Two tests (VI, rRT-PCR)	0.64	0.37, 0.95	--	--	0.947	0.81, 0.99
Two tests (DFA, rRT-PCR)	--	--	0.57	0.39, 0.79	0.93	0.75, 0.99

Evaluation of FD in detecting CIV. Of the 1372 shelter dog nasal swab samples for evaluation of the FD test compared to rRT-PCR, only 3 of the 38 tests that were positive by rRT-PCR were also positive by FD, while FD did not detect influenza virus in 35 nasal swab samples that were positive by rRT-PCR. Conversely, the FD test was positive for 218 of the 1334 samples that were negative by rRT-PCR. Using traditional calculations for a 2 x 2 contingency table with rRT-PCT as the gold standard, the FD had a sensitivity of 7.9% (3/38), specificity of 83.7% (1116/1334), positive predictive value of 1.4% (3/221), and negative predictive value of 97.0% (1116/1151). The three nasal swabs positive by both FD and rRT-PCR had Ct values ranging from 26 to 33. Nasal swabs with negative FD results included samples that had rRT-PCR Ct values as low as 26. All six shelters included in the study showed similar inconsistencies between the rRT-PCR and FD test results.

DISCUSSION

These studies are the first to describe the development of an rRT-PCR-based assay that can be used specifically for detecting CIV in canine nasal and pharyngeal swabs and that evaluate common diagnostic methods for detecting CIV shedding in both CIRD and healthy dogs. As CIV can cause subclinical infection, it is imperative for disease control to correctly diagnose CIV in sick dogs that reside in humane shelters, are boarded, or attend canine day care.[128] It is also well known that dogs shed influenza virus several days before any noticeable illness. Therefore, the relatively short window of opportunity to identify CIV-infected dogs necessitates the rapid diagnosis of CIV, even before CIRD is observed, to prevent any further CIV transmission.

The finding that there is no overall statistical difference in the rRT-PCR Ct values among paired nasal and pharyngeal swabs samples (Figure 3-1c) suggests that collection from nasal mucosa, a less

invasive procedure than swabbing the pharynx, is sufficient for diagnosing influenza infection in dogs. Furthermore, as there were several negative pharyngeal samples that did not correspond to respective paired positive nasal swab samples, the nares appear to be the better site to swab when suspecting influenza infection. However, sampling protocols should be specific as to how deep within the nasal cavity a swab needs to be inserted to get an optimal mucosal sample. Canine nasal anatomy might pose a problem for the inexperienced as well, as the swab needs to clearly bypass the alar cartilage at the nostril entrance to reach the mucosa deep within the canal.

From properly collected swabs, the rRT-PCR assay is highly sensitive in detecting CIV. The three-test model showed 96% sensitivity for rRT-PCR, which was much higher compared to traditional VI (72%) and the patient-side RIDT DFA (65%). There are several assumptions for the stochastic latent class analysis that should be addressed, however. The first two, (1) there were two dependent tests and one independent test and (2) there were two different populations, were met. DFA and PCR both detect the same biological phenomenon (gene or gene products), while VI relies on the presence of infectious virus and ability of the virus to infect and replicate in eggs or cell culture. Although the goal of humane shelters is to adopt dogs from the facility out into the community, there is a clear environmental difference between dogs residing in households versus dogs that are located within humane shelters, where they often co-mingle and are co-housed with other dogs. Additionally, the constant introduction of new dogs (often not quarantined for a sufficient period to allow for cessation of viral shedding) poses a much higher threat to dogs in shelters compared to dogs that live in one-dog households or that share the same space with familiar dogs. Thus, treating shelter and household dogs as two different populations in our stochastic models is valid. Indeed, seroprevalence studies conducted in shelters reporting CIRD,[7,112] show much higher seropositivity compared to the few studies done in dogs residing in homes.[128,130] The third, and final, assumption is that there is constant sensitivity and specificity of the tests. As there is no indication that any of the three tests is more or less sensitive or specific for the populations sampled in these studies, this assumption has been met as well.

The high sensitivity of rRT-PCR is not surprising. Other rRT-PCR assays used in influenza virus detection show similar sensitivities.[120,123] As virus isolation has traditionally been considered the gold standard in influenza virus diagnostics, the discrepancies between isolating virus in embryonated chicken

eggs or cell culture and detecting CIV on rRT-PCR cannot be simply dismissed. In this study, virus isolation was carried out on samples that were frozen within 12 hours of collection and had subsequently been thawed. This single freeze-thaw may have resulted in a reduction of infectious virus titer and, thus, reduction in the sensitivity of virus detection by isolation in cell culture compared to antigen detection by DFA (generally performed as soon as the sample was delivered to the laboratory) and by rRT-PCR. It is also interesting that egg culture was more successful in isolating canine influenza viruses than was MDCK cell cultures. VI from embryonated chicken eggs is still considered the gold standard for equine influenza virus (CIV's nearest ancestor) diagnosis.[116] Accordingly, avian, equine, and canine influenzas have all been shown to preferentially bind receptors with glycans having sialic acid linked to galactose by an α2,3 linkage.[28,32,105] Moreover, laboratory experience suggests that growing CIV in embryonated chicken eggs is not always foolproof. Indeed, a recent EIV and CIV molecular evolution study found that neither embryonated chicken eggs nor MDCK cell cultures were better able than the other to grow CIV.[106] What this means for CIV diagnosis is still unknown. Clearly, however, VI in eggs or MDCK cells cannot solely be relied upon to determine CIV infection. Therefore, as RT-PCR has been shown to be able to detect influenza viruses even in samples repeatedly frozen and thawed,[117] the relatively high sensitivity of rRT-PCR and its rapid turn-around makes it the ideal test for first-step CIV analysis. VI would be the next logical step for sequencing and genetic characterization of positive rRT-PCR samples.

Rapid influenza diagnostic tests might also be considered as bedside or on-site tests for CIV if rRT-PCR or VI is not available due to financial, geographical, or time restraints. However, the limitations of these RIDTs need to be understood by the user. Although the DFA was clearly better than the FD test at diagnosing influenza in dogs, the 95% PI for DFA sensitivity ranged from 48% to 80%, suggesting true positive samples in our studies might have been misdiagnosed as negative. Similarly, the FD test is very poor for CIV diagnosis, and, in fact, was more likely to give a false positive rather than a true positive result. It should be noted, however, that PPV is generally low when there is low prevalence of disease. As the true prevalence of CIV is currently unknown in all dog populations, it is difficult to accurately assess PPV, and the estimated 1.4% from the 2 x 2 calculations might actually be higher (or lower). Despite this, however, a practical approach for a humane shelter or a geographically remote clinic might be proper isolation and quarantine measures for every dog entering the facility if none of the CIV diagnostic tests

can be performed. Undoubtedly, there is still a need for the development of a fairly cost-effective, highly sensitive RIDT.

In conclusion, the findings in these studies suggest that rRT-PCR has the highest sensitivity of the three methods evaluated for detecting CIV nasal shedding and can be used as a rapid diagnostic test for CIV. As the cost for rRT-PCR might be more than several RIDTs, facilities with financial constraints might want to consider testing in series, starting with a RIDT performed on-site. Any samples positive by a RIDT could then be sent to a veterinary diagnostic center for confirmation by rRT-PCR, while the potentially infected dog is isolated to minimize CIV transmission to naïve dogs. As turn-around time for rRT-PCR is ~1day, the quarantine period might be as short as 48 hrs, if the test is negative, or as long as seven days to account for viral shedding, if the test is positive. Overall, however, rRT-PCR is a practical, relatively inexpensive, and quick test that can be used in CIV diagnosis.

CHAPTER FOUR: SEROPREVALENCE AND RISK FACTORS FOR CANINE H3N8 INFLUENZA VIRUS EXPOSURE IN HOUSEHOLD DOGS IN COLORADO*

INTRODUCTION

Influenza A viruses infect a wide range of species, including humans, horses, pigs, sea mammals and birds.[1] Historically, dogs were not considered to be natural hosts for influenza. However, in 2004, an outbreak of respiratory disease in racing greyhounds at a track in Florida provided evidence of transmission of an influenza virus closely related to the contemporary equine H3N8 "Florida" lineage.[7] Over the course of that same year, outbreaks of canine influenza occurred at 14 greyhound racetracks in six states, and by 2005 the virus had spilled over into the non-greyhound dog population.[7,8] To date, canine influenza virus (CIV) infections have been detected in at least 27 States and the District of Columbia.[114] Because the virus represents a novel pathogen, all dogs irrespective of age, breed, and sex are potentially susceptible to infection with CIV. The virus is shed in respiratory secretions and transmitted in aerosols created by coughing and sneezing. As a consequence, close contact and closed environments appear to favor virus transmission among susceptible animals.[8,104]

While it is impossible to determine the total number of CIV infections that have occurred since first identification of the virus, a recent one-year study performed in our laboratory demonstrated that CIV was found in five of five Colorado humane shelters that experienced outbreaks of canine respiratory disease.[131] These results suggest that CIV is a frequently detected pathogen in Colorado humane shelters that have a history of canine respiratory disease. To date, only a few studies have investigated CIV seroprevalence in non-shelter dog populations[132-134] and no information is available on the prevalence of CIV infection in the household dog population of Colorado. To better understand the extent of CIV infection in household dogs in a state with a presumed high incidence of CIV infection,[114] we sought to estimate the rates of CIV seropositivity among dogs from non-shelter environments that were

* This chapter was published as a brief communication in the Journal of Veterinary Internal Medicine: Barrell EA, Pecoraro HL, Torres-Henderson C, Morley PS, Lunn KF, Landolt GA. (2010) JVIM 24:1524-1527. Barrell and Pecoraro contributed equally to the paper and are co-first authors.

seen at Colorado State University's Veterinary Medical Center (CSU VTH). In addition, as identification of factors that increase the risk of CIV infection would provide a factual foundation on which to base disease prevention and control measures, a second objective of this study was to investigate risk factors for CIV infection in Colorado household dogs.

MATERIALS AND METHODS

Study population. For this study, we utilized a prospective convenience sampling method in which eligible households were defined as a domestic unit located in the state of Colorado consisting of the members (or a member) of a family who live together along with a dog (or dogs) of undefined age, breed, vaccination status, or sex. While dogs that qualified under this definition were eligible for inclusion regardless of the reason for presentation, they were excluded from the study if their owner declined to provide consent. In addition, dogs were excluded if the clinician determined that blood collection would have caused undue stress to the animal or if the dog had received CIV vaccination. Based on the results of a serological surveillance study for swine influenza virus infections among unvaccinated pigs in the north-central United states,[135] the sample size was calculated using an estimated seroprevalence of 20%. Prior to initiation, this study was reviewed and approved for conduct by the Colorado State University Institutional Animal Care and Use Committee.

Animals. Serum samples were collected from three different household dog populations presented to the CSU VTH. Over a collection period of 9 months (March to December 2009), 140 serum samples were obtained from household dogs admitted to the Community Practice service at the CSU VTH. These dogs were generally in good health and were presented for reasons such as wellness examinations, preventive health care, dentistry, and heartworm testing. In addition, serum samples were obtained from 110 dogs that had been submitted by CSU VTH clinicians to the Clinical Pathology laboratory from dogs of various ages, breeds, and clinical histories. The blood samples were submitted as part of the diagnostic investigation of a range of clinical conditions. Lastly, 75 serum samples collected prior to 2004 from Colorado household dogs of all ages obtained from a serum bank maintained at the Animal Cancer Center at CSU VTH (kindly provided by Dr. S. Lana, Animal Cancer Center, CSU) were used for the estimation of the pre-2004 CIV seroprevalence.

Data collection. A questionnaire was administered to the 140 dog owners that presented their animals to the Community Practice service at CSU VTH at the time of sample collection. The questionnaire included the dog's signalment (age, breed, sex), vaccination status, and past and current medical histories. In addition, the questionnaire addressed the length of ownership of the dog, the number of other dogs living in the same household, the dog's travel history, and information about social interactions with non-household dogs, such as visits and/or stays at dog parks (including the number of visits), day-care facilities (including frequency of stays), and boarding facilities (including number and length of stays).

Serology. The levels of hemagglutination inhibiting antibodies in serum samples were determined by HI assays that were performed as previously described[135,136] and in accordance with procedures recommended by the World Organization for Animal Health (OIE). Briefly, sera were incubated overnight at 37ºC with four volumes of receptor destroying enzyme (RDE; Denka Seiken Co, Tokyo, Japan) prepared from *Vibrio cholera*. After inactivation of the RDE enzyme by incubation of the samples at 56°C for 60 min, twofold serial dilutions of sera were mixed with four hemagglutinin units of CIV in 96-well microtiter plates. The assays were developed by adding 0.5% (vol/vol) chicken red blood cells (cRBCs), and the HI antibody titers were defined as the reciprocal of the highest dilution causing complete inhibition of agglutination. To account for potential antigenic drift the following CIV strains were used as antigens: A/canine/Florida/43/04 (H3N8) (kindly provided by Dr. P.C. Crawford, University of Florida), A/canine/CO-1/224986/06 (H3N8), and A/canine/CO-5/234550/09 (H3N8). Each sample was run in duplicate and each assay included both positive (positive control canine serum) and negative (physiological saline only) controls. Seropositivity was defined at a HI titer >1:8.

Statistical Analyses. Data from the questionnaires were collated with patient signalment information and results of serological testing. These data were validated and entered in a computer spreadsheet and data were summarized using contingency tables. 'Plus four' confidence intervals were calculated for the proportions of seropositive dogs by adding two successes to the seropositivity outcome and four to our sample size for computation of a more appropriate 95% confidence interval.[137] Associations between seropositivity and potential risk factors such as age (≥ 2 years [immature], < 2 years [mature]), sex (male, female), history of cough in the past 6 months (yes, no), other dogs living in

the same household (yes, no), dog acquired from shelter or rescue (yes, no), dog attended day care in the past 6 months prior to sample collection (yes, no), dog was boarded in the past 6 months prior to sample collection (yes, no), dog greeted other non-household dog in the past 6 months (yes, no), and dog visited dog park in the past 6 months prior to sample collection (yes, no), were investigated using the Fisher's Exact Test with a critical alpha of 0.05.

RESULTS

CIV H3N8 seroprevalence. A total of 325 canine serum samples were tested. All dogs included in the study lived in the state of Colorado at the time of sample collection. One-hundred and forty serum samples were collected from dogs seen at the Community Practice service at CSU VTH between March and December 2009 (mean age 4.8 yrs [range 6 months - 15 years]; 73 males, 67 females). One-hundred and 10 samples were obtained through the Clinical Pathology laboratory from dogs that were seen by other clinical services at CSU VTH for various reasons between March and December 2009 (mean age 8 yrs [range 3 months – 16 years]; 51 males, 59 females). Lastly, 75 serum samples that had been collected prior to 2004 were obtained. Of all serum samples collected from patients in 2009, 3.6% (9/250; 95% CI = 1.1%, 6.1%) were antibody positive, including 2.9% (4/140; 95% CI = 0.01%, 6.1%) of dogs seen by the Community Practice service and 4.5% (5/110; 95% CI = 0.1%, 9.0%) of dogs seen by other services at CSU VTH. This difference in seroprevalence between patient groups was not statistically significant (P=0.48). All serum samples collected from dogs prior to 2004 were seronegative. The antibody titers to A/canine/Florida/43/04 were similar to those for the 2006 and 2009 isolates. The mean geometric log base 2 HI antibody titer for the positive serum samples was 7.5 (1:176), ranging from 5.8 to 8.4 (1:58 to 1:341). These patterns of inhibition demonstrated the antigenic similarly between the CIV isolates tested and indicate that measurable antigenic drift has not yet occurred.

Risk factor analysis. One hundred and forty questionnaires were completed by owners presenting their dogs to the Community Practice service at CSU VTH. The only factor statistically associated with CIV H3N8 seropositivity was attendance at canine day-care within the last 6 months (OR=53.8; 95% CI = 6.2, 463.6; P <0.001) (Table 4-1). Additionally, although not statistically significant, dogs boarded in kennels within 6 months prior to serum sample collection appeared to be predisposed to

CIV infection (OR=8.5; 95% CI=1.4, 53.0; *P*=0.06). No other factors related to the dog's signalment (age, breed, sex), vaccination status, medical and travel histories, or other social interactions with non-household dogs, such as at dog park visits, were associated with CIV seropositivity.

Table 4-1. Results of analyses of various potential risk factors for association with CIV seropositivity among dogs presented to the Community Practice service at Colorado State University Veterinary Medical Center.

Variable	Categories	No of CIV positive dogs	No of CIV negative dogs	OR*	95% CI**	P value
Age of dog						
	≥ 2 years	4	85	5.4	1.1-26.6	0.16
	< 2 years	0	51	Reference		
Sex						
	Female	2	65	1.1	0.8-1.6	0.66
	Male	2	71	Reference		
History of cough in past 6 months prior to sample collection						
	Yes	0	18	0.7	0.1-3.6	1.00
	No	4	118	Reference		
Other dogs living in the same household						
	Yes	3	82	1.5	1.5-1.6	0.49
	No	1	54	Reference		
Dog acquired from shelter or rescue						
	Yes	2	40	2.4	1.7-3.4	0.35
	No	2	96	Reference		
Dog attended day care in the past 6 months prior to sample collection						
	Yes	2	2	53.8	37.2-77.8	<0.001
	No	2	134	Reference		
Dog boarded in the past 6 months prior to sample collection						
	Yes	2	14	8.5	6.5-10.9	0.06
	No	2	122	Reference		
Dog has greeted other non-household dog in the past 6 months						
	Yes	4	111	2.1	0.4-10.3	0.45
	No	0	25	Reference		
Dog visited dog park in the past 6 months prior to sample collection						
	Yes	0	26	0.5	0.1-2.3	1.00
	No	4	110	Reference		

OR = Odds ratio, CI = Confidence interval

* OR adjusted for zero values = $[(A+0.5)\times(D+0.5)]/[(B+0.5)\times(C+0.5)]$

** 95% CI adjusted for zero values = $\exp(\ln(OR) +/- 1.96 \times \ln[(1/[A+0.5])+(1/[B+0.5])+(1/[C+0.5])+(1/[D+0.5])]^{0.5})$

DISCUSSION

Findings of the present study suggest that CIV H3N8 seroprevalence in household dogs in Colorado is lower compared to CIV seroprevalence in humane shelters,[7,112] although CIV seroprevalence has increased since 2004. Despite the fact that the total number of canine influenza infections that have

occurred since first identification of CIV is unknown, a recent one-year study conducted by our laboratory found that five of five Colorado humane shelters that experienced outbreaks of canine respiratory disease ("kennel cough") housed at least one CIV real-time PCR positive dog.[131] Similarly, an earlier study found a 97% CIV seroprevalence in shelter dogs in Florida and in dogs housed at veterinary clinics in Florida and New York that had a recent history of a respiratory disease outbreak.[7] Taken together, these data suggest that CIV plays an important role in dogs with clinical respiratory disease that are housed in closed environments in Colorado, Florida, and New York.

Given the presumed high prevalence of CIV exposure in Colorado shelter dogs, it was surprising that the seroprevalence found in Colorado pet dogs was so low. As the canine sera demonstrated similar titers to canine influenza viruses isolated in 2004, 2006, and 2009, indicating a lack of measurable antigenic drift, the small number of seropositive dogs found in our study cannot be explained by a change in antigenicity of the virus. While other studies conducted in non-shelter dogs have demonstrated similarly low seroprevalences (0.4% and 1.2%),[132,134] these studies were conducted in geographic areas where CIV prevalence is thought to be low (Ontario, Canada; Iowa).[114] Considering the frequent detection of CIV in Colorado humane shelters, a potential explanation for the assumed discrepancy in the frequencies of CIV exposure is that influenza virus ecology is different in shelter dogs than in household dogs and spillover of virus from the shelter environment may not pose a common risk for most household pets presented to the CSU VTH.

Another possible reason for the low number of CIV H3N8 positive dogs found in this study is that the current study was a population prevalence study and not a study focusing on animals with clinical signs of respiratory disease. Despite this, the low seroprevalence in this study suggests that canine influenza virus is not a common respiratory pathogen encountered by the majority of pet dogs in Colorado. This notion seems to be further supported by the finding that a history of coughing within 6 months prior to sample collection was not statistically associated with CIV seropositivity. In contrast, our study identified attendance at canine day-care as a risk factor for CIV exposure, supporting the notion that commingling of animals in closed environments favors virus transmission. This result may be used to target preventive strategies (e.g., vaccination) at dogs that have the greatest risk of CIV exposure, such

as dogs attending day-care or dogs that are being boarded. However, controlled field trials are needed to evaluate the value of such preventative measures in these groups of animals.

We recognize several limitations of this study. First, as the number of seropositive animals was small, the power to define risk factors for CIV exposure is low and, therefore, the data should be weighed carefully. Moreover, the small number of positive serum samples does increase the potential for Type II statistical error (not finding a statistical difference when one truly exists). As a result, it is possible that, although no significance was found between CIV seropositivity and other risk factors analyzed in our questionnaire, there may actually have been associations present. Secondly, a potential bias relating to the sample population also has to be considered. This study involved dogs presented to a primary (Community Practice Service at CSU VTH) and secondary (other veterinary services at CSU VTH) veterinary care facilities. This population is likely biased as there is an expected subset of animals that are rarely or never presented for veterinary care. As the majority of dogs included in this study originated from a limited geographical area, one cannot draw conclusions as to the true CIV H3N8 seroprevalence in pet dogs in the state of Colorado. Lastly, while published data report detection of antibodies in banked dog sera more than five years after collection,[138] the duration of persistence of CIV antibodies in individual animals has not been well defined. It is possible that some of the dogs tested in this study had previously been infected but no longer had detectable antibody titers, which may have resulted in an underestimation of CIV seroprevalence in this study. Nevertheless, while it remains to be determined how long CIV antibodies persist in canine serum, the discrepancy between the frequent detection of CIV in Colorado shelters and the low seroprevalence in household dogs merits further investigation.

CHAPTER FIVE: SEROPREVALENCE OF CANINE INFLUENZA VIRUS H3N8 IN IDITAROD RACING SLED DOGS [*]

INTRODUCTION

Influenza A viruses infect many different species, including various birds and mammals. Historically, dogs were not considered natural hosts for influenza, despite limited cases of infection.[9,95] In 2004, however, influenza virus (H3N8) was isolated from racing greyhounds at a Florida racetrack and the virus subsequently spread to 14 racetracks located throughout 6 states.[7] By 2005, CIV was detected in pet and shelter dog populations as was evidenced by a study that reported 97% influenza seropositivity in dogs with respiratory disease at several veterinary clinics and shelters in New York and Florida.[7] Moreover, since first isolation of the virus in 2004, the Animal Health Diagnostic Center at Cornell University has reported CIV H3N8 infection and/or detected antibodies in dogs sampled in at least 28 states and the District of Columbia.[114] Interestingly, CIV has yet to be reported in the Canadian provinces except for an Ontario study, which detected CIV serum antibodies in only one dog – a greyhound from Florida.[132]

As influenza virus replicates in the respiratory epithelium, respiratory secretion droplets generated by coughing and sneezing, aerosolized secretions, and direct physical contact between infected and naïve individuals constitute potential routes of transmission.[139] Results from several recent studies suggest that dogs housed in close proximity in closed-air environments are at greater risk for CIV exposure.[112,128] For example, a CIV surveillance study at a Philadelphia shelter found 42% CIV seropositivity in dogs.[112] In another study from Colorado, 100% (5/5) of humane shelters experiencing canine respiratory disease had at least one dog at the facility shedding influenza virus.[131] Further studies found an increased risk for CIV seropositivity in Colorado household dogs that had attended canine day-care or had been boarded within six months prior to serum sample collection.[128] Additionally, stress might increase risk for influenza infection in dogs, as has been observed in other species.[140-142]

---

[*] This chapter was published as a scientific article in the Canadian Veterinary Journal: Pecoraro HL, Lee JS, Achenbach JE, Nelson JR S, Landolt GA. (2012) CVJ 54:1091-1094

Like shelter dogs, sled dogs are housed in close proximity with one another. They also experience additional stressors compared to household dogs, such as travel, prolonged endurance exercise in inclement weather, and exposure to harsh terrain. As such, sled dogs represent a population that might be particularly vulnerable to CIV infection. To determine the seroprevalence of CIV in this population, we sought to estimate the rate of CIV seropositivity in dogs racing at the 2010 Iditarod and to investigate any risk factors associated with CIV infection in racing sled dogs.

MATERIALS AND METHODS

Sample population. We utilized a cross-sectional convenience sampling method for this study. All sled dogs racing in the Iditarod received a pre-race blood draw for a chemistry panel and complete blood count conducted by the laboratory at Providence Alaska Medical Center in Anchorage. Following clinical pathology analysis, laboratory personnel chose ten serum samples from each team for this study. Each of the 71 Iditarod teams was able to enroll up to 24 dogs for pre-race screening. Thus, a total of 1704 dogs were eligible to participate in the current study. The sample size for a seroprevalence estimation of 5% was 70 dogs, assuming a 5% margin of error and a 95% confidence level.[128,143]. Before initiation, this study was reviewed and approved for conduct by the Colorado State University Institutional Animal Care and Use Committee.

Hemagglutination inhibition (HI) assay. Serum antibody titers from all samples were determined by HI assay, as previously described,[128,136] and in accordance with procedures recommended by the World Organization for Animal Health (OIE). Briefly, one volume of serum was incubated at 37ºC for 16-20 hours with three volumes of receptor destroying enzyme (Denka Seiken Co, Tokyo, Japan) prepared from *Vibrio cholera*, which was thereafter deactivated at 56°C for 30-60 minutes. Twenty-five μl of treated serum was diluted with phosphate buffered saline (PBS) across a 96-well plate and mixed with four hemagglutinin units of a CIV isolated in Colorado in 2006 (A/canine/Colorado/148902/2006 [H3N8]). Chicken red blood cells at a 0.5% (v/v) were added to develop the assays. HI antibody titers were determined as the reciprocal of the highest dilution causing complete inhibition of agglutination. Sera were tested in duplicate and each assay included both positive (CIV seropositive canine serum) and negative (PBS) controls. For serum samples collected from dogs reported as CIV-vaccinated, HI assays

were conducted using two additional CIV isolates: A/canine/Colorado/224986/2006 [H3N8] and A/canine/Florida/43/2004 [H3N8] (kindly provided by Dr. P.C. Crawford, University of Florida) to account for any potential antigenic drift. For all samples, seropositivity was defined as a HI titer > 1:8, as previously described in other seroprevalence studies.[128]

Virus neutralization assay. Serum samples collected from dogs that had been reported as having received CIV vaccination were further tested for CIV antibodies by virus microneutralization assay. The assay was performed as previously described with slight modifications.[144] Three serum samples from CIV-infected dogs positive for CIV antibodies on HI assay were included as positive controls, while H3 influenza-negative canine, ferret, and mouse sera served as negative controls. Briefly, receptor destroying enzyme-treated serum samples were diluted across a 96-well plate two-fold in a 50 μl volume of diluent consisting of Dulbecco's modified eagle medium (Sigma-Aldrich, St. Louis, MO), 1% albumin from bovine serum (Sigma-Aldrich), penicillin 100U/ml, and streptomycin 50 μg/ml. The diluted sera were incubated with an equal volume of virus (A/canine/Colorado/224986/2006 [H3N8]) at a titer of 10^2 50% tissue culture infectious dose ($TCID_{50}$) per 50 μl. The 96-well plates were then incubated at 37°C, 5% CO_2 for one hour. Madin-Darby canine kidney cells were added at a concentration of 1.5 x 10^5 cells/ml in diluent with 1 μg/ml of TPCK trypsin from bovine pancreas (Sigma-Aldrich) to all wells of the 96-well plate. The plates were incubated at 37ºC, 5% CO_2 for 20 hours before they were washed with PBS and fixed with 80% acetone in PBS for 10 minutes and allowed to air-dry. Plates were subjected to an ELISA assay to detect the presence of the viral nucleoprotein (NP) utilizing a monoclonal mouse antibody blend (clone A1 and A3) to influenza A NP (Millipore, Billerica, MD) and a secondary antibody of horseradish peroxidase-labeled goat anti-mouse immunoglobulin G (Kirkegaard & Perry, Gaithersburg, MD). The color was developed with 10mg of *o*-phenylenediamine dihydrochloride chromogen (Sigma-Aldrich) dissolved in 20 ml of phosphate citrate buffer with sodium perborate (Sigma-Aldrich). Plates were incubated approximately four to five minutes before the reaction was stopped with 1N sulfuric acid. Plates were read at 490 nm using a Bio-Rad Microplate reader. Results were calculated as previously described.[144] Sera were considered positive for H3 antibody if antibody titers were ≥ 40. Negative sera at the lowest dilution of 1/20 were reported as ≤ 20.

Collection and analysis of team data. Each musher completed a questionnaire for his/her racing team which requested information on the team's medical history, such as any coughing within the last six months and CIV vaccination status, background on the size and the location of the kennel, and travel history. Additionally, questions addressed any potential interaction with other dogs, such as breeding and participation in sled dog competitions. Questionnaires were collected at the time of the team's pre-race screening and a veterinary technician or study author was available to answer any questions. Descriptive statistics were compiled for each team from which serum samples were tested.

RESULTS

Seroprevalence of CIV H3N8 in sled dogs. A total of 419 samples from 42 sled dog teams were tested for the presence of CIV antibodies. However, because there were matching questionnaires for only 40 teams, the final sample size was 399 sled dogs, or 23.4% of the total 1704 dogs screened for the 2010 Iditarod race. Surprisingly, none of the 399 serum samples tested were positive for CIV H3N8 antibodies based on HI assay, including sera from 39 dogs reported as vaccinated for CIV. Indeed, none of the serum samples exhibited hemagglutination inhibition at any of the dilutions tested (starting at the serum-receptor destroying enzyme dilution of 1:4). Seronegativity for the presumably CIV-vaccinated dogs was confirmed by HI assay using two additional CIV isolates to account for antigenic variation, as well as by the more sensitive virus microneutralization assay. Interestingly, medical histories revealed that 10% (4/40) of the teams reported their dogs to be CIV-vaccinated in the months prior to the 2010 Iditarod and 22.5% (9/40) teams indicated that at least one dog on their team had been coughing within 6 months of the race.

Team questionnaire data. As there were no positive samples, risk factor analysis for CIV in sledding dogs was not possible. However, descriptive statistics were compiled to characterize the social interactions between dogs and any points of contact among teams. The sled dogs in this study originated from eight states (Alaska, Colorado, Idaho, Minnesota, Michigan, Montana, Washington, and Wyoming) and three Canadian provinces (British Columbia, Saskatchewan, and Ontario). Additionally, 52.5% (21/40) of sled dog teams travelled outside their home state or province within 6 months of the 2010 Iditarod, including to states where CIV has been detected (Colorado, Washington, and Wyoming). The

majority of sled dogs (62.5%; 25/40 teams) were housed with 50 dogs or more, and 87.5% of teams (35/40) were kenneled at only one location. Housing of sled dogs ranged from teams that allowed free roaming access inside the musher's home, to teams that separated their dogs in designated crates within an enclosed building, to teams that housed sled dogs outdoors with dogs tethered next to individual housing structures according to guidelines established by Mush with P.R.I.D.E. (http://www.mushwithpride.org). During travel to racing competitions, dog confinement also varied widely. While many teams transported their teams in enclosed trailers, with internal dog crates facing one another, other teams' traveling systems consisted of trailers with individual compartments facing outward to the open air, completely separating dogs from one another. Breeding, another potential point of direct contact among dogs, was reported in 35% (14/40) of teams, although the questionnaire did not specify whether breeding was within the teams' own kennel or with dogs from an outside kennel. Interestingly, at least three 2010 Iditarod teams were known to include dogs acquired from humane shelters (from Alaska, Montana, and the Yukon Territory). Finally, all teams tethered dogs to a common chain (or cable run) before, during, and after competitions, allowing for additional nose-to-nose contact among team dogs.

Team-to-team interactions. Contact among different sled dog teams primarily consisted of any breeding between dogs from separate kennels (information was not requested on the questionnaire, though it was related to the authors during blood collection), acquisition of racing dogs from other sled dog teams (also reported during blood collection), and any interactions among dogs during racing competitions. As each team must qualify to race at the Iditarod, there is a minimum of two interactions with other sled dog teams a year (the qualifying and the Iditarod races). Sled dog teams typically were separated before the start of racing competitions and at checkpoints during races, although contact among teams potentially occurred along racing trails. In the year prior to the Iditarod, 52.5% of sled dog teams (21/40) took part in at least three long distance race competitions where other sled dog teams were present. In total, there were 27 races in Canada and the United States in which the racing sled teams from this study participated.

DISCUSSION

The results of this study demonstrate that none of the sled dogs sampled had been recently exposed to CIV and subsequently produced detectable antibodies to the virus. This finding is surprising given the fact that several of the sled dog teams that were tested are living in large, shared housing, reside in and travel to states where CIV infections have been reported (i.e., California, Colorado, Oregon, Utah, Washington, Wisconsin, and Wyoming),[114] frequently compete in races where they come into contact with other sled dog teams, and include team members that have been adopted from humane shelters. Several recent studies conducted in Ontario, Iowa, Colorado, and Pennsylvania indicate that CIV seroprevalence in non-shelter pet dogs without a history of respiratory disease is low [0.4%,[132] 0.5%,[134] 3.0%,[130] and 3.6%,[128] respectively], compared to 42% seropositivity in the Philadelphia humane shelter study that tested sera from dogs with and without clinical signs of respiratory disease. Therefore, CIV seroprevalence in sled dogs appears to be closer to that found in household dogs rather than to the higher level of seropositivity seen in shelter dogs.

It is clear that sled dogs come into direct contact with one another (both intra- and inter-team), as well as in indirect contact (sharing of leashes, crates, etc.). Thus, it is not understood why exactly no CIV positive dogs were detected. As the majority of sled dogs included in this study originated from states and provinces where CIV infection has yet to be reported,[114] geographical location cannot be discounted. In spite of this limitation, CIV has been repeatedly detected in states where sled dogs that were included in this study live, train, travel, and race (such as Colorado and Wyoming). One important difference may be that, unlike shelter dogs, many sled dogs are confined outdoors where they are tethered next to separate housing structures. While favorable environmental temperatures (i.e., 5ºC)[145] exist for influenza virus survival in all the states where the tested sled dogs reside, other factors such as exposure to open air and solar radiation might account for the lack of CIV infection in the sledding dogs tested in this study. More ecological studies are needed to elucidate the CIV transmission dynamics within different environmental settings, although it is well-known that in open-air environments, viruses and other microorganisms are susceptible to inactivation by a multitude of reactive chemicals.[146,147]

Another interesting finding is the fact that all the dogs reported by the mushers to be vaccinated for CIV also tested negative for CIV antibodies. These results were consistently negative even when

employing three different viruses as antigens to account for antigenic drift and two different serological assays (HI and virus microneutralization assays). It is important to note that it is currently unknown how long CIV antibodies persist in individual dogs following natural exposure or vaccination to CIV, and, therefore, any of the sled dogs tested in this study would have no detectable antibody if CIV antibodies are indeed short-lived. Despite this caveat, however, the finding that none of the reportedly CIV-vaccinated dogs were CIV seropositive is unexpected and may be explained by several possibilities. Firstly, perhaps these dogs were not actually vaccinated for CIV. Although teams reported vaccinating their dogs in the months prior to the 2010 Iditarod race, confusion regarding whether CIV is included in a standard vaccination regimen is not uncommon. Secondly, it is possible that these dogs did not receive the recommended second booster dose or received it within days of the pre-screening blood draws when antibodies might not yet be present (dates of vaccination were not reported). During a recent CIV vaccine study, there was an average 2.5-fold geometric mean HI antibody titer difference after initial CIV vaccination compared to after the second CIV booster.[111] Clearly, the second CIV booster induces a stronger antibody response than receiving only one CIV vaccine. Thirdly, as sled dogs are the equivalent of ultra or elite marathon runners, extreme exercise in these animals might lead to catabolism of endogenous protein (such as globulins) for energy, which could cause a decrease in overall serum antibody concentration. This notion is supported by the finding that Alaskan sled dogs exposed to prolonged endurance exercise demonstrated significantly decreased levels of protein concentrations in serum after the onset of long-distance racing compared to serum levels before the race.[148] Therefore, it would be worth investigating differences in CIV-induced immunity among dogs of various athletic conditioning. Finally, it is possible that vaccination, or even past infection, did not induce an antibody response detectable by HI or microneutralization assay. Although the HI assay is considered the test of choice for virus surveillance and serological diagnosis of influenza A virus infections in animals,[116,149] it is not the most sensitive assay for detecting influenza antibodies.[150] While microneutralization assays are more sensitive than HI assays, the single radial hemolysis assay is still judged to be the most sensitive of the three serological assays.[150] Future seroprevalence studies might consider using this last method if known vaccinates consistently test negative using the other two serological methods utilized in this study.

In conclusion, this study found no CIV antibodies in serum samples collected from the racing sled dogs tested, despite shared housing among dogs, travel to states were CIV has been reported, and contact with dogs from humane shelters. However, given the potential for introduction of CIV into the sled dog population, continued studies are warranted to assess CIV infection among racing sled dogs and to further evaluate the ecology of CIV transmission and vaccine efficacy within this particular population.

CHAPTER SIX: EPIDEMIOLOGY AND ECOLOGY OF CANINE INFLUENZA VIRUS H3N8 IN U.S. SHELTER DOGS

INTRODUCTION

Canine influenza virus (CIV) (H3N8) was first detected in Florida racing greyhounds in 2004 and quickly spread into New York and Florida shelter dog populations.[7] The transmission of influenza A viruses is primarily thought to be by direct or indirect contact, deposition of infectious droplets (≥ 5 μm) on nasal or oral mucosa, or inhalation of infectious particles (< 5 μm).[139] Recent CIV seroprevalence studies suggest that there is an increased risk of CIV infection for dogs housed in closed-air communal environments,[128] where promotion of efficient virus transmission by any of the aforementioned routes of infection is possible. Indeed, humane shelters have consistently reported high rates of CIV in dogs exhibiting signs of canine infectious respiratory disease or CIRD. In a New York and Florida humane shelter study conducted shortly after CIV emergence, up to 97% of dogs were seropositive for CIV.[7] Similarly, dogs from humane shelters in Colorado were infected with CIV in five out of five humane shelters reporting CIRD outbreaks in 2006 and 2007.[131] More recently, in Pennsylvania, 42% of dogs with and without clinical signs of CIRD were CIV seropositive.[112]

Besides respiratory illness signaled by fever (>103°F), nasal/ocular discharge, non-productive, persistent cough, anorexia, lethargy, and weight loss,[104] CIV infection in dogs might account for significant delays between intake into a shelter and discharge.[151] In turn, increased length of stay in a humane shelter further impacts a pet's chance for adoption, as the longer an animal remains in a facility, the less socialized the pet might become and the greater the risk of pathogen transmission.[152] Considering the threat CIV poses to canine health and adoptability, persistence of the virus within humane shelters is worrisome, especially as CIV transmission dynamics are virtually unknown. Studies conducted in non-shelter dog populations find very little CIV seropositivity in household, racing sled, and flyball tournament dogs,[128,130,153] suggesting CIV infection to be relatively lower in dogs from the non-shelter community. In contrast, CIV is routinely detected in humane shelters throughout the U.S., especially in Colorado and New York, where surveillance studies have been recently performed.[106,108,131] The discrepancy between CIV in community versus shelter dogs leads to more questions than answers. Particularly, what role does

dogs entering a facility play in CIV introduction? What factors contribute to the establishment of CIV within humane shelters once the virus has been introduced? Furthermore, and perhaps the most significant biological question, are humane shelters centers for CIV amplification? Given these questions and the unknown transmission dynamics of CIV, greater knowledge of true CIV prevalence in shelter dogs, as well as a greater understanding of the epidemiological and ecological factors contributing to CIV persistence within humane shelters, would help shelter veterinarians and personnel identify strategies for CIV prevention. For this purpose, we sought to compare CIV shedding and seroprevalence in shelter dogs upon shelter admission and discharge and to identify any correlates of CIV infection in U.S. humane shelters.

METHODS AND MATERIALS

Animals. Prior to initiation, all studies described were reviewed and approved for conduct by the Colorado State University Institutional Animal Care and Use Committee. For these studies, we utilized a prospective convenience sampling method. The number of nasal swabs and serum samples required for estimation of infection rates and seroprevalence was 4680[143] among six participating shelters: Sacramento, CA (SSPCA); Pikes Peak Region, CO (HSPPR); Tampa Bay, FL (HSTB); New York, NY (ASPCA); Charlestown, SC (CAS); and Austin, TX (TLAC). Shelters were chosen based on expected CIV rates (e.g., CA, TX, and SC had relatively lower expected CIV compared to CO, FL, and NY). To meet this goal, each shelter collected blood and nasal swab samples from 20 admitted and 20 discharged dogs each month. Generally, sampling would be performed on designated day(s) and collection occurred sequentially from every dog admitted to and discharged from the humane shelter until the monthly goal was met. All dogs, irrespective of age, breed, sex, and vaccination or health status were included, unless the primary caretaker determined that blood collection would be unsafe for the dog. Discharged dogs were dogs that had resided at the shelter for at least seven days and included dogs that were adopted out into the community, as well as dogs that were scheduled by the humane shelter for euthanasia. Between December 2009 and January 2012, over 5160 samples were collected from the six shelters.

Clinical samples. Matching nasal swab and serum samples were collected from shelter dogs (although occasional single blood or nasal swab samples were also processed). Briefly, a sterile

polyester-tipped swab was inserted deep into one of the nostrils and rotated to collect mucus. The swab samples were immediately placed in 1 ml of viral transport medium (phosphate buffered saline, 0.5% bovine serum albumin, 2,000 U/ml potassium penicillin G, 4 mg/ml streptomycin, 16 µg/ml gentamicin, and 100 U/ml nystatin) and stored at 4°C. Additionally, ~2ml of blood were drawn from either the cephalic, saphenous, or jugular veins, placed in an additive-free tube, and stored at 4°C. Once all samples were collected for the month, they were shipped to the laboratory at Colorado State University on ice overnight. At the laboratory, samples were immediately processed for further analysis.

Hemagglutination inhibition (HI) assay. In accordance with procedures recommended by the World Organization for Animal Health (OIE) and from previous studies,[128,136] sera from blood samples were used to determine CIV H3N8 antibody titers by HI assay. Briefly, one volume of serum was incubated at 37ºC for 20 hours with three volumes of *Vibrio cholera* receptor destroying enzyme (Denka Seiken Co; Tokyo, Japan). After a deactivation period at 56°C for 60 minutes, 25 µl of treated serum was diluted in duplicate with phosphate buffered saline across a 96-well plate. Four hemagglutinin units of A/canine/CO/224986/2006 (H3N8) were incubated with the diluted sera for 45-60 minutes. To develop the assays, 0.5% (v/v) chicken red blood cells were added to each well and allowed to agglutinate for 25-30 minutes. HI antibody titers were determined as the reciprocal of the highest dilution causing complete agglutination inhibition. Seropositivity was defined as a HI antibody titer of equal to or greater than 1:16 in accordance with the OIE.

Real time reverse transcriptase-polymerase chain reaction (rRT-PCR). Viral ribonucleic acid was extracted from 200 µl of viral transport medium and eluted in a final volume of 60 µl RNase-free water using an automated ribonucleic acid isolation system (Qiagen Qiaxtractor; Hilden, Germany) before storage at -80°C. The one-step rRT-PCR assay was performed as previously described.[131] Briefly, a 5 µl-aliquot of RNA template was mixed with 20 µl of mixture containing iScript One-Step RT-PCR Kit reagents (BioRad; Hercules, CA), 200 nmol each of the forward (5' GAA CAC CGA TCT TGA GGC ACT C 3') and reverse (5' GGC ATT TTG GAC AAA GCG TCT AC 3') primers to amplify 144bp of the influenza virus matrix gene, and 80 nmol of probe. Water and viral transport medium were used as negative controls, while the positive control consisted of 10 TCID50 of A/canine/CO/224986/2006 (H3N8). For consistency, the epMotion Ep5070p (Eppendorf; Hamburg, Germany) automated system was used to

load 96 well plates before amplification and detection by Mastercycler Realplex (Eppendorf) using previously described conditions.[131]

Prevalence estimates. True prevalence was estimated by stochastic latent class analysis, as described by Branscum et al.[126] using Gibbs sampling,[127] in WinBUGS statistical software.[129] Assumptions for this method include constant sensitivity and specificity of the diagnostic test used, as well as only one population sampled. For prior distributions, the rRT-PCR mode was set at 0.96 for sensitivity and 0.74 for specificity at a 95% probability interval, based on previous studies.[121,131] As each shelter is located in a distinct geographical region constituting a unique population, true prevalence was determined separately for each shelter.

Model development and analysis. Logistic regression was used to evaluate possible risk factors for CIV shedding and seropositivity as indicated by positive nasal swabs by rRT-PCR and HI assay, respectively. As temporal, spatial, and seasonal patterns have been observed with human influenza A virus,[154,155] variables of interest included number of days in the shelter before sample was taken, the geographical region of the shelter, the study year, and the sampling month. Other data collected for analysis included the type of sample (admission/discharge), whether the dog received a CIV vaccination upon admission, whether the shelter quarantined incoming dogs for over seven days, whether the shelter accepted dogs transferred from out of state, whether the shelter isolated apparently sick dogs, whether the shelter adhered to an all-in/all-out room protocol, whether the dogs were allowed to co-mingle or were ever housed with one-another, and the size of the shelter.

As the emerging consensus is that CIV is being propagated in endemic regions due to repeated introduction of CIV-infected dogs into humane shelters and subsequent dog-to-dog transmission within the facility,[108] we proposed two sets of hypotheses to explain CIV risk in shelter dogs. The first model set included 14 a priori models (including an intercept-only model as a model of no effect) to assess the associations between CIV and region and time. Recent research suggests that increased length of stay within a shelter is related to CIV seropositivity within a metropolitan humane shelter,[112] while, regionally, CIV has been consistently isolated from shelter dogs in Colorado, New York, and Florida.[106,108,131] Furthermore, Hayword et al.[108] reported genetic distinction of CIV isolates from humane shelters located within the same city. Proposed hypotheses for explaining CIV shedding and seropositivity status within

the dataset included geographical region (west=1, east=2, southeast U.S.=3), vaccination status (no=0, yes=1), days in the shelter before sample collection (continuous variable), year of the study (year 1=1, year 2=2), sampling month (1-12, corresponding to month of year), as well as additive effects and interactions among these variables. The second model set consisted of 9 a priori models (including an intercept-only model) used to assess associations between CIV and within-shelter dog-interactions, as previous studies have suggested CIV transmission among dogs to be due to direct contact.[113,115] Proposed hypotheses included co-mingling/co-housing (no=0, yes=1), days in the shelter (continuous), and sampling month (1-12), plus additive effects and interactions between co-mingling/co-housing and days and month. The specific shelter itself was not considered in any of the models to avoid collinearity with other explanatory variables. Additionally, there was lack of variability among shelters for several of the factors of interest, including the length of time each dog was quarantined upon admission, shelter size, isolation protocols, and other shelter practices. We, therefore, did not evaluate the effect these variables on CIV outcomes. As the Colorado and New York shelters were the only shelters within their respective regions with positive results, the Florida and South Carolina shelters were used to represent the southeast region for the within-shelter dog-interaction models.

All together, four model sets were analyzed by logistic regression using ProcLOGISTIC in SAS 9.3:[156] (1) temporal-spatial effects on CIV-shedding, (2) temporal-spatial effects on CIV-seropositivity, (3) within-shelter dog-interaction effect on CIV-shedding, and (4) within-shelter dog-interaction effect on CIV-seropositivity. Logistic regression models in all model sets were estimated from the second order Akaike information criterion (AICc),[157,158] calculated using the following equation:

$$AICc = [-2(\text{log-likelihood}) + 2K] + [2K(K+1)/n-K-1],$$

where *K* represents the number of estimators and *n* the number of samples in the model. After calculation of the AICc estimates, model selection and multi-model inference were performed as described below.

Model selection and multi-model inference. We used an information-theoretic approach[159] using AIC[160,161] for model selection and multi-model inference. AICc values were ranked low to high and the model with the lowest AICc was considered to have the best strength of evidence. Each candidate model (*i*) was compared and weighted (ω) to estimate the probability that the top-ranking model was the best model given the dataset. Additionally, parameter estimates were averaged across all models for

variables in the top models to calculate the parameter estimates ($\beta[\simeq]$), unconditional standard errors (SE), and 95% confidence intervals (CI).[159] Finally, to explain model variance in the dataset, we include the re-scaled R-square from each of the models analyzed by logistic regression.[156]

RESULTS

Geographic distribution. There were a total number of 5161 serum and 5182 nasal swab samples from the six humane shelters (Table 6-1). Samples per shelter ranged from ~700 (South Carolina) to ~1000 (Colorado) and all shelters, except California and Texas, submitted more intake samples than discharge samples. As expected, the New York and Colorado shelters had the highest numbers of both CIV seropositivity and nasal shedding (Figure 6-1). The Florida humane shelter, however, had much lower than expected CIV nasal shedding, given the fact that CIV first emerged in the state and previous Florida shelter studies showed relatively higher prevalence.[7] On the other hand, the humane shelter in South Carolina had more than expected CIV nasal shedding, which can be attributed to a CIV outbreak during the first months of sample collection. After these initial CIV positive nasal swabs, only two CIV positive serum samples were collected from South Carolina throughout the remainder of the study. Both California and Texas had no

Table 6-1. Number of tested nasal and serum samples from admitted and discharged shelter dogs, Dec 2009 to Jan 2012.

Shelter	Nasal swabs		Serum samples	
	Admitted	Discharged	Admitted	Discharged
CA	411	443	420	442
CO	573	462	564	460
FL	575	376	574	374
NY	498	368	489	363
SC	419	288	419	287
TX	299	470	298	471
OVERALL	2775	2407	2764	2397

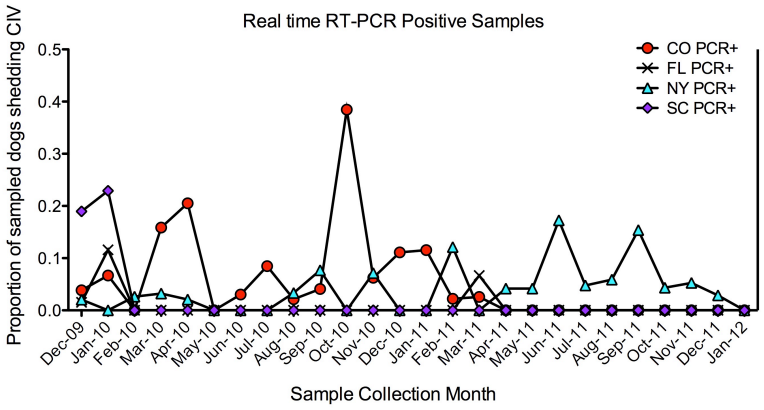


Figure 6-1. Proportion of dogs sampled each month of the study that were shedding CIV detected by real time RT-PCR (PCR+).

Table 6-2. True prevalence median probability estimates (95% probability intervals) based on the proportion of CIV shedding in U.S. shelter dogs.

Shelter	Crude prevalence probability estimate	True prevalence probability estimate
CA	0.001	0.002 (0.0-0.009)
CO	0.047	0.027 (0.020-0.085)
FL	0.013	0.007 (0.0-0.027)
NY	0.044	0.025 (0.020-0.082)
SC	0.032	0.019 (0.010-0.062)
TX	0.000	0.001 (0.0-0.007)

CIV seropositive samples and California had only one dog shedding CIV upon admission into the shelter (April 2010) throughout the entire study. True prevalence estimates (Table 6-2) corroborate other reports suggesting CIV is endemic in Colorado and New York[106,108,131] with prevalence estimates of 2.7% and 2.5% for these shelters, respectively. The CIV positive nasal swabs collected from South Carolina in the first two months of the study may explain the higher CIV shedding prevalence estimates for the South Carolina shelter (1.9%) compared to the Florida shelter (0.7%). The two shelters with one or no CIV shedding dogs (Texas and California) had the lowest estimates for CIV prevalence (0.12-0.16%).

Community-shelter dog transmission. Differences between intake and discharge CIV shedding rates depended on the humane shelter sampled (Figure 6-2). For Colorado shelter dogs, there were six times more dogs (8.9%) leaving the facility shedding CIV than dogs that entered (1.4%). In contrast, New York dogs were more likely to be shedding virus upon admittance (6.9%) rather than upon discharge (1.1%). In the other two shelters (Florida and South Carolina) where CIV was detected in nasal swab samples, intake and discharge dogs had similar rates of shedding (ranging from 1.2% to 3.8%). In total, 66/2768 (2.4%) dogs shedding CIV were admitted into the shelters and 57/2418 (2.4%) shelter dogs were discharged while shedding the virus. These numbers included dogs from all four positive shelters, plus the one positive dog at the California shelter. There were a total of 92 seropositive dogs upon admission (of 2765 – 3.3%) and 97 dogs (of 2408 – 4.0%) upon discharge. Three hundred twenty five dogs were known vaccinates, and received at least one dose of the CIV vaccine upon admittance into the shelter. Of these,

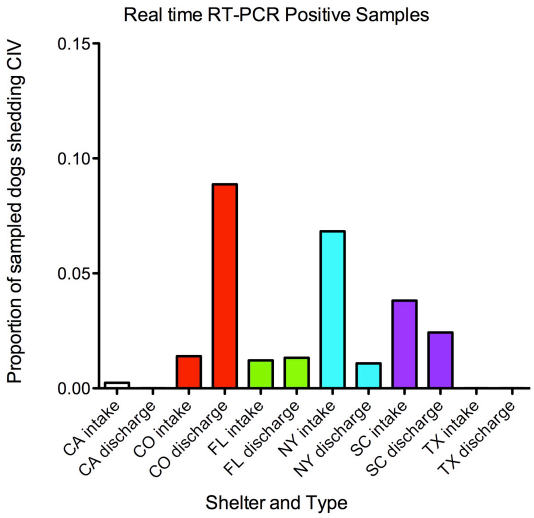


Figure 6-2: Proportion of sampled admitted and discharged dogs in U.S. humane shelters.

125 were sampled on discharged. Interestingly, only five of the 125 vaccinated discharged dogs were seropositive (three dogs that received two doses and two dogs that received only one vaccine dose). Additionally, 51 dogs received both the initial CIV vaccination and a booster 7-14 days later while residing in the shelter. All but one of these were seronegative upon discharge, while three of these were shedding CIV upon discharge.

Risk factor analysis. After excluding dogs from the California and Texas shelters, any samples with missing information, and 148 duplicate samples (dogs that had been sampled both on intake and discharge), there were a total of 3407 nasal swab and 3385 serum samples for the temporal-spatial analyses. There were 1608 nasal swab and 1604 serum samples from the Southeast shelters. For the first model set, temporal-spatial effects contributing to CIV shedding, the year x region interaction was ranked the highest and, thus, appears to have the best strength of evidence with a model weight (ωi) of 0.93 for explaining nasal viral shedding in shelter dogs from 2009-2012 (Table 6-3). This model indicates CIV shedding was highly associated with both the region where the dog resided and the year of sampling. The model-averaged estimate for the effect of year suggests shedding was more likely during the first

Table 6-3. Candidate model set and ranking of models examining temporal-spatial factors contributing to CIV-shedding in U.S. shelter dogs.

Model (i)	R^2	log(L)	K	AICc	Δ_i	ω_i
year*region	0.04	-480.18	4	968.37	0.00	0.93
vax+region+days+year+month	0.04	-481.30	5	974.62	6.25	0.04
vax+region+days+year	0.04	-482.80	5	975.61	7.23	0.03
vax+region+days	0.03	-487.53	4	983.08	14.70	0.00
year+month	0.02	-490.40	3	986.80	18.43	0.00
year	0.02	-491.49	2	986.99	18.62	0.00
vax*days	0.02	-490.01	4	988.03	19.66	0.00
days+region	0.02	-492.43	3	990.87	22.50	0.00
region	0.01	-494.95	2	993.90	25.52	0.00
vax	0.01	-494.96	2	993.92	25.55	0.00
month+region	0.01	-493.98	3	993.97	25.60	0.00
days	0.01	-496.03	2	996.06	27.68	0.00
Intercept-only	--	-499.37	1	1000.75	32.37	0.00
month	0.00	-498.45	2	1000.90	32.53	0.00

R^2: Adjusted R^2
log(L): Log likelihood
K: Number of parameters
AICc: Second order Akaike information criterion
Δ_i: Difference between model and best model
ω_i: Weight of model

year of the study (β[≃]=0.55, unconditional SE=0.06, 95% CI=0.43, 0.66), while for region there was more shedding in the eastern shelters as a whole compared to the Colorado shelter (β[≃]=0.07, unconditional SE=0.67, 95% CI=-1.38, 1.24). The next best model (i.e., next highest ranked model) for CIV nasal shedding included all of the variables of interest (vaccination status, region, days in shelter, year of study, and month) and had a ω*i* of 0.04. It is important to note that for both of these models, the adjusted R-square[162] value is low (0.04). For CIV-seropositivity in U.S. shelter dogs, the highest-ranking model contained vaccination status (β[≃]=0.04, unconditional SE=0.13, 95% CI=-0.21, 0.28), region (β[≃]=-0.73, unconditional SE=0.53, 95% CI=-1.76, 0.30), days in shelter (β[≃]=0.02, unconditional SE=0.01, 95% CI=0.01, 0.04; Figure 6-3), and year of study (β[≃]=0.11, unconditional SE= 0.09, 95% CI=-0.07, 0.28), and had the best strength of evidence with a ω*i* of 0.42 (Table 6-4).

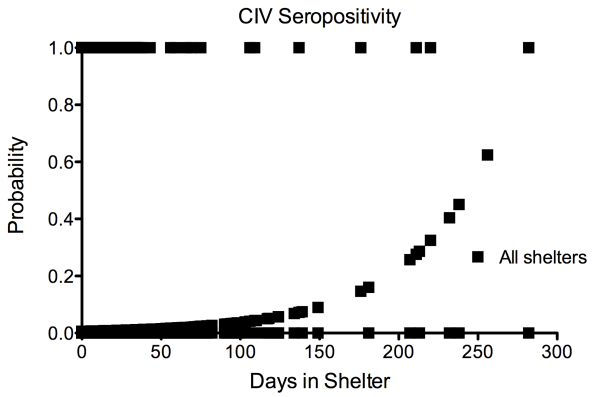


Figure 6-3. Probability of seropositivity increased with increased length of stay within humane shelters.

Table 6-4. Candidate model set and ranking of models examining temporal-spatial factors contributing to CIV-seropositivity in U.S. shelter dogs.

Model (*i*)	R^2	log(*L*)	*K*	AICc	Δ_i	ω_i
vax+region+days+year	0.09	-652.05	5	1314.12	0.00	0.42
days+region	0.09	-654.62	3	1315.25	1.13	0.24
vax+region+days+year+month	0.09	-651.77	6	1315.57	1.45	0.20
vax+region+days	0.09	-654.09	4	1316.20	2.08	0.15
year*region	0.00	-669.36	4	1346.73	32.61	0.00
region	0.06	-672.29	2	1348.58	34.46	0.00
month+region	0.06	-672.02	3	1350.05	35.93	0.00
vax*days	0.02	-694.81	4	1393.64	79.52	0.00
days	0.02	-695.75	2	1395.51	81.39	0.00
year	0.00	-703.92	2	1411.84	97.72	0.00
year+month	0.00	-703.68	3	1413.37	99.25	0.00
Intercept-only	--	-706.15	1	1414.30	100.18	0.00
vax	0.00	-705.79	2	1415.59	101.47	0.00
month	0.00	-705.86	2	1415.73	101.61	0.00

Table 6-5. Candidate model set and ranking of models examining within shelter dog interactions contributing to CIV-shedding in U.S. shelter dogs.

Model (*i*)	R^2	log(*L*)	*K*	AICc	Δ_i	ω_i
month*cohm	0.0700	-154.27	4	316.55	0.00	0.92
days+month+cohm	0.0484	-157.52	4	323.06	6.50	0.04
cohm+month	0.0396	-158.83	3	323.67	7.12	0.03
days+cohm	0.0320	-159.98	3	325.97	9.42	0.01
cohm	0.0235	-161.24	2	326.49	9.94	0.01
days*cohm	0.0322	-159.95	4	327.92	11.37	0.00
month	0.0133	-162.77	2	329.55	12.99	0.00
days	0.0124	-162.91	2	329.82	13.27	0.00
Intercept-only	--	-164.76	1	331.51	14.96	0.00

Table 6-6. Candidate model set and ranking of models examining within shelter dog interactions contributing to CIV-seropositivity in U.S. shelter dogs.

Model (*i*)	R^2	log(*L*)	*K*	AICc	Δ_i	ω_i
days	0.1039	-40.47	2	84.94	0.00	0.53
days+cohm	0.1094	-40.22	3	86.46	1.52	0.25
days+month+cohm	0.1103	-40.18	4	88.38	3.45	0.10
days*cohm	0.1094	-40.22	4	88.47	3.53	0.09
Intercept-only	--	-45.03	1	92.05	7.12	0.02
month	0.0022	-44.93	2	93.86	8.93	0.01
cohm	0.0000	-45.03	2	94.06	9.12	0.01
cohm+month	0.0022	-44.93	3	95.87	10.93	0.00
month*cohm	0.0043	-44.84	4	97.70	12.76	0.00

R^2: Adjusted R^2
log(*L*): Log likelihood
K: Number of parameters
AICc: Second order Akaike information criterion
Δ_i: Difference between model and best model
ω_i: Weight of model

In this analysis, the top four models explain 95% of the variation (i.e., the top four ωi's have a sum of ≥95%), and, again include a combination of the variables from the highest-ranked model. The last two model sets examining the effects of within-shelter dog-interactions on CIV -shedding and -seropositivity, exhibited similar patterns. In the analysis for CIV-shedding, the month x co-mingling/co-housing interaction was the highest-ranking model with a model weight (ωi) of 0.92 and had an R-square of 0.07 (Table 6-5). The model-averaged estimate for the effect of co-mingling/co-housing suggests shedding was more likely in shelters that allowed dogs to interact ($\beta[\simeq]$=0.25, unconditional SE=0.34, 95% CI=-0.42, 0.92) and during certain months of the year ($\beta[\simeq]$=-0.20, unconditional SE=0.08, 95% CI=-0.35, -0.04). The CIV seropositivity model set had slightly higher R-square values (Table 6-6). Here, four models contributed to 95% of the variation, and included an additive or interactive combination of days in

shelter ($\beta[\simeq]$=0.05, unconditional SE=0.03, 95% CI=-0.02, 0.11), co-mingling/co-housing ($\beta[\simeq]$=-0.04, unconditional SE=0.27, 95% CI=-0.58, 0.49), and month ($\beta[\simeq]$= 0.00, unconditional SE=0.01, 95% CI=-0.02, 0.02). However, the results of the within-shelter dog-interaction model sets should be interpreted cautiously, as the total numbers of CIV-shedding and -seropositive dogs were only 34 and 7, respectively.

DISCUSSION

CIV continues to be a threat to the health and welfare of shelter dogs. The studies described here are among the first to evaluate the CIV status of community dogs entering humane shelters and shelter dogs being discharged back out into the community. It is clear that CIV is endemic in the areas where the Colorado and New York humane shelters are located, namely, the greater Colorado Springs and New York City regions.[106,108,131] What is less apparent is to what extent CIV affects the other regions in our study. Despite not detecting CIV from any of the dogs sampled at the Texas shelter, there were PCR-confirmed CIV cases in Texas near the end of the sampling period.[114] Interestingly, CIV was last confirmed in Florida dogs by the Cornell Diagnostic Laboratory in 2008,[106,114] while a CIV isolate was sequenced from a dog in California in 2006.[106] Therefore, despite the results from this study, whether negative in the cases of California and Texas or positive in the case of Florida, no region should be considered entirely safe from the threat of a potential CIV epidemic. The true prevalence estimates reflect this as well, as none of the shelters were completely CIV-free.

The intake and discharge data from each shelter suggest that the transmission dynamics between the community and shelter dog populations vary among individual shelters. Clearly, CIV is entering humane shelters from community dogs; all of the shelters, except Texas, had at least one intake dog that was shedding CIV. Similarly, the shelters with consistently positive nasal swabs released CIV shedding dogs back into the community. It follows that humane shelters likely facilitate the amplification of CIV, as has been suggested in other studies examining enclosed-environments.[128] As such, the need for an effective vaccine becomes undeniable. However, due to the fact that only 325 dogs in the study received one or more CIV vaccinations, evaluating the effect the vaccine had on the shelter dog population is difficult. It is interesting that only one of the twice-vaccinated dogs was seropositive upon

discharge from the shelter and three discharged dogs were shedding CIV after receiving two CIV vaccine doses. The negative HI titers might be due to early sampling before CIV antibodies are detectable. Furthermore, two of the three vaccinated dogs shedding CIV upon discharge might have shed less CIV than if they had not been vaccinated, as the virus detected in their nasal swab samples was close to the negative threshold point (1000 matrix gene copies) for rRT-PCR. However, the date these dogs were infected is unknown, as is when during the course of CIV-infection they were sampled. It is also important to note that the best candidate models containing vaccine variables were in combination with other temporal-spatial variables. The models with only vaccine status have very little evidence of effect for our datasets. This suggests that receiving the vaccine alone is not associated with increased or decreased risk of CIV. Therefore, especially as dogs may not reside in the shelter long enough to receive the recommended CIV vaccine booster and as CIV isolates are known to cause subclinical infection in experimental dogs,[115] further research on the potential of the CIV vaccine to reduce CIV infection in shelters is warranted.

In dogs that were shedding CIV, several risk factors have been identified. For CIV-shedding in U.S. shelters, there was evidence suggesting CIV depends on space (region) and time (year). From our data, most of the positive Colorado nasal swabs were collected during the first year of the study, before detectable shedding tapered off by early study year two. The Colorado shelter sampled 90% (44/49) of their CIV-shedding dogs during the first year and submitted the last positive nasal swab March 2011. The opposite is true for the New York shelter. Few shedding dogs were sampled in study year one, while positive swabs gradually increased over time (in fact, 25 of the 36 total positive samples for New York were collected after January 2011). The South Carolina and Florida shelters show a similar pattern as well. South Carolina submitted all of their positive nasal swab samples during a very short period in study year one (December 2009 through January 2010), while the majority (9/12) of Florida's positive swab samples were collected during those same two months.

Spatio-temporal patterns for influenza A viruses are not new,[154,155,163] and true prevalence rates estimated over the entire study period might not accurately reflect the trends seen in the data. It appears that CIV, instead of being a pathogen persisting at low levels in endemic shelters, actually falls more in line with an ebb and flow pattern – where outbreaks occur at one shelter in one region and wane before

CIV emerges in an entirely different shelter in another region. Such a pattern also takes into account the movement of infected dogs along a network, as seen during the emergence of CIV,[7] as well as the co-mingling/co-housing x month effect seen in the within-shelter dog-interaction models. During months when CIV-shedding was detected in the Southeast region, separating dogs appeared to decrease CIV-infection. This is also reflected by the odds ratio: no co-mingling/co-housing during months of CIV-infection reduced risk by 33% (OR 0.67; 95% Confidence Interval [CI] 0.51, 0.89).

CIV-seropositivity was also associated with the additive effects of space (region) and time (year and month), as well as vaccine status and number of days spent in the shelter. However, odds ratios from the multivariable model for the last two factors suggest that, individually, they neither increase nor decrease CIV-seropositivity: vaccination (OR 1.00, 95% CI 0.50, 2.00) and days (OR 1.02, 95% CI 1.01, 1.02). Days in shelter is particularly interesting, as it has been previously associated with CIV in shelter dogs.[112] However, when the dataset is narrowed to evaluate the within-shelter dog-interaction effect, days in the shelter before sampling actually becomes the common variable among the best models. Thus, in shelters where dogs are allowed to interact for longer periods of time, the more risk they have of becoming CIV-seropositive. As CIV antibodies in experimentally-infected and sentinel dogs have been noted between days 7-12 post-challenge,[115] a length of shelter stay over 7 days allows time for seroconversion. This might account for some of the seropositive discharged dogs, especially in the New York shelter where dogs infected upon intake would have seroconverted if they were in the shelter for seven days or longer.

Although several risk factors identified here have been reported elsewhere,[112,113] other variables not included in these models are likely contributors to CIV within shelters. The low R-square values suggest that some other factors are in play. A number of variables could not be modeled in our analysis, including quarantine, isolation, and all-in/all-out practices, as well as the effect of accepting dogs transferred from out of state and size of the shelter. For example, New York was the only shelter that quarantined their animals for seven days or more. Coincidentally, New York was also the only shelter that saw a six-fold decrease in CIV-positive dogs upon discharge compared to intake. It should also be noted that New York was the smallest shelter in the study (750-800 annual dog intake). On-the-other-hand, Colorado, which was one of the largest participating shelters (11,000 annual dog intake), had only a 24-

48 hour quarantine in place during the study period and had a six-fold increase of CIV-positive dogs upon discharge compared to intake. The effects of size and quarantine could not be modeled here due to lack of intra- and inter-shelter variability. However, these possible effects should be taken into account during the research design process of any future studies.

In conclusion, our results suggest that community dogs are a source of CIV introduction into humane shelters and that, once CIV has become established, dog-to-dog transmission maintains CIV within the shelter for a short-period of time (1-9 months), as evidenced by CIV-positive discharged dogs from the shelters sampled in our study. As a disease of space and time, modeling could be helpful for predicting future CIV outbreaks; however, other risk factors are still in need of investigation. Ultimately, it is hoped that this report will be used to begin the process of identifying preventative and control measures for CIV in humane shelters, and, thus, reduce risk of CIV within one of the most vulnerable dog populations.

CHAPTER SEVEN: GENOME EVOLUTION AND ANTIGENIC VARIATION OF CANINE INFLUENZA VIRUSES H3N8 IN DOGS

INTRODUCTION

Although serological evidence suggests canine influenza virus (CIV) (H3N8) has been circulating in U.S. dogs since as early as 1999,[138] it was not until 2004 that the virus was first isolated from Florida racing greyhounds exhibiting signs of infectious respiratory disease.[7] Early phylogenetic analyses showed CIV, an influenza A virus in the family *Orthomyxoviridae*, to be closely related to contemporary equine influenza viruses (EIV) of the Florida Clade 1 sublineage.[7,8] From the emerging CIVs, ten amino acid substitutions within the hemagglutinin (HA), neuraminidase, nucleoprotein, and polymerase proteins were identified.[8] Unlike other horse-to-dog EIV transmission events in which dogs have been only incidentally infected,[9,98] CIV represented a new threat to canine health, as the virus rapidly spread to naïve dogs throughout the racing track circuit,[7,103] animal shelters,[7,112,131] and other boarding facilities.[104] Studies have shown that, in spite of successful experimental EIV and CIV infections in both equine and canine hosts,[105,109,110,115] influenza species specificity is currently being maintained in naturally-infected horses and dogs.[106] Indeed, CIV isolates sequenced between 2005 and 2008 share the same ten amino acid substitutions originally identified in early Florida CIV, as well as an additional nine amino acid substitutions among the influenza proteins[106] (three within the HA alone). These findings suggest that CIV continues to evolve from both its canine and equine influenza predecessors.

Hemagglutinin, one of two surface glycoproteins on influenza A viruses, has several important functions, including mediation of receptor binding, host cell entry, and membrane fusion.[164] Given its vital role during influenza infection, it is not surprising that the HA protein is also the main target for antibody attachment and virus neutralization.[107,165] Five antigenic regions (sites A-E) have been identified in the HA subtype 3 (H3) HA1 subunit protein.[82,83,166-168] As influenza is a negative-sense single-stranded RNA virus, amino acid substitutions are common throughout the hemagglutinin protein. However, mutations within the antigenic regions have been associated with immune escape or antigenic drift,[84] which can result in vaccine failure and increased host susceptibility to influenza infection. Historically, antigenic drift has had epidemiological impact when four or more amino acid substitutions are located within at least two

of these antigenic regions.[84] Antigenic drift has been reported in human H3 influenza viruses,[166,168] with influenza strains clustering into antigenic groups over time. For example, influenza A H3N2 viruses isolated from humans between 1968 and 2003 formed 11 distinct antigenic clusters.[168] Equine influenza viruses, in contrast, clustered into only three antigenic groups over the same time period,[24] while antigenic drift has yet to be detected in CIVs isolated from dogs.[128,169]

However, with the widespread use of a recently developed killed CIV vaccine,[111] continual molecular evaluation of circulating CIVs is necessary for monitoring antigenic drift, caused either by herd immunity of naturally infected and vaccinated animals, and by the high mutation rate inherent to RNA virus replication. Furthermore, as CIV emerged only within the last decade, evolutionary relationships among CIV isolates, as well as between CIV and other influenza A viruses, are continually being evaluated and characterized.[7,8,106,108] By analyzing the HA nucleotide and protein sequences of the first CIVs isolated from Florida dogs, other published CIV H3N8 strains isolated from across the U.S., and newly isolated CIVs from CIV-epidemic and -endemic U.S. regions, we are able to further describe the evolution of CIV since it first emerged in 2004, as well as report antigenic variations that could possibly result in the first clustering of antigenically distinct CIVs. The studies described here add to our general knowledge of influenza A virus evolution and, in particular, expand our understanding of CIV and its threat to canine health.

METHODS AND MATERIALS

Animals. Dogs residing in six U.S. shelters participated in this prospective study. The six shelters were located near or within metropolitan centers (Sacramento, CA; Colorado Springs, CO; Tampa Bay, FL; New York, NY; Charlestown, SC; and Austin, TX) and were part of a larger CIV surveillance study conducted December 2009 to January 2012.[170] Shelters were chosen in regions that were considered CIV-endemic (CO, FL, NY) and -epidemic (CA, SC, TX). Nasal swabs were taken from approximately 40 dogs every month per shelter and all dogs, irrespective of age, breed, sex, and vaccination or health status, were considered eligible to participate. Prior to initiation, all studies were reviewed and approved by CSU IACUC.

Clinical samples. Nasal swab samples were collected from dogs on-site at each humane shelter, as previously described.[131,170] Samples were refrigerated at 4°C and shipped monthly to Colorado State University Veterinary Teaching Hospital (CSU VTH). Upon arrival in the laboratory, 200µl of nasal swab viral transport medium was removed from each sample and placed in a 96-well block for later ribonucleic acid (RNA) isolation. The remaining sample, along with the samples in the 96-well block, were immediately stored at -80°C until further processing.

Real time RT-PCR assay. Using an automated RNA isolation system (Qiagen Qiaxtractor; Hilden, Germany), viral RNA was extracted from the 200µl viral transport medium aliquots and eluted in a final volume of 60µl water. A one-step real time RT-PCR assay developed by members of or laboratory was performed to identify any CIV-positive clinical samples, as previously described.[131] Briefly, a 5 µl-aliquot of RNA template was mixed with 20 µl of mixture containing iScript One-Step RT-PCR Kit reagents (BioRad; Hercules, CA), 80 nmol of probe, and 200 nmol of forward (5' GAA CAC CGA TCT TGA GGC ACT C 3') and 200 nmol of reverse (5' GGC ATT TTG GAC AAA GCG TCT AC 3') primers to amplify 144bp of the conserved influenza A virus matrix (M) gene. Water and viral transport medium were used as negative controls, while the positive control consisted of 10 TCID50 of A/canine/CO/224986/ 2006 (H3N8). An epMotion Ep5070p (Eppendorf; Hamburg, Germany) automated system was used to load 96 well plates before amplification and detection by Mastercycler Realplex (Eppendorf), using previously described conditions.[131]

Virus isolation. All real-time RT-PCR CIV-positive nasal swabs were stored at -80°C until virus isolation could be performed. Aliquots of 150-200 µl thawed viral transport medium were injected by candlelight into the allantoic cavities of three 10-day old embryonated chicken eggs. In addition, 50 µl of nasal swab sample was inoculated into MDCK cells on 6-well plates in duplicate. Inoculation medium contained both antibiotic (gentamicin for the egg inoculation and penicillin and streptomycin for the cell culture inoculations) and antifungal (amphotericin B) agents, as previously described.[131] Each isolate was blind-passaged twice (for a total of three passages) using 200 µl of the previous passage and evaluated for CIV by hemagglutination inhibition (HI) assays. All first-passaged and HI-positive isolates were additionally tested for presence of the influenza M gene by real-time RT-PCR.

Hemagglutination Inhibition Assays. In accordance with procedures recommended by the World Health Organization,[171] and as described in previous studies,[128,136,170] receptor-destroying enzyme-treated sera from blood samples were used to determine CIV H3N8 antibody titers by HI assay. Briefly, four hemagglutinin units per 25μl of H3N8 antigen viruses were incubated with diluted sera for 45-60 minutes and then developed using 0.5% (v/v) chicken red blood cells for 30 minutes. HI antibody titers were determined as the reciprocal of the last dilution of antiserum that completely inhibited agglutination. Seropositivity was defined as a HI titer of greater than or equal to 16, as in previous seroprevalence studies.[170]

Sequencing. The full-length HA protein-coding region for each isolated virus was amplified by a two-step RT-PCR. Briefly, extracted viral RNA was reverse transcribed using SZAHA primers[125] and SuperScript™ III reverse transcriptase (Gibco/Invitrogen, Carlsbad, CA) before hemagglutinin cDNAs were amplified by PCR using Platinum® *Taq* DNA polymerase high fidelity (Gibco/Invitrogen). PCR products were obtained by direct cycle sequencing using ABI Big Dye (PE Applied Biosystems, Foster City, CA) and sequenced at the Proteomic and Metabolomics Facility at Colorado State University (http://www.dnatools.com). Nucleotide sequences of RNA from the shelter dog study, as well as several Colorado and Wyoming CIVs isolated in our laboratory since 2006, were compiled de novo using Genious Pro software[172] and deposited into GenBank under accession numbers JX235372 through JX235394.

Genome and antigenic evolutionary analyses. Each nucleotide sequence was translated to protein using the ExPASy Bioinformatics Resource Portal (http://web.expasy.org/translate/) before both nucleotide and amino acid alignments were assembled using the Kyoto University Bioinformatics Center multiple sequence alignment on-line tool (http://www.genome.jp/tools/clustalw/). All available CIV (H3N8) full-length HA sequences published up until 25-Jun-2012 were downloaded from the Influenza Virus Resource, National Center for Biotechnology Information (NCBI) database. Additionally, HAs of viruses previously sequenced by members of our laboratory (GenBank accession numbers HQ917678–HQ917681) were included. In total, there were 62 (19 new shelter, 9 Colorado and Wyoming strains previously isolated by members of our laboratory, and 34 published) HA amino acid sequences imported into MEGA 5.05[173] for phylogenetic analysis. Neighbor-joining trees were constructed using a 1000-replicate bootstrap analysis. To determine selection pressure, the Datamonkey webserver[174,175] using the

HyPhy package[176] was utilized to evaluate the nucleotide sequences from 58 (17 shelter, 8 previously sequenced in our laboratory, and 33 published) isolates; nine nucleotide sequences were identical to other isolates and were excluded from analysis. As viral populations often do not meet the assumption for using the ratio of non-synonymous to synonymous calculation (dN/dS), where a dN/dS ≥ 1 indicates positive selection pressure, because more than two mutations might segregate at a single site in viral populations,[177] we used the mixed-effects model for evolution (MEME),[178] using the HKY85 nucleotide substitution bias model. This method accounts for episodic selection at each individual amino acid site, rather than at only the node or branch alone.[178]

Antigenic regions were identified according to sites reported for human and equine influenza H3 viruses.[82,83,166-168] Additionally, to assess antigenic variability among CIV isolates within U.S. dogs, we performed a series of HI assays. Serum samples tested included CIV-seropositive serum samples from the Colorado, Florida, New York, and South Carolina humane shelters. Five different antigens were included: one isolate from emerging CIV (A/canine/FL/43/2004), two CIVs isolated during outbreaks from 2006- to 2007 in Colorado and Wyoming (A/canine/CO/224986/2006 and A/canine/WY/86033/2007), and recent CIV isolates from humane shelters located in two geographically distinct regions (A/canine/CO/863160/2010 and A/canine/NY/6977983/2010).

RESULTS

Virus isolation. Over 5100 nasal swab samples were collected from shelter dogs entering and exiting the six participating humane shelters over the study period. Of these, 111 were positive upon real time RT-PCR for the influenza M gene (Table 7-1). After three passages in both embryonated chicken eggs and MDCK cells, 19 viruses (17%) were isolated and their HA genes sequenced. The majority of these (11/19) were grown in allantoic fluid only, while one was grown in cell culture only, and seven viruses grew in both culture

Table 7-1. Viruses isolated from dogs residing in humane shelters December 2009 through January 2012.

Shelter Location	Samples collected	Swabs inoculated	Isolates sequenced
California	854	1	0
Colorado	1037	38	7
Florida	953	12	0
New York	863	37	11
South Carolina	708	23	1
Texas	771	0	0
TOTAL	5186	111	19

systems. Most of the isolates were from the Colorado and New York shelters, although one virus each was isolated from the Florida and South Carolina shelters. Interestingly, all of the Colorado isolates were obtained in the first year of the study (2009-2010), while all of the New York viruses were from nasal swabs collected during study year two (2010-2011) (Table 7-2). Additionally, the South Carolina virus was collected during the CIV-outbreak that occurred in the South Carolina shelter the first month of the study (December 2009). The other viruses listed in Table 7-2 were isolated from either pet dogs seen at the CSU VTH or were collected from dogs residing at Colorado and Wyoming humane shelters during reported outbreaks of canine infectious respiratory disease from 2006 to 2007.

Table 7-2. Canine influenza viruses isolated from nasal swabs collected from dogs 2006- 2011.

Strain	Date collected	Passage*	Location
A/canine/CO/224986/2006	06-Jun-06	C3	CSU VTH†
A/canine/CO/148902/2006	30-Oct-06	C2	Humane shelter
A/canine/CO/3/2006	28-Apr-06	C3	CSU VTH
A/canine/CO/224766/2006	24-May-06	C2	CSU VTH
A/canine/WY/86033/2007	14-Feb-07	E3; C3	Humane shelter
A/canine/CO/231256/2007	08-Mar-07	C6	Humane shelter
A/canine/WY/86955/2007	27-Feb-07	E1; C3	Humane shelter
A/canine/CO/2025974/2007	14-Mar-07	E1; C3	Humane shelter
A/canine/CO/234550/2009	27-Feb-09	C6	CSU VTH
A/canine/SC/89215/2009	05-Dec-09	E3	Humane shelter
A/canine/CO/850078/2009	31-Dec-09	E3	Humane shelter
A/canine/CO/861142/2010	03-Mar-10	E3	Humane shelter
A/canine/CO/861997/2010	09-Mar-10	E3	Humane shelter
A/canine/CO/863160/2010	20-Mar-10	E3	Humane shelter
A/canine/CO/866907/2010	10-Apr-10	E3	Humane shelter
A/canine/CO/884753/2010	21-Jul-10	C1	Humane shelter
A/canine/CO/886050/2010	27-Jul-10	C3	Humane shelter
A/canine/NY/6977983/2010	05-Sep-10	E3	Humane shelter
A/canine/NY/12370090/2011	12-Feb-11	E4	Humane shelter
A/canine/NY/13258337/2011	05-Jun-11	E3	Humane shelter
A/canine/NY/13454104/2011	30-Jun-11	E1	Humane shelter
A/canine/NY/13453967/2011	30-Jun-11	C1	Humane shelter
A/canine/NY/3821631/2011	06-Jul-11	E3	Humane shelter
A/canine/NY/13693247/2011	03-Aug-11	E1	Humane shelter
A/canine/NY/13752393/2011	12-Aug-11	E3	Humane shelter
A/canine/NY/13889381/2011	25-Aug-11	E3	Humane shelter
A/canine/NY/13949315/2011	01-Sep-11	E1	Humane shelter
A/canine/NY/13949306/2011	01-Sep-11	E2	Humane shelter

*Passage used for sequencing. Either from cell culture (C) or embryonated chicken eggs (E). Number indicates passage.
†Colorado State University Veterinary Teaching Hospital

Genetic evolution of CIV. The phylogenetic analysis of HA amino acid sequences suggests that CIV is diverging into two genetically distinct clades (Figure 7-1). From the bootstrap consensus, the New

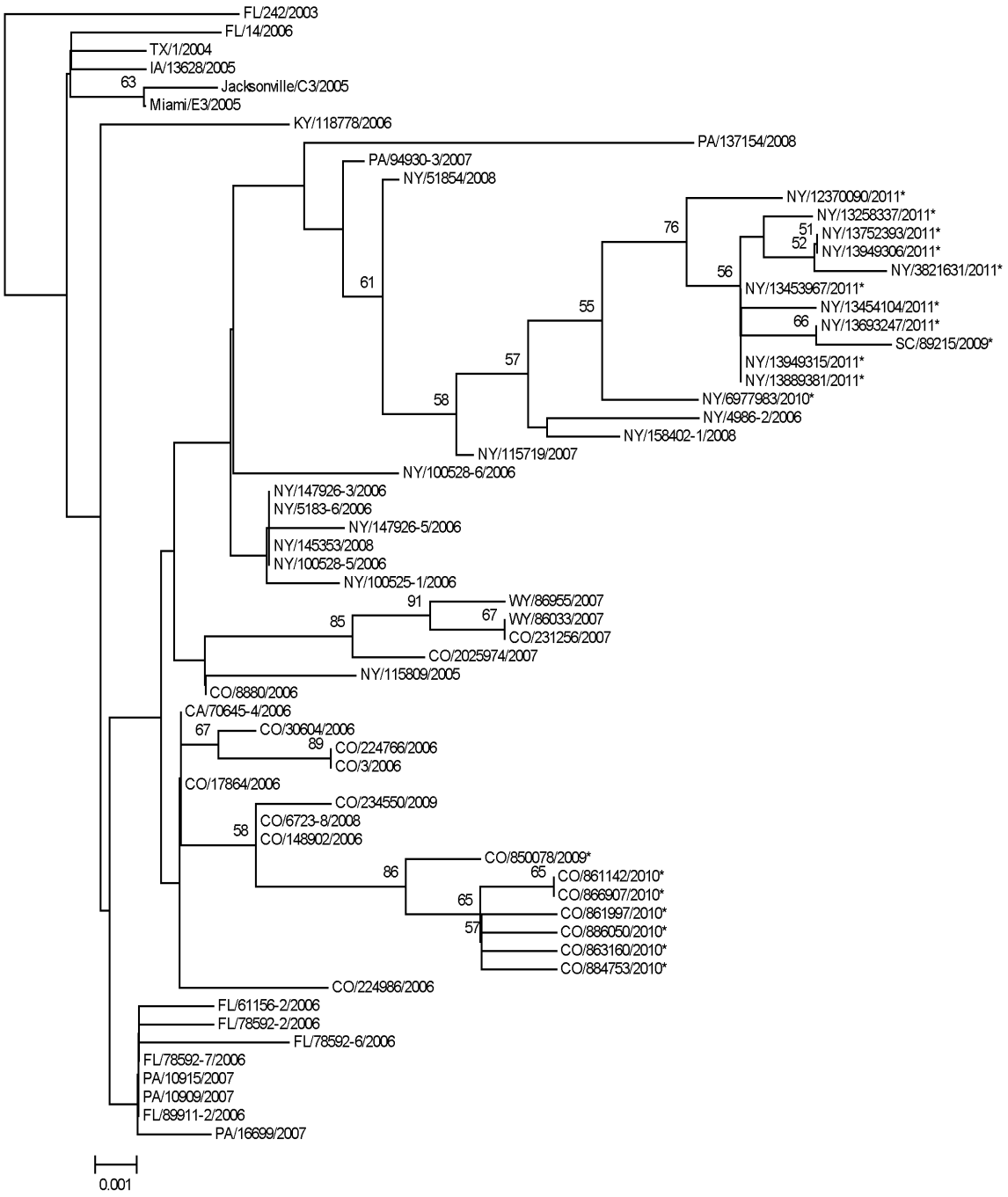


Figure 7-1: Neighbor-joining tree with 1000 bootstrap replicates for all published canine influenza virus (H3N8) full-length HA protein sequences. Labels are included for branches with over 50% bootstrap consensus. Asterisks denote isolates from humane shelters collected 2009-2011.

York/South Carolina and the Colorado CIV isolates cluster separately from one another. Although viruses from these two regions share eight of the original nine adaptations from EIV and emerging CIV identified by Payungporn et al.[8], as well as the three HA substitutions reported by Rivallier et al.,[106] there were an additional 11 amino acid differences among Colorado and New York CIV isolates (I58V, D/E77D, I112V, G/E124G, K172K/E, Y174F, V223I, T/I242I, M268I, G/D464N/D, and L496I). Interestingly, five of these eleven newly identified differences were within H3 antigenic regions (Table 7-3). Moreover, viruses isolated from within the Colorado region also appear to be evolving. Nine amino acid differences appear among the viruses isolated during early infections in Colorado and Wyoming (2006-2007) and the isolates collected during the shelter study (2009-2010): D/E77E, S/L107L, V112I, G/E124G, F174Y, G/R218G, T/I242I, G/D464D, and E479K. This last site is of particular interest as it was one of the earliest CIV substitutions identified.[7,8] The MEME analysis revealed five sites exhibiting evidence of episodic diversifying selection at a significance level of $p<0.1$. These included H3 amino acid residues 107, 169, 216, 453, and 464. Site 107 is likely involved in cellular immunity,[179] while sites 169 and 216 are located in or near antigenic sites. Amino acid residues 453 and 464 are located on the highly conserved HA2 subunit of the HA protein.

Antigenic variation in CIV isolates. Overall, there were eight amino acid residue substitutions within three antigenic sites (A, D, and E) among all the isolates (Table 7-3).

Table 7-3: Amino acid differences within antigenic regions among early CIV and contemporary CIV (H3N8) isolates. Shading denotes consistent differences between emerging CIV and later CIV isolates.

H3 amino acid residue	H3 antigenic region[82,166,167,168]	Early FL CIV[106]	Early CO/WY CIV	Recent CO CIV	Recent NY/SC CIV
75*	E	H	H	H	Q
124	A	G	G/E	G	G
172	D	K	K	K	K/E
174	D	F	F	Y	F
216*	D	N	N	N	N/H
223	D	V	V	V	I
242	D	I	T/I	I	I
262*	E	T	T	T	P

*Sites previously identified[7,8,106,108]

Due to suspected serum degradation issues and low numbers of positive samples from South Carolina (n=3) and Florida (n=4), only a few sera from each shelter were tested against all five antigens with HIA. Even with the limited data, however, several trends emerge (Figure 7-2). Although sera from the A/canine/WY/86033/2006-infected and Colorado shelter dogs have little variation among HIA titers

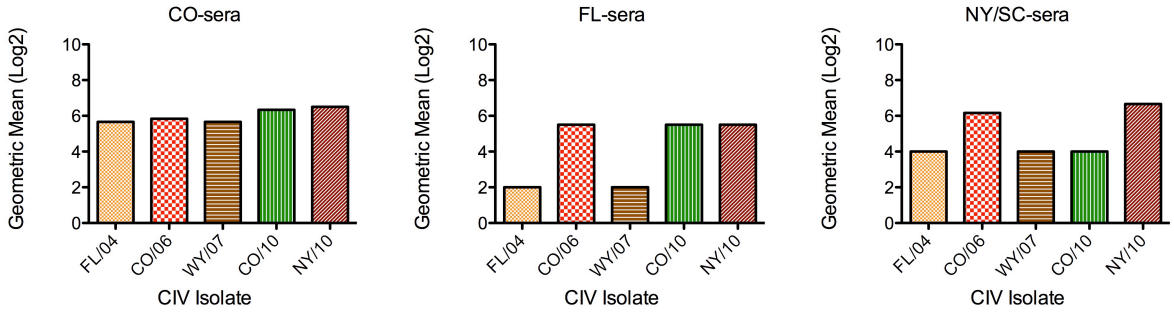


Figure 7-2: Cross-reactivity (hemagglutination inhibition antibody titers) between canine influenza virus (CIV) H3N8 isolates and CIV-positive sera collected from dogs 2009-2012. Hemagglutination inhibition titers are shown as geometric (log2) means (±SEM) from dogs residing at the Colorado (n=6), Florida (n=2), and New York and South Carolina (n=6) humane shelters.

(± 1-log difference), samples from Florida, South Carolina and New York had 2- to 3-log differences among geometric mean titers. The older Colorado virus (A/canine/CO/224986/2006) and the New York isolate (A/canine/NY/6977983/2010) both induced higher antibody titers for all three groups, suggesting potential antigenic drift from the early CIV isolate (A/canine/FL/43/2004) and the Wyoming virus (A/canine/WY/86033/2007). Interestingly, the Florida sera also had higher geometric mean titers to the newer Colorado virus (A/canine/CO/863160/2010).

DISCUSSION

Since the emergence of CIV nearly nine years ago, the virus has circulated in a number of dog populations, most notably in dogs residing in humane shelters.[7,112] Indeed, CIV is routinely isolated from such facilities.[106,108,131] Whether this is because there are actually more CIV infections in shelter dogs compared to non-shelter dogs or because there is more surveillance in this particular population is a matter under current study.[170] Nevertheless, the frequent isolation of CIVs in shelter dogs presents a unique opportunity to characterize genetic evolution and antigenic variation of a relatively novel influenza A virus “in situ.”

It is evident from these studies and others[106,108] that CIV is diverging into two genetically distinct clades: Colorado and New York. Although tempting to consider these Western and Eastern CIV clades, the lack of sequences from other geographical regions (e.g., South Carolina and Florida in the East and California and Texas in the West) makes such a designation premature. It is interesting that the recent

New York viruses and the one South Carolina isolate are more related to older CIVs isolated in Colorado and Wyoming than to the newer Colorado CIV isolates (Figure 7-1). Additionally, it appears that CIV has undergone several evolutionary "dead ends." For example, isolates from Cheyenne Animal Shelter (A/canine/WY/86955/2007 and A/canine/WY/86033/2007), were collected several months after severe CIV outbreaks in Cheyenne caused the shelter to reportedly euthanize 70-80 dogs.[180] These three CIVs do not appear to be closely related to any of the newer Colorado isolates. Instead, the recent Colorado CIVs are genetically similar to viruses isolated from dogs residing at animal shelters located in Fort Collins and Boulder, CO. In fact, all of the Colorado CIVs are nearer phylogenetically to the emerging Florida isolates (as previously reported[106,108]), while the New York shelter isolates appear to have evolved from the same ancestor as the Wyoming isolates from 2006-2007.

The MEME analysis found five sites in the HA coding region undergoing episodic diversifying selection: 107, 169, 216, 453, 464. Three of these sites (107, 169, 216) are located on the HA1 protein (HA sites 1 through 328), including one site located in an antigenic region, while two of these (453, 464) are on the highly conserved HA2 protein (HA sites 330 through 550). Substitutions at site 107 were reported in 2008 as a replacement of serine in the conserved EIV and early Florida CIV isolates by a proline in the A/canine/Jacksonville/2005 strain.[8] However, recent isolates from both New York and Colorado show a leucine substitution at this site instead. Site 107 is part of a peptide region (105-140) that stimulates proliferation of T helper cells.[179] Isolates substituting serine at position 107 change the amino acid from one with a polar uncharged side chain to either one with a secondary amine (proline) or one with a hydrophobic side chain (leucine). Such substitutions might result in steric and/or conformational changes of the peptide, resulting in lack of T-helper cell recognition, and, thus, reduced CIV-specific immune response. Likewise, mutations leading to conformational change at sites 216 (antigenic region D) and 169 (near antigenic region D) might alter antibody-binding affinity. Interestingly, a P169L mutation was seen in two H3N2-vaccinated guinea pigs from post-challenge isolates,[181] suggesting a mutation at this site might be involved in immune escape. The sites identified as undergoing selection pressure on the HA2 protein – HA 453 (HA2 site 124) and 464 (HA2 site 135) – contribute to protein stabilization before virion envelope and endosome membrane fusion.[182] Additionally, although not identified as a site undergoing selection pressure, residue 479 is also located in this HA2

peptide region. The canine adaptation at site 479 (HA2 site 150)[8,106,108] from glycine in EIV and emerging CIV to glutamic acid in 2005-2008 canine isolates has been observed before in human influenza H3 viruses,[183] and is in the same soluble region (HA2 38-175) as HA sites 453 and 463. It is conceivable that these three mutations might either hinder or help conformational stability of the HA2 protein before fusion of the viral and endosomal membranes, enabling release of viral ribonucleoproteins into the cytoplasm and subsequent virus transcription and replication. Further investigation into the role these sites play on CIV stability might aid our understanding of how CIV emerged from EIV to become a canine-specific influenza A virus.

With eight amino acid substitutions within three antigenic regions, antigenic drift might also be occurring for the newer CIV isolates. However, it is important to note that E124 (site A) was only observed in two of the viruses isolated after the Wyoming 2006 outbreak (A/canine/CO/231256/2007 and A/canine/WY/86033/2007). Other mutations seen in only a few isolates included two clinical samples from the CSU VTH with I242T (A/canine/CO/3/2006 and A/canine/CO/224766/2006) and two shelter CIVs with K172E (A/canine/SC/89215/2009 and A/canine/NY/13693247/2011). Therefore, five consistent substitutions were observed in either Colorado or New York isolates. In antigenic site D, two amino acid substitutions (N216H and V223I) were identified in the New York isolates – only one New York isolate (A/canine/NY/12370090/2011) shared the N216 motif with the Colorado isolates. Also within site D, another substitution (F174Y) was seen in the CIVs isolated from the Colorado shelter 2009-2010. For site E, two amino acid residue mutations were seen in the New York isolates (H75Q and P262T). Although HI assay results should be cautiously interpreted because of the small sample size and unknown CIV strain inducing serum antibodies, there is some serological evidence supporting antigenic drift of CIVs. For example, there was a four-fold HI titer difference for serum samples from New York between the A/canine/NY/6977983/2010 and A/canine/CO/863160/2010 antigens. It is interesting that the older Colorado strain (A/canine/CO/224986/2006) had a similar HI titer as the New York strain. The only amino acid residue from Table 7-3 common to the 2006-2007 Colorado and 2010-2011 New York isolates, that is not shared with the 2009-2012 Colorado isolates, is F174. The biochemical difference between phenylalanine and tyrosine is the polar side group (OH) on the latter. What conformation change this would cause in CIV has yet to be examined.

As the analyses presented here are based on genomic and antigenic sites described for human and equine H3 influenza A viruses,[82,83,166-168] it is possible that the CIV HA has different antigenic regions that have yet to be defined. Additionally, more research on CIV is warranted to confirm the structure of the CIV HA protein, map antigenic regions associated with virus neutralization and antigenic drift, and further investigate antigenic variation leading to CIV immune escape. This latter point is of particular import, as influenza vaccines are well recognized for inducing antigenic drift. Finally, one limitation of our study was the possible degradation of nasal swab samples between collection, transport, and processing. As samples were stored on-site at humane shelters before being shipped to the CSU laboratory, infectious virus might have been compromised, which could account for our low number of isolates. Future studies utilizing virus isolation may want to find a quicker, yet practical, protocol for sending samples to the laboratory soon after collection.

In conclusion, the findings reported here support previous studies[106,108] that suggest CIV is diverging into two genetically distinct clades – Colorado and New York. More research is needed to determine how far geographically this divergence extends. Examination of 58 CIV HA nucleotide sequences isolated between 2003 and 2011 found five amino acid residues under selection pressure – one within an antigenic region. Additionally, a total of five amino acid changes were observed in two antigenic sites (D and E) for CIVs isolated from Colorado and New York humane shelters from 2009 to 2011. Finally, although preliminary studies suggest that the New York clade is evolving into a distinct antigenic cluster, controlled experiments are required to determine true extent of antigenic drift occurring within circulating CIV.

CHAPTER EIGHT: CONCLUSIONS AND FUTURE DIRECTIONS

Conclusions

The studies conducted here support the main hypothesis that CIV is being transmitted and maintained in U.S. dogs, which is further supported by the evolution of the canine-specific influenza A lineage. Additionally, these reports contribute several important findings to our understanding of CIV and its impact on dog populations: (1) Real-time RT-PCR assays should be used as a rapid test for detecting CIV and can minimize false negatives, allowing a more accurate representation of CIV infection in dogs. (2) Both CIV shedding and seroprevalence in the dog populations sampled, including from CIV-endemic and -epidemic shelters, are not nearly as high as first reported in racing greyhounds and New York and Florida shelters in 2004-2005, suggesting either there are other genetic or environmental factors contributing to fatal CIV infection or there are some host-immune response mechanisms limiting severe CIV disease in some dogs. (3) Despite low CIV prevalence, control measures (i.e., minimizing dog interactions within a facility and use of an effective vaccine) should still be implemented for at-risk dogs, such as dogs in boarding facilities, attending canine day care, and residing in shelters. (4) Additionally, CIV, like other influenza A viruses, appears to be a disease of space and time. Therefore, modeling could be helpful for predicting future CIV outbreaks. (5) Finally, CIV is evolving into two genetically distinct clades, one of which is antigenically different from previous CIVs. Controlled experiments are required to determine true extent of antigenic drift occurring within circulating CIV and, if necessary, CIV vaccines should be updated accordingly.

Future Directions

Although there are many follow-up studies that may support the evidence presented in this dissertation, efforts would be better served if future research is focused on three specific areas: (1) the efficacy of the CIV vaccination in a field setting, (2) understanding the CIV-dog pathogen-host interactions, and (3) comparing what we know about H3N8 CIV in the United States to emerging H3N2 IAV in dogs in southeastern Asia.

For the first, it is interesting that in several studies dogs that were reported to be vaccinated did not seroconvert. This observation has been noted in laboratory experiments with sera from six fully-vaccinated dogs as well (unpublished data). Naturally, there are several possibilities for these results: the dogs were not vaccinated or not vaccinated correctly, the samples were taken before the onset of the host immune response, no vaccine booster was given, or the vaccine does not induce a detectable antibody response. Even vaccinates in the study conducted by the vaccine manufacturer[111] report a mean HI titer of 138 (it is stated to be a geometric mean but this is likely an error as pre-vaccination titers were reported as <10). Although follow-up studies demonstrate more convincingly that the vaccine reduced severity of illness in dogs co-infected with CIV and *Streptococcus equi* spp. *zooepidemicus*,[184] the lack of seroconversion seen in field samples needs to be further investigated. The biggest question at hand is whether or not vaccinated dogs are protected against CIV. While true that the vaccine manufacturer found lessened disease severity in their experiments, both studies were conducted in pups six to ten weeks old. Without controlled and field experiments to examine the efficacy of the CIV vaccine in adult dogs, it is difficult to recommend its use outside at-risk dog populations (such as shelter, day care, and boarding dogs). Even then, the gap of knowledge regarding its efficacy tempers enthusiasm for the vaccine.

The second focus of future research, understanding the CIV-dog pathogen-host interaction, relates to how an IAV jumped the species barrier. Studies in this area might also shed light on why CIV-infection was so fatal in the beginning of the outbreak. Results from Appendix A and Quintana et al.[109] show that members of our laboratory have developed an *in vitro* cell culture system (respiratory epithelial cells grown at the air-fluid interface) that mirrors infection and replication of EIV and CIV observed in the equine and canine host. Using this system, it is possible to determine cell signals inducing interferon and other cytokine production during early respiratory infection.[185] Such experiments with equine herpes virus-infected equine respiratory cells have been underway, and EIV infection experiments were recently begun to examine mRNA expression and protein production during early IAV infection. A similar experiment using canine respiratory epithelial cells from various dog breeds and different CIV isolates might answer the question of why greyhounds were particularly afflicted with high case fatality rates. Even more promising is what the mRNA and protein expression comparisons of chemokines induced by EIV- and

CIV-infection might reveal. Research here (Appendix A) and elsewhere[105] suggest that receptor binding preference is the same for both CIVs and EIVs. Determining what happens after viral entry in early infection, therefore, is a logical next step for trying to elucidate how an EIV was able to sustain infections in dogs.

Finally, it has been reported that an avian IAV H3N2 is being transmitted among dogs in South Korea and China.[186-188] Experimentally, avian IAV H3N2 is able to be transmitted from infected dogs to susceptible dogs via direct contact.[189] Transmission among dogs in an experimental setting, however, does not necessarily mean there is a new CIV emerging. A major criterion for designation of a host-specific IAV lineage is sustained transmission among the new host species and subsequent maintenance. Consistent amino acid substitutions within the proteome would also suggest adaptation to a new host. Although more surveillance studies are needed to confirm sustained transmission, viruses from 6 of 13 nasal swabs collected during an IAV outbreak were isolated and found to contain the same W222L mutation found in CIV H3N8 isolates.[188] Additionally, 19% (68/361) of farmed dogs in South Korea were seropositive on anti-NP ELISA.[187] It thus appears that sustained transmission of an avian IAV is occurring and that there are amino acid adaptations to the canine host. However, further complicating designation of a H3N2 canine-specific IAV is the fact that cats have also been reported to be infected.[190] Despite this caveat, the transmission of avian IAV in dogs and cats is a great opportunity for research to re-examine the emergence of CIV H3N8 to find any similarities and differences between the two IAVs, both genetically or epidemiologically.

REFERENCES

1. Webster RG, Bean WJ, Gormon OT, et al. Evolution and ecology of influenza A viruses. Microbiology Reviews 1992;56:152-179.
2. Lipatov AS, Govorkova EA, Webby, et al. Influenza: emergence and control. Journal of Virology 2004;78:8951-8959.
3. Sovinova O, Tumova B, Pouska F, et al. Isolation of virus causing respiratory disease in horses. Acta Virology 1958;2:52-61.
4. Webster RG. Are equine 1 influenza viruses still present in horses? Equine Veterinary Journal 1993;25:537-538.
5. Olusa TAO, Adegunwa AK, Aderonmu AA, et al. Serological evidence of equine H7 influenza virus in polo horses in Nigeria. Science World Journal 2010;5:17-19.
6. Waddell GH, Eigland MB, Sigel MM. A new influenza virus associated with equine respiratory disease. Journal of the American Veterinary Medical Association 1963;143:587-590.
7. Crawford PC, Dubovi EJ, Castleman WL, et al. Transmission of equine influenza virus to dogs. Science 2005;310:482-485.
8. Payungporn S, Crawford PC, Kouo TS, et al. Influenza A virus (H3N8) in dogs with respiratory disease, Florida. Emerging Infectious Diseases 2008;14:902-908.
9. Daly JM, Blunden AS, MacRae S, et al. Transmission of equine influenza virus to English foxhounds. Emerging Infectious Diseases 2008;14:461-462.
10. Newton R, Cooke A, Elton D, et al. Canine influenza virus: cross-species transmission from horses (letter). Veterinary Record 2007;161:142-143.
11. Palese P, Shaw ML. Orthomyxoviridae: the viruses and their replication. In: Knipe DM, Howley PM, eds. Fields Virology, 5 ed. Philadelphia: Lippincott Williams and Wilkins; 2007.
12. Ohishi K, Ninomiya A, Kida H, et al. Serological evidence of transmission of human influenza A and B viruses to Caspian seals (*Phoca caspica*). Microbiology and Immunology 2002;46:639-644.
13. Kimura H, Abiko C, Peng G, et al. Interspecies transmission of influenza C virus between humans and pigs. Virus Research 1997;48:71-79.
14. Haig DA, Woodall JP, Danskin D. Thogoto virus: a hitherto undescribed agent isolated from ticks in kenya. Journal of General Microbiology 1965;38:389-394.
15. Kibenge FS, Munir K, Kibenge MJ, et al. Infectious salmon anemia virus: causative agent, pathogenesis and immunity. Animal Health Research Reviews 2004;5:65-78.
16. Webster RG, Laver WG, Air GM, et al. Molecular mechanisms of variation in influenza viruses. Nature 1982;296:115-121.
17. Smith GJD, Vijaykrishna D, Bahl J, et al. Origins and evolutionary genomics of the 2009 swine-origin H1N1 influenza A epidemic. Nature 2009;459:1122-1126.
18. Nayak DP, Balogun RA, Yamada H, et al. Influenza virus morphogenesis and budding. Virus Research 2009;143:147-161.
19. Radyukhin V. The concept of transmembrane asymmetry of lateral domains in biomembranes and influenza virus envelope fine structure. Molecular Biology 2009;43.
20. Ye Z, Liu T, Offringa DP, et al. Association of influenza virus matrix protein with ribonucleoproteins. Journal of Virology 1999;73:7467-7473.
21. Hampson AW. Influenza virus antigens and 'antigenic drift'. In: Potter CW, ed. Influenza, First ed. Amsterdam, The Netherlands: Elsevier Science B. V.; 2002:49-85.
22. Wright PF, Neumann G, Kawaoka Y. Orthomyxoviruses. In: Knipe DM, Howley PM, eds. Fields Virology, 5 ed. Philadelphia: Lippincott Williams and Wilkins; 2007.
23. Tong S, Li Y, Rivailler P, et al. A distinct lineage of influenza A virus from bats. PNAS 2012;109:4269-4274.
24. Lewis NS, Daly JM, Russell CA, et al. Antigenic and genetic evolution of equine influenza A (H3N8) virus from 1968 to 2007. Journal of Virology 2011;85:12742-12749.
25. Kelkar SD, Kadam SS, Banerjee K. Hemagglutination inhibition antibodies against influenza virus in bats. Indian Journal of Medical Research 1981;74:147-152.
26. Lvov DK, Easterday B, Hinshaw W, et al. Isolation of strains of Hong Kong complex (H3N2) influenza virus from *Nyctalus noctula* bats in Kazakhstan. Vopr Virusol 1979;4:338-341.

27. Vines A, Wells K, Matrosovich M, et al. The role of influenza A virus hemagglutinin residues 226 and 228 in receptor binding specificity and host range restriction. Journal of Virology 1998;72:7626-7631.
28. Connor RJ, Kawaoka Y, Webster RG, et al. Receptor specificity in human, avian, and equine H2 and H3 influenza virus isolate. Virology 1994;205:17-23.
29. Ito T, Suzuki Y, Suzuki T, et al. Recognition of N-glycolylneuraminic acid linked to galactose by the alpha2,3 linkage is associated with intestinal replication of influenza A virus in ducks. Journal of Virology 2000;74:9300-9305.
30. Gambaryan AS, Matrosovich MN. A solid-phase enzyme-linked assay for influenza virus receptor-binding activity. Journal of Virological Methods 1992;39:111-123.
31. Matrosovich M, Tuzikov A, Bovin N, et al. Early alterations of the receptor-binding properties of H1, H2, and H3 avian influenza virus hemagglutinins after their introduction into mammals. Journal of Virology 2000;74:8502-8512.
32. Matrosovich MN, Gambaryan AS, Teneberg S, et al. Avian influenza A viruses differ from human viruses by recognition of sialyloligosaccharides and gangliosides and by a higher conservation of the HA receptor-binding site. Virology 1997;23:224-234.
33. Couceiro JN, Paulson JC, Baum LG. Influenza virus strains selectively recognize sialyloligosaccharides on human respiratory epithelium: the role of the host cell in selection of hemagglutinin receptor specificity. Virus Research 1993;29:155-165.
34. Ito T. Interspecies transmission and receptor recognition of influenza A viruses. Microbiology and Immunology 2000;44:423-430.
35. Shinya K, Ebina M, Yamada S, et al. Influenza virus receptors in the human airway. Nature 2006;440:435-436.
36. Suzuki T. Gangliosides as influenza virus receptors. Variation of influenza viruses and their recognition of the receptor sialo-sugar chains. Progress in Lipid Research 1994;33:429-457.
37. Suzuki Y, Ito Y, Suzuki T, et al. Sialic acid species as a determinant of the host range of influenza A viruses. Journal of Virology 2000;74:11825-11831.
38. Matrosovich MN, Matrosovich TY, Gray T, et al. Human and avian influenza viruses target different cell types in cultures of human airway epithelium. PNAS 2004;101:4620-4624.
39. Matlin KS, Reggio H, Helemius A, et al. Infectious entry pathway of influenza virus in a canine kidney cell line. Journal of Cell Biology 1985;91:601-613.
40. Sieczkarski SB, Whittaker GR. Influenza virus can enter and infect cells in the absence of clathrin-mediated endocytosis. Journal of Virology 2002;76:10455-10464.
41. Klenk HD, Rott R, Orlich M, et al. Activation of influenza A viruses by trypsin treatment. Virology 1975;68:426-439.
42. Kido H, Yokogoshi Y, Sakai K, et al. Isolation and characterization of a novel trypsin-like protease found in rat bronchiolar epithelial Clara cells. A possible activator of the viral fusion glycoprotein. Journal of Biological Chemistry 1992;267:13573-13579.
43. Lazarowitz SG, Choppin PW. Enhancement of the infectivity of influenza A and B viruses by proteolytic cleavage of the hemagglutinin polypeptide. Virology 1975;68:440-454.
44. Skehel JJ, Wiley DC. Receptor binding and membrane fusion in virus entry: the influenza hemagglutinin. Annual Reviews in Biochemistry 2000;69:531-569.
45. Cross KJ, Burleigh LM, Steinhauer DA. Mechanisms of cell entry by influenza virus. Expert Reviews in Molecular Medicine 2001;3:1-18.
46. Hernandez LD, Hoffman LR, Wolfsberg TG, et al. Virus-cell and cell-cell fusion. Annual Reviews in Cell and Developmental Biology 1996;12:627-661.
47. Jahn R, Sudhof TC. Membrane fusion and exocytosis. Annual Reviews in Biochemestry 1999;68:863-911.
48. Lindau M, Almers W. Structure and function of fusion pores in exocytosis and ectoplasmic membrane fusion. Current Opinion in Cell Biology 1995;7:509-517.
49. Tse FW, Iwata A, Almers W. Membrane flux through the pore formed by a fusogenic viral envelope protein during cell fusion. Journal of Cell Biology 1993;121:543-552.
50. Kozlov MM, Chernomordik LV. A mechanism of protein-mediated fusion: coupling between refolding of the influenza hemagglutinin and lipid rearrangements. Biophysical Journal 1998;75:1384-1396.
51. Cros JF, Palese P. Trafficking of viral genomic RNA into and out of the nucleus: influenza, Thogoto, and Borna disease viruses. Virus Research 2003;95:3-12.

52. Gabriel G, Klingel K, Otte A, et al. Differential use of importin-α isoforms governs cell tropism and host adaptation of influenza virus. Nature Communication 2011;2:156.
53. Guilligay D, Tarendeau F, Resa-Infante P, et al. The structural basis for cap binding by influenza virus polymerase subunit PB2. Nature Structural and Molecular Biology 2008;15:500-506.
54. Dias A, Bouvier D, Crépin T, et al. The cap-snatching endonuclease of influenza virus polymerase resides in the PA subunit. Nature 2009;458:914-918.
55. Braam J, Ulmanen I, Krug RM. Molecular model of a eucaryotic transcription complex: functions and movements of influenza P proteins during capped RNA-primed transcription. Cell 1983;34:609-618.
56. Hughey PG, Compans RW, Zebedee SL, et al. Expression of the influenza A virus M2 protein is restricted to apical surfaces of polarized epithelial cells. Journal of Virology 1992;66:5542–5552.
57. Whittaker GR, Digard P. Entry and intracellular transport of influenza virus. In: Kawaoka Y, ed. Influenza Virology: Current Topics. Norfolk: Caister Academic Press; 2006:37-56.
58. Gottschalk A. Neuraminidase: the specific enzyme of influenza virus and *Vibrio cholerae*. Biochimica et Biophysica Acta 1957; 23:645–646.
59. Palese P, Ueda M, Tobita K, et al. Characterization of temperature sensitive influenza virus mutants defective in neuraminidase. Virology 1974;61:397-410.
60. Pahl HL. Activators and target genes of Rel/NF-κB transcription factors. Oncogene 1999;18:6853-6866.
61. Nimmerjahn F, Dudziak D, Dirmeier U, et al. Active NF-κB signalling is a prerequisite for influenza virus infection. Journal of General Virology 2004;85:2347-2356.
62. Flory E, Kunz M, Scheller C, et al. Influenza virus-induced NF-κB-dependent gene expression is mediated by overexpression of viral proteins and involves oxidative radicals and activation of IκB kinase. Journal of Biological Chemistry 2000;275:8307-8314.
63. Kumar N, Xin ZT, Liang Y, et al. NF-κB signaling differentially regulates influenza virus RNA synthesis. Journal of Virology 2008;82:9880-9889.
64. Wurzer WJ, Ehrhardt C, Pleschka S, et al. NF-κB-dependent induction of tumor necrosis factor-related apoptosis-inducing ligand (TRAIL) and Fas/FasL is crucial for efficient influenza virus propogation. Journal of Biological Chemistry 2004;279:30931-30937.
65. Pauli EK, Schmolke M, Wolff T, et al. Influenza A virus inhibits type I IFN signaling via NF-kB dependent induction of SOCS-3 expression. PLoS Pathogens 2008;4:Epub.
66. Faleiro L, Lazebnik Y. Caspases disrupt the nuclear-cytoplasmic barrier. Journal of Cell Biology 2000;151:951-959.
67. Wurzer WJ, Planz O, Ehrhardt C, et al. Caspase 3 activation is essential for efficient influenza virus propagation. European Molecular Biology Organization 2003;22:2717-2728.
68. Ehrhardt C, Marjuki H, Wolff T, et al. Bivalent role of the phosphatidylinositol-3-kinase (PI3K) during influenza virus infection and host cell defence. Cellular Microbiology 2006;8:1336-1348.
69. Ehrhardt C, Wolff T, Pleschka S, et al. Influenza A virus NS1 protein activates the PI3K/Akt pathway to mediate antiapoptotic signaling responses. Journal of Virology 2007;81:3058-3067.
70. Ehrhardt C, Ludwig S. A new player in a deadly game: influenza viruses and the PI3K/Akt signalling pathway. Cellular Microbiology 2009;11:863-871.
71. Marjuki H, Alam MI, Ehrhardt C, et al. Membrane accumulation of influenza A virus hemagglutinin triggers nuclear export of the viral genome via protein kinase Cα-mediated activation of ERK signaling. Journal of Biological Chemistry 2006;281:16707-16715.
72. Pleschka S. RNA viruses and the mitogenic Raf/MEK/ERK signal transduction cascade. Biological Chemistry 2008;389:1273-1282.
73. Pleschka S, Wolff T, Ehrhardt C, et al. Influenza virus propogation is impaired by inhibition of the Raf/MEK/ERK signalling cascade. Nature Cell Biology 2001;3:301-305.
74. Diebold SS, Kaisho T, Hemmi H, et al. Innate antiviral responses by means of TLR7-mediated recognition of single-stranded RNA. Science 2004;303:1529-1531.
75. Lund JM, Alexopoulou L, Sato A, et al. Recognition of single-stranded RNA viruses by Toll-like receptor 7. PNAS 2004;101:5598-5603.
76. Kato H, Sato S, Yoneyama M, et al. Cell type-specific involvement of RIG-I in antiviral response. Immunity 2005;23:19-28.
77. Li X, Lu C, Stewart M, et al. Structural basis of double-stranded RNA recognition by the RIG-I like receptor MDA5. Archives of Biochemistry and Biophysics 2009;488:23-33.

78. Ludwig S, Ehrhardt C, Neumeier ER, et al. Influenza virus-induced AP-1-dependent gene expression requires activation of the JNK signaling pathway. Journal of Biological Chemistry 2001;276:10990-10998.
79. Randall RE, Goodbourn S. Interferons and viruses: an interplay between induction, signalling, antiviral responses and virus countermeasures. Journal of General Virology 2008;89:1-47.
80. McGill J, Heusel JW, Legge KL. Innate immune control and regulation of influenza virus infections. Journal of Leukocyte Biology 2009;86:803-812.
81. Wolff T, Zielecki F, Abt M, et al. Sabotage of antiviral signaling and effectors by influenza viruses. Biological Chemistry 2008;389:1299-1305.
82. Wiley DC, Wilson IA, Skehel JJ. Structural identification of the antibody-binding sites of Hong Kong influenza haemagglutinin and their involvement in antigenic variation. Nature 1981;289:373-378.
83. Skehel JJ, Stevens DJ, Daniels RS, et al. A carbohydrate side chain on hemagglutinins of Hong Kong influenza viruses inhibits recognition by a monoclonal antibody. PNAS 1984;81:1779-1783.
84. Wilson IA, Cox NJ. Structural basis of immune recognition of influenza virus hemagglutinin. Annual Reviews in Immunology 1990;8:737-771.
85. Shil P, Chavan SS, Cherian SS. Antigenic variability in neuraminidase protein of Influenza A/H3N2 vaccine strains (1968 - 2009). Bioinformation 2011;7:76-81.
86. Jelley-Gibbs DM, Strutt TM, McKinstry KK, et al. Influencing the fates of CD4 T cells on the path to memory: lessons from influenza. Immunology and Cellular Biology 2008;86:343-352.
87. Castrucci MR, Kawaoka Y. Biological importance of neuraminidase stalk length in influenza A virus. Journal of Virology 1993;67:759-764.
88. Murphy BR, Buckler-White AJ, London WT, et al. Characterization of the M protein and nucleoprotein genes in avian influenza A virus which are involved in host range restriction in monkeys. Vaccine 1989;7:557-561.
89. Scholtissek C, Burger H, Kistner O, et al. The nucleoprotein as a possible major factor in determining host specificity of influenza H3N2 viruses. Virology 1985;147:287-294.
90. Snyder MH, London WT, Maassab HF, et al. A 36 nucleotide deletion mutation in the coding region of the NS1 gene of an influenza A virus RNA segment 8 specifies a temperature-dependent host range phenotype. Virus Research 1990;15:69-83.
91. Landolt GA, Olsen CW. Up to new tricks - a review of cross-species transmission of influenza A viruses. Animal Health Research Reviews 2007;8:1-21.
92. Wan H, Perez DR. Quail carry sialic acid receptors compatible with binding of avian and human influenza viruses. Virology 2006;346:278-286.
93. Ito T, Couceiro JN, Kelm S, et al. Molecular basis for the generation in pigs of influenza A viruses with pandemic potential. Journal of Virology 1998;72:7367-7373.
94. Rogers GN, Paulson JC. Receptor determinants of human and animal influenza virus isolates: differences in receptor specificity of the H3 hemagglutinin based on species of origin. Virology 1983;127:361-373.
95. Chang CP, New AE, Taylor JF, et al. Influenza virus isolations from dogs during a human epidemic in Taiwan. International Journal of Zoonoses 1976;3:61-64.
96. Pysina RV, Surin NG. Isolation from dogs of an influenza virus similar to A (Hong Kong) 68. Vopr Virusol 1972;17:245-248.
97. Houser RE, Heuschele WP. Evidence of prior infection with influenza A/Texas/77 (H3N2) virus in dogs with clinical parainfluenza. Canadian Journal of Comparative Medicine 1980;44:396-402.
98. Crispe E, Finlaison DS, Hurt AC, et al. Infection of dogs with equine influenza virus: evidence for transmission from horses during the Australian outbreak. Australian Veterinary Journal 2011;89:27-28.
99. Lee YN, Lee HJ, Lee DH, et al. Severe canine influneza in dogs correlates with hyperchemokinemia and high viral load. Virology 2011;417:57-63.
100. Powe JR, Castleman WL. Canine influenza virus replicates in alveolar macrophages and induces TNF-alpha. Veterinary Pathology 2009;46:1187-1196.
101. Castleman WL, Powe JR, Crawford PC, et al. Canine H3N8 influenza virus infection in dogs and mice. Veterinary Pathology 2010;47:507-517.
102. de Jong MD, Simmons CP, Thahn TT, et al. Fatal outcome of human influenza A (H5N1) is associated with high viral load and hypercytokinemia. Nature Medicine 2006;12:1203-1206.

103. Yoon K-J, Cooper VL, Schwartz KJ, et al. Influenza virus infection in racing greyhounds [letter]. Emerging Infectious Diseases 2005;11:1974-1976.
104. Dubovi EJ, Njaa BL. Canine influenza. Veterinary Clinics Small Animal Practice 2008;38:827-835.
105. Yamanaka T, Tsujimura K, Kondo T, et al. Infectivity and pathogenicity of canine H3N8 influenza A virus in horses. Influenza and Other Respiratory Viruses 2010;4:345-351.
106. Rivailler P, Perry IA, Jang Y, et al. Evolution of canine and equine influenza (H3N8) viruses co-circulating between 2005 and 2008. Virology 2010;408:71-79.
107. Hoelzer K, Murcia PR, Baillie GJ, et al. Intrahost evolutionary dynamics of canine influenza virus in naïve and partially immune dogs. Journal of Virology 2010;84:5329-5335.
108. Hayward JJ, Dubovi EJ, Scarlett JM, et al. Microevolution of canine influenza virus in shelters and its molecular epidemiology in the United States. Journal of Virology 2010;84:12636-12645.
109. Quintana AM, Hussey SB, Burr EC, et al. Restricted infectivity of a canine lineage H3N8 influenza A virus in ponies. American Journal of Veterinary Research 2011;72:1071-1078.
110. Yamanaka T, Nemoto M, Tsujimura K, et al. Interspecies transmission of equine influenza virus (H3N8) to dogs by close contact with experimentally infected horses. Veterinary Microbiology 2009;139:351-355.
111. Deshpande MS, Jirjis FF, Tubbs AL, et al. Evaluation of the efficacy of a canine influenza virus (H3N8) vaccine in dogs following experimental challenge. Veterinary Therapy 2009;10:103-112.
112. Holt DE, Moyer MR, Cimino Brown D. Serologic prevalence of antibodies against canine influenza virus (H3N8) in dogs in a metropolitan animal shelter. Journal of the American Veterinary Medical Association 2010;237:71-73.
113. Jirjis FF, Deshpande MS, Tubbs AL, et al. Transmission of canine influenza virus (H3N8) among susceptible dogs. Veterinary Microbiology 2010;144:303-309.
114. Cornell Animal Health Diagnostic Center. Test summary for canine influenza virus in dogs not affiliated with greyhound racetracks. 2012. Available at http://ahdc.vet.cornell.edu/docs/Statistics_for_Canine_Influenza_Virus.pdf
115. Pecoraro HL, Bennett S, Quintana AM, et al. Comparison of the infectivity and transmission of contemporary canine and equine H3N8 influenza viruses in dogs. Submitted 2012.
116. Office International des Epizooties. Manual of diagnostic tests and vaccines for terrestrial animals. Paris, France: OIE; 2008.
117. Herrmann B, Larsson C, Zweygberg BW. Simultaneous detection and typing of influenza viruses A and B by a nested reverse transcription-PCR: comparison to virus isolation and antigen detection by immunofluorescence and optical immunoassay (FLU OIA). Journal of Clinical Microbiology 2000;39:134-138.
118. Liao RS, Landt O, Hill JT. Comparison of a laboratory-developed RT-PCR and the CDC RT-PCR protocol with rapid immunodiagnostic testing during the 2009 H1N1 influenza A pandemic. Diagnostic Microbiology and Infectious Disease 2011;70:236-239.
119. Gröndahl B, Puppe W, Weigl J, et al. Comparison of the BD Directigen Flu A+B Kit and the Abbott TestPack RSV with a multiplex RT-PCR ELISA for rapid detection of influenza viruses and respiratory syncytial virus. Clinical Microbiology and Infection 2005;11:848-850.
120. Landolt GA, Karasin AI, Hofer C, et al. Use of real-time reverse transcriptase polymerase chain reaction assay and cell culture methods for detection of swine influenza A viruses. American Journal of Veterinary Research 2005;66:119-123.
121. Lu Z, Chambers TM, Boliar S, et al. Development and evaluation of one-step TaqMan real-time reverse transcription-PCR assays targeting nucleoprotein, matrix, and hemagglutinin genes of equine influenza virus. Journal of Clinical Microbiology 2009;47:3907-3913.
122. Munch M, Nielsen LP, Handberg KJ, et al. Detection and subtyping (H5 and H7) of avian type A influenza virus by reverse transcription-PCR and PCR-ELISA. Archives of Virology 2001;146:87-97.
123. Chidlow G, Harnett G, Williams S, et al. Duplex real-time reverse transcriptase PCR assays for rapid detection and identification of pandemic (H1N1) 2009 and seasonal influenza A/H1, A/H3, and B viruses. Journal of Clinical Microbiology 2010;48:862-866.
124. Roa PL, Catalán P, Giannella M, et al. Comparison of real-time RT-PCR, shell vial culture, and conventional cell culture for the detection of the pandemic influenza A (H1N1) in hospitalized patients. Diagnostic Microbiology and Infectious Disease 2011;69:428-431.

125. Zou S. A practical approach to genetic screening for influenza virus variants. Journal of Clinical Microbiology 1997;35:2623-2627.
126. Branscum AJ, Gardner IA, Johnson WO. Estimation of diagnostic-test sensitivity and specificity through Bayesian modeling. Preventive Veterinary Medicine 2005;68:145-163.
127. Spiegelhalter D, Thomas A, Best N, et al. BUGS: Bayesian inference using Gibbs sampling. Version 0.50. Cambridge: MRC Biostatistics Unit; 1996.
128. Barrell EA, Pecoraro HL, Torres-Henderson C, et al. Seroprevalence and risk factors for canine influenza virus (H3N8) exposure in household dogs in Colorado. Journal of Veterinary Internal Medicine 2010;24:1524-1527.
129. Lunn DJ, Thomas A, Best N, et al. WinBUGS -- a Bayesian modelling framework: concepts, structure, and extensibility. Statistics and Computing 2000;10:325--337.
130. Serra VF, Stanzani G, Smith G, et al. Point seroprevalence of canine influenza virus H3N8 in dogs participating in a flyball tournament in Pennsylvania. Journal of the American Veterinary Medical Association 2011;238:726-730.
131. Pecoraro HL, Spindel ME, Bennett S, et al. Evaluation of virus isolation, one-step real-time reverse transcriptase-PCR assay, and rapid influenza diagnostic tests for detecting canine influenza virus (H3N8) infection in dogs. Submitted 2012.
132. Kruth SA, Carman S, Weese JS. Seroprevalence of antibodies to canine influenza virus in dogs in Ontario. Canadian Veterinary Journal 2008;49:800-802.
133. Knesl O, Allan FJ, Shields S. The seroprevalence of canine respiratory coronavirus and canine influenza virus in dogs in New Zealand. New Zealand Veterinary Journal 2009;57:295-298.
134. Liu S, Kim WI, Johnson J. Characterization and surveillance of canine influenza virus in Iowa. American Society for Virology 28th Annual Meeting Vancouver, B.C. 2009;326.
135. Landolt GA, Karasin AI, Phillips L, et al. Comparison of the pathogenesis of two genetically different H3N2 influenza A viruses in pigs. Journal of Clinical Microbiology 2003;41:1936-1941.
136. Palmer DF, Dowdle WR, Coleman MT, et al. Advanced laboratory techniques for influenza diagnosis. In: Health USDo, ed. Washington, D. C.: 1975.
137. Baldi B, Moore DS. The Practice of Statistics in the Life Sciences. New York, NY: W.H. Freeman Company; 2009.
138. Anderson TC, Bromfield CR, Crawford PC, et al. Serological evidence of H3N8 canine influenza-like virus circulation in USA dogs prior to 2004. Veterinary Journal 2012;191:312-316.
139. Brankston G, Gitterman L, Hirji Z, et al. Transmission of influenza A in human beings. Lancet Infectious Diseases 2007;7:257-265.
140. Lunn DP, Hussey S, Sebing R, et al. Safety, efficacy, and immunogenicity of a modified-live equine influenza virus vaccine in ponies after induction of exercise-induced immunosuppression. Journal of the American Veterinary Medical Association 2001;218:900-906.
141. Murphy EA, Davis JM, Carmichael MD, et al. Exercise stress increases susceptibility to influenza infection. Brain, Behavior, and Immunity 2008;22:1152-1155.
142. Avitsur R, Hunzeker J, Sheridan JF. Role of early stress in the individual differences in host response to viral infection. Brain, Behavior, and Immunity 2006;20:339-348.
143. Raosoft. Sample size calculator. 2004. Available at http://www.raosoft.com/samplesize.html
144. Rowe T, Abernathy RA, Hu-Primmer J, et al. Detection of antibody to avian influenza A (H5N1) virus in human serum by using a combination of serologic assays. Journal of Clinical Microbiology 1999;37:937-943.
145. Lowen AC, Mubarenka S, Steel J, et al. Influenza virus transmission is dependent on relative humidity and temperature. PLoS Pathogens 2007;3:1470-1476.
146. Druett HA, May KR. Unstable germicidal pollutant in rural air. Nature 1968;220:395-396.
147. Kekez MM, Sattar SA. A new ozone-based method for virus inactivation: preliminary study. Physics in Medicine and Biology 1997;42:2027-2039.
148. McKenzie EC, Jose-Cunilleras E, Hinchcliff KW, et al. Serum chemistry alterations in Alaskan sled dogs during five successive days of prolonged endurance exercise. Journal of the American Veterinary Medical Association 2007;230:1486-1492.
149. World Health Organization. Manual on animal influenza diagnosis and surveillance. Geneva, Switzerland: World Health Organization Global Influenza Programme; 2002.

150. Yamagishi H, Nagamine T, Shimoda K, et al. Comparative measurement of equine influenza virus antibodies in horse sera by single radial hemolysis, neutralization, and hemagglutination inhibition tests. Journal of Clinical Microbiology 1982;15:660-662.
151. Litster A, Allen J, Mohamed A, et al. Risk factors for delays between intake and veterinary approval for adoption on medical grounds in shelter puppies and kittens. Preventive Veterinary Medicine 2011;101:107-112.
152. Newbury S, Blinn MK, Bushby PA, et al. Guidelines for standards of care in animal shelters. The Association of Shelter Veterinarians. 2010.
153. Pecoraro HL, Lee JS, Achenbach J, et al. Seroprevalence of canine influenza virus (H3N8) in Iditarod racing sled dogs. Canadian Veterinary Journal 2012;In press.
154. Bonabeau E, Toubiana L, Flahault A. The geographical spread of influenza. Proceeding of the Royal Society Biological Sciences 1998;265:2421-2425.
155. Lemey P, Suchard M, Rambaut A. Reconstructing the initial global spread of a human influenza pandemic. PLoS Currents 2009;1:RRN1031.
156. SAS. SAS (r) Proprietary Software 9.3. In. Cary, NC: SAS Institute Inc.; 2002-2010:Copyright, SAS Institute Inc. SAS and all other SAS Institute Inc. product or service names are registered trademarks or trademarks of SAS Institute Inc., Cary, NC, USA.
157. Sugiura N. Further analysis of the data by Akaike's information criterion and the finite corrections. Communications in Statistics - Theory and Methods 1978;A7:13-26.
158. Hurvich CM, Tsai C-L. Regression and time series model selection in small samples. Biometrika 1989;76:297-307.
159. Burnham KP, Anderson DR. Model Selection and Multimodel Inference: A Practical Information-theoretic Approach, 2nd ed. New York, NY: Springer-Verlag; 2002.
160. Akaike H. A new look at the statistical model identification. IEEE Transactions on Automatic Control 1974;19:716-723.
161. Konishi S, Kitagawa G. Information criteria and statistical modeling. New York, NY: Springer; 2008.
162. Shtatland ES, Moore S, Barton MB. Why we need an R^2 measure of fit (and not only one) in PROC LOGISITC and PROC GENMOD. In: SAS Users Group International. Indianapolis, IN: Statistics and Data Analysis; 2000.
163. Pfeiffer DU, Minh PQ, Martin V, et al. An analysis of the spatial and temporal patterns of highly pathogenic avian influenza occurrence in Vietnam using national surveillance data. Veterinary Journal 2007;174:302-309.
164. Wiley DC, Skehel JJ. The structure and function of the hemagglutinin membrane glycoprotein of influenza virus. Annual Reviews in Biochemestry 1987;56:365-394.
165. Couch RB, Kasel JA. Immunity to influenza in man. Annual Reviews in Microbiology 1983;37:529-549.
166. Both GW, Sleigh MJ, Cox NJ, et al. Antigenic drift in influenza virus H3 hemagglutinin from 1968 to 1980: multiple evolutionary pathways and sequential amino acid changes at key antigenic sites. Journal of Virology 1983;48:52-60.
167. Stray SJ, Pittman LB. Subtype- and antigenic site-specific difference in biophysical influences on evolution of influenza virus hemagglutinin. Virology 2012;9:91.
168. Smith DJ, Lapedes AS, de Jong JC, et al. Mapping the antigenic and genetic evolution of influenza virus. Science 2004;305:371-376.
169. Anderson TC, Crawford PC, Katz JM, et al. Diagnostic performance of the canine influenza A virus subtype H3N8 inhibition assay. Journal of Veterinary Diagnostic Investigation 2012;24:499-508.
170. Pecoraro HL, Bennett S, Huyvaert KP, et al. Epidemiology and ecology of canine influenza viruses (H3N8) in U.S. shelter dogs. Submitted 2012.
171. World Health Organization. WHO manual on animal influenza diagnosis and surveillance. In: Response DoCDSa, ed. World Health Organization; 2002.
172. Drummond AJ, Ashton B, Buxton S, et al. Geneious v5.4. 2011. Available at http://www.geneious.com/
173. Tamura K, Peterson D, Peterson N, et al. MEGA5: Molecular Evolutionary Genetics Analysis using Maximum Likelihood, Evolutionary Distance, and Maximum Parsimony Methods. Molecular Biology and Evolution Submitted 2011.
174. Kosakovsky Pond SL, Frost SD. Datamonkey: rapid detection of selective pressure on individual sites of codon alignments. Bioinformatics 2005;21:2531-2533.

175. Delport W, Poon AP, Frost SD, et al. Datamonkey 2010: a suite of phylogenetic analysis tools for evolutionary biology. Bioinformatics 2010;26:2455-2457.
176. Kosakovsky Pond SL, Frost SD, Muse SV. HyPhy: hypothesis testing using phylogenies. Bioinformatics 2005;21:676-679.
177. Kryazhimskiy S, Plotkin JB. The population genetics of dN/dS. PLoS Pathogens 2008;4:1-10.
178. Kosakovsky Pond SL, Murrell B, Fourment M, et al. A random effects branch-site model for detecting episodic diversifying selection. Molecular Biology and Evolution 2011;28:3033-3043.
179. Lamb JR, Green N. Analysis of the antigen specificity of influenza haemagglutinin-immune human T lymphocyte clones: identification of an immunodominant region for T cells. Immunology 1983;50:659-666.
180. Trib.com. Shelter will destroy dogs. In: Trib.com. Wyoming: Trib.com; 2006. Available at http://trib.com/news/state-and-regional/shelter-will-destroy-dogs/article_be937afd-acd8-55c9-b107-b16a25199ce7.html
181. Long J, Bushnell RV, Tobin JK, et al. Evolution of H3N2 influenza virus in a guinea pig model. PLoS One 2011;6:1-9.
182. Chen J, Wharton SA, Weissenhorn W, et al. A soluble domain of the membrane-anchoring chain of influenza virus hemagglutinin (HA2) folds in *Escherichia coli* into the low-pH-induced conformation. PNAS 1995;92:12205-12209.
183. Ward CW, Dopheide TA. Amino acid sequence and oligosaccharide distribution of the haemagglutinin from an early Hong Kong influenza virus variant A/Aichi/2/68 (X-31). Biochemistry Journal 1981;193:953-962.
184. Larson LJ, Henningson J, Sharp P, et al. Efficacy of the canine influenza virus H3N8 vaccine to decrease severity of clinical disease after cochallenge with canine influenza virus and *Streptococcus equi* subsp. *zooepidemicus*. Clinical and Vaccine Immunology 2011;18:559-564.
185. Quintana AM, Landolt GA, Annis KM, et al. Immunological characterization of the equine airway epithelium and of a primary equine airway epithelial cell culture model. Veterinary Immunology and Immunopathology 2011;140:226-236.
186. Song D, Kang B, Lee C, et al. Transmission of avian influenza virus (H3N2) to dogs. Emerging Infectious Diseases 2008;14:741-746.
187. Lee C, Song D, Kang B, et al. A serological survey of avian orgin canine H3N2 influenza virus in dogs in Korea. Veterinary Microbiology 2009;137:359-362.
188. Lin Y, Zhao Y, Zeng X, et al. Genetic and pathobiologic characterization of H3N2 canine influenza viruses isolated in the Jiangsu Province of China in 2009–2010. Veterinary Microbiology 2012;158:247-258.
189. Song D, Lee C, Kang B, et al. Experimental infection of dogs with avian-origin canine influenza A virus (H3N2). Emerging Infectious Diseases 2009;15:56-58.
190. Song DS, An DJ, Moon HJ, et al. Interspecies transmission of the canine influenza H3N2 virus to domestic cats in South Korea, 2010. Journal of General Virology 2011;92:2350-2355.
191. Tumpey TM, Basler CF, Aguilar PV, et al. Characterization of the reconstructed 1918 Spanish influenza pandemic virus. Science 2005;310:77-80.
192. Hatta M, Halfmann P, Wells K. Human influenza a viral genes responsible for the restriction of its replication in duck intestine. Virology 2002;295:250-255.
193. Dalton RM, Mullin AE, Amorim AJ. Temperature sensitive influenza A virus genome replication results from low thermal stability of polymerase-cRNA complexes. Virology 2006;3:58.
194. Hiromoto Y, Yamazaki Y, Fukushima T. Evolutionary characterization of the six internal genes of H5N1 human influenza A virus. Journal of General Virology 2000;81:1293-1303.
195. Subbarao EK, London W, Murphy BR. A single amino acid in the PB2 gene of influenza A virus is a determinant of host range. Journal of Virology 1993;67:1761-1764.
196. Tian SF, Buckler-White AJ, London WT. Nucleoprotein and membrane protein genes are associated with restriction of replication of influenza A/Mallard/NY/78 virus and its reassortants in squirrel monkey respiratory tract. Journal of Virology 1985;53:771-775.
197. Bender C, Hall H, Huang J. Characterization of the surface proteins of influenza A (H5N1) viruses isolated from humans in 1997-1998. Virology 1999;254:115-123.
198. Suarez DL, Perdue ML, Cox N. Comparisons of highly virulent H5N1 influenza A viruses isolated from humans and chickens from Hong Kong. Journal of Virology 1998;72:6678-6688.

199. Zhou NN, Shortridge KF, Claas EC. Rapid evolution of H5N1 influenza viruses in chickens in Hong Kong. Journal of Virology 1999;73:3366-3374.
200. Fang R, Min Jou W, Huylebroeck D. Complete structure of A/duck/Ukraine/63 influenza hemagglutinin gene: animal virus as progenitor of human H3 Hong Kong 1968 influenza hemagglutinin. Cell 1981;25:315-323.
201. Gething MJ, Bye J, Skehel J. Cloning and DNA sequence of double-stranded copies of haemagglutinin genes from H2 and H3 strains elucidates antigenic shift and drift in human influenza virus. Nature 1980;287:301-306.
202. Kawaoka Y, Krauss S, Webster RG. Avian-to-human transmission of the PB1 gene of influenza A viruses in the 1957 and 1968 pandemics. Journal of Virology 1989;63:4603-4608.
203. Dillion S, Spindel ME, Landolt GA. Genetic characterization of canine and equine H3N8 influenza viruses isolated in Colorado and Wyoming between 2006 and 2007. In: American Society for Virology 27th Annual Meeting. Ithaca, NY: 2008.
204. Lunn DP, Soboll G, Schram BR, et al. Antibody responses to DNA vaccination of horses using the influenza virus hemagglutinin gene. Vaccine 1999;17:2245-2258.
205. Karasin AI, Brown IH, Carman S, et al. Isolation and characterization of H4N6 avian influenza viruses from pigs with pneumonia in Canada. Journal of Virology 2000;74:9322-9327.
206. Landolt G, Karasin AI, Hofer C, et al. Detection of swine influenza A viruses in pooled nasal swab samples using real-time TaqMan RT-PCR. American Journal of Veterinary Research 2005;66:119-124.
207. Spindel MF, Dillon S, Lunn KF, et al. Detection and quantification of canine influenza virus by one-step real-time reverse transcription PCR. Journal of Veterinary Internal Medicine 2007;21:576.
208. Bateman AC, Busch MG, Karasin KI, et al. Amino acid 226 in the hemagglutinin of H4N6 for α2,6-linked sialic acid and infectivity levels in primary swine and human respiratory epithelial cells. Journal of Virology 2008;82:8204-8209.
209. Hall JS, Bentler KT, Landolt G, et al. Influenza infection in wild raccoons. Emerging Infectious Diseases 2008;14:1842-1848.
210. Eisen MB, Sabesan S, Skehel JJ, et al. Binding of the influenza A virus to cell-surface receptors: structures of five hemagglutinin-sialyloligosaccharide complexes determined by X-ray crystallography. Virology 1997;232:19-31.
211. Weis W, Brown JH, Cusack S, et al. Structure of the influenza virus hemagglutinin complexed with its receptor, sialic acid. Nature 1988;333:426-431.
212. Wang Q, Tian X, Chen X, et al. Structural basis for receptor specificity of influenza B virus hemagglutinin. PNAS 2007;104:16874-16879.
213. Masunaga K, Mizumoto K, Kata H, et al. Molecular mapping of influenza virus RNA polymerase by site-specific antibodies. Virology 1999;256:130-141.

APPENDIX A: COMPARISON OF THE INFECTIVITY AND TRANSMISSION OF CONTEMPORARY CANINE AND EQUINE H3N8 INFLUENZA VIRUSES IN DOGS

INTRODUCTION

Due to the partial host range restriction of influenza A viruses, transmission of an influenza virus from one species to another is relatively rare. However, such cross-species transmission events do occur and have generated severe disease outbreaks in the new host species. The 1918 "Spanish Flu" is a classic example of cross-species transmission with devastating results, as the influenza virus that caused the pandemic was likely transmitted directly from birds to humans.[191] Therefore, understanding the molecular mechanisms that allow these viruses to cross the species barrier and adapt to new hosts is crucial for identifying influenza viruses that could potentially threaten both human and animal health. While evidence has accumulated over the years indicating contributions by all eight gene segments,[87-90,192-196] the examination of the impact of individual viral proteins to host range restriction is complicated by several factors. For instance, mutations often occur in multiple gene segments during the process of virus adaptation to a new species,[194,197-199] and, while some of these mutations may indeed reflect adaptation of the virus to the new host, others may be introduced in response to host immune pressure or they might simply represent spurious mutations. Furthermore, cross-species transmission of influenza is frequently preceded by an exchange of gene segments between two viruses ("genetic reassortment"), resulting in even greater genetic variability.[200-202]

Historically, dogs were not considered to be natural hosts for influenza despite occasional transmission of viruses to dogs from humans,[95,96] birds,[186] and horses.[9,10] In 2004, however, influenza was reported in racing greyhounds and the virus has since been maintained in dog populations throughout the U.S.[7,8] Phylogenetic analyses reveal that canine influenza virus (CIV) isolates are closely related to, and evolved from, the equine influenza virus (EIV) H3N8 Florida sublineage.[7] Amino acid sequence analyses demonstrate that the CIV isolates consistently differ from contemporary equine-lineage H3 viruses at five amino acid residues in the hemagglutinin protein (HA), including a tryptophan (W) to leucine (L) substitution at residue 222 located near the receptor binding pocket[7,8] and seven amino acid mutations within the internal genes.[7,8,203] Moreover, as the equine H3N8 virus was transmitted to

dogs without prior genetic reassortment,[7] it is possible that specific constellations of gene segments do not play a role in controlling the equine-to-canine species barrier.

Interestingly, results from two recent studies demonstrate that CIV isolates are unable to infect, replicate, and spread among susceptible horses.[105,109] Moreover, inoculation of horses with canine influenza virus did not result in clinical disease in either study, indicating the existence of genetic differences in the horse that resulted in an "all or nothing" infection when inoculated with EIV or CIV, respectively. To determine whether contemporary equine viruses are similarly restricted in dogs, we evaluated the infectivity and transmission of a recent EIV isolate in dogs. Additionally, we sought to determine the receptor binding affinity of recent CIV isolates to examine whether the HA W222L mutation has resulted in an alteration in receptor binding affinity of canine isolates.

MATERIALS/METHODS

Influenza Viruses. For the binding assays, field isolates of A/equine/CO/10/07 (Eq/CO) (H3N8), A/canine/CO/224986/06 (Ca/CO-1) (H3N8), A/canine/WY/86033/07 (Ca/WY) (H3N8), A/canine/CO/2025974/07 (Ca/CO-2) (H3N8), A/equine/KY/1/81(H3N8) (Eq/KY; provided as allantoic fluid stock from the University of Wisconsin-Madison's Influenza Virus Repository), and A/Sydney/05/97 (H3N2) (A/Syd; provided as allantoic fluid stocks from the CDC) were cultivated in embryonated chicken eggs or MDCK cells as previously described.[135,204] The Eq/CO and Ca/WY isolates were used for inoculation of dogs in the *in vivo* challenge and were passaged in 10-day old embryonated chicken eggs and MDCK cells. All gene segments from the Eq/CO and Ca/WY isolates used for inoculation were sequenced and compared with their respective parental virus sequences.

Sequence Analyses. To confirm the presence of the amino acid differences between the equine and canine H3 viruses, the full-length protein coding regions of the HA genes of Eq/CO, Ca/CO-1, Ca/WY, and Ca/CO-2 were amplified by reverse transcriptase (RT)-PCR, as previously described.[205] Sequence comparisons of these viruses, as well as published equine and canine influenza virus H3 sequences (obtained from the BLAST database [http://blast.ncbi.nlm.nih.gov]), were made using Clustal W alignment (http://www.genome.jp/tools/clustalw/).

Equine and Canine Influenza Challenge. Twenty 12-month old dogs were obtained from a commercial laboratory animal vendor and assigned to one of five groups: two inoculation groups (five dogs/group), one mock-inoculation control group (four dogs/group), or two sentinel groups (three dogs/group). Study groups were housed separately in nearby research facilities. Serum samples from each dog were confirmed to be CIV- and EIV-negative using hemagglutination inhibition (HI) assay before inoculation. The animals were examined prior to inoculation and found to be clinically healthy and in good body condition. Dogs were maintained in accordance with guidelines of the Colorado State University Research and Animal Resources Committee. One dog from the Ca/WY inoculation group was withdrawn due to behavioral issues with other dogs in the group. Inoculated groups were infected with either Ca/WY or Eq/CO via intra-tracheal aerosol exposure with 10^7 50% tissue culture infectious dose ($TCID_{50}$) after they were pre-medicated with midazolam and butorphanol, anesthetized by intravenous administration of 4-6 mg/kg of propofol, and intubated. Sentinel groups were introduced and housed with each infection group two days post inoculation. Before initiation, this study was reviewed and approved for conduct by the Colorado State University Institutional Animal Care and Use Committee.

Clinical Scoring. For 14 days following inoculation, each dog was observed 20 minutes for clinical signs of infection. Clinical scoring consisted of numerical scoring based on observations of lethargy, anorexia, sneezing, coughing, ocular and nasal discharge, respiratory rate, and temperature, as previously described.[109] Briefly, parameters assessed included general attitude, cough, nasal discharge (these were quantified on a scale of 0 to 3, with 0 indicating a clinically normal animal and 3 indicating a severely abnormal animal), food intake (with 0 indicating that the animal was eating and 1 indicating that the animal was anorexic), respiratory rate (number of breaths per minute), and rectal temperature.

Assessment of Viral Shedding. Nasal swabs were collected daily one day before and for 21 days after inoculation. The swabs were placed in 1 ml of viral transport medium and stored at -80°C until they could be processed for influenza virus isolation. RNA was extracted from 140μl of viral transport medium using the QIAamp® Viral RNA Mini Kit (QIAGEN, Hilden, Germany). Real-time RT-PCR assays were performed using previously established cycling conditions.[206] Briefly, for virus quantification, purified full-length influenza A matrix (M) gene RNA was used as a standard for calibration of the M gene copy number. Each nasal swab sample was run in duplicate. Negative controls included neat transport medium

processed as for the nasal swab specimens. The positive controls consisted of 10^1 to 10^6 $TCID_{50}$ of A/canine/CO/224986/06 in distilled water.

Serological Analysis. Sera from blood samples taken from inoculated dogs on days 7, 12, and 19 post inoculation and from sentinel dogs on days 7, 12, and 19 post introduction were treated with receptor destroying enzyme prepared from *Vibrio cholera* before they were tested for hemagglutination inhibiting antibodies via HI assay.[135] Briefly, two-fold serial dilutions of sera were mixed with four hemagglutination units of Eq/CO and Ca/WY. The assays were developed by adding 0.5% (vol/vol) chicken red blood cells, and the HI antibody titers were read as the reciprocal of the highest dilution causing complete inhibition of hemagglutination.

Virus Binding Affinities. To account for any cell-culture induced mutations, sialic acid (SA) binding affinities of both MDCK cell- and embryonated chicken egg-grown stocks of Ca/CO-1, Ca/WY, Ca/CO-2, and Eq/CO were determined using a solid-phase binding assay,[30,31] with slight modification of the original protocol to equilibrate the viruses assayed to ~20,000 matrix gene copies.[207,208] The biotinylated glycopolymers tested included Neu5Acα2-3Galβ1-4Glc-PAA[1000]-biot (2,3SL), Neu5Acα2-3Galβ1-4GlcNAcβ-PAA[1000]-biot (2,3SLN), Neu5Acα2-6Galβ1-4GlcN-PAA[1000]-biot (2,6SL), and Neu5Acα2-6Galβ1-4GlcNAcβ-PAA[1000]-biot (2,6SLN) (Syntesome, Moscow, Russia). All polymers had a molecular weight of 1MDa and were diluted 1:500 prior the experiments. Both fetuin- and non-fetuin-coated plates were utilized, as conditions for performing the solid-phase binding assay have not yet been described for use with CIV and EIV isolates. Each assay was performed four times in duplicate, including positive (Eq/KY and Eq/CO for SAα2,3 binding and A/Syd for SAα2,6 binding) and negative (working buffer) controls.

Sialic Acid Staining. To investigate whether the receptor binding specificity of the canine viruses reflect complementary SA receptor expression in the respiratory tract of dogs, we stained sections of canine nasal mucosa, larynx, trachea, bronchus, and lung tissue with SAα2,3-gal-specific and SAα2,6-gal specific lectins.[35,209] Fluorescein isothiocyanate (FITC)-labeled *Sambucus nigra* lectin (Vector Laboratories, Burlingame, CA) was used to indicate the presence of SAα2,6-gal, while biotinylated *Maackia amurensis* lectin (Vector Laboratories) detected with Alexa Fluor 594-streptavidin complex (Molecular Probes/Invitrogen, Carlsbad, CA) was used to stain SAα2,3-gal receptors. Tissues were

counterstained with DAPI (4,6-diamidino-2-phenylindole). Equine airway tissues collected from the same anatomical locations were included in the staining procedure to serve as controls, as the pattern of SA expression in the horse trachea has been previously defined.[37]

Statistical Analyses. To analyze the overall mean differences in the levels of virus nasal shedding, HI antibody titers, clinical scores, and body temperatures between the infected groups and the mock-inoculated controls, we performed generalized estimating equations, as previously described.[109] Briefly, adjusted mean differences were clustered on repeated measures for each outcome. Other outcome variables (except clinical scores, which were ranked prior to analysis) were log transformed to meet the major assumptions of linearity and normality. For data transformation, HI antibody titers and M gene copy numbers with values of zero were converted to one. For the receptor-binding assays, dissociation constants (K_D) for viruses were determined by linear regression analysis of Scatchard plots performed using Prism software (GraphPad, La Jolla, CA).

RESULTS

For the *in vivo* challenge, both Ca/WY and Eq/CO inoculated dogs showed little to no evidence of clinical disease. None of the dogs had a temperature over 103°F during the course of the study. One sentinel dog and one inoculated dog in the Ca/WY group were observed coughing, sneezing, and with serous ocular discharge between days 5 and 12 post inoculation (between days 3 and 10 post introduction for the sentinel). From the Eq/CO group, one inoculated dog had periodic anorexia and sneezing between days 3 and 13 post inoculation and one sentinel showed signs of lethargy on day 3 post introduction. Although little clinical disease was evident, serologically, 4/4 (100%) of the Ca/WY-inoculated dogs seroconverted by day 12 after

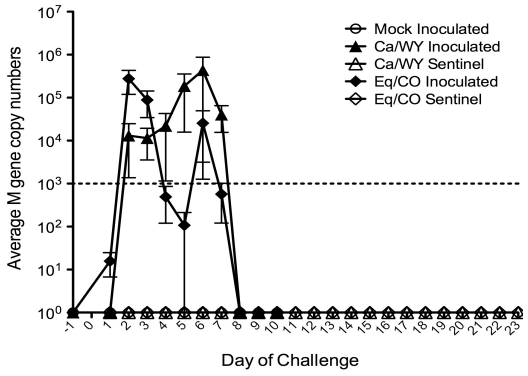


Figure A-1: The M gene copy number mean ± SEM of virus shed in nasal passages for mock-inoculated (○), Ca/WY-inoculated (▲), Ca/WY sentinel (□), Eq/CO-inoculated (□), and Eq/CO sentinel (□) dogs. The minimum detection level of the real-time RT-PCR was 1000 M gene copies per reaction corresponding to 10^3 $TCID_{50}$ of Ca/CO-1 and is represented by the dashed line.

inoculation (mean titer 1:896 ± 368 SEM) and 2/3 (67%) of the CIV-infected dog sentinels had positive HI assays (mean titer 1:1365 ± 557 SEM) by day 12 after introduction. Of the Eq/CO-inoculated dogs, 80% (4/5) seroconverted (mean titer 1:461 ± 152 SEM) by day 12 after inoculation, although none of the sentinels had a positive HI assay through day 21. Real-time RT-PCR data resemble the HI data for the inoculated dogs from both groups (Figure A-1). Interestingly, influenza virus was not detected by real time RT-PCR in neither the Ca/WY nor the Eq/CO sentinel groups. The negative controls did not shed detectable virus, as expected. Generalized estimating equations revealed that, compared to the negative controls, only nasal shedding and antibody titers were significantly higher for both the Ca/WY and Eq/CO inoculated dogs (Table A-1) compared to the mock-inoculated negative controls.

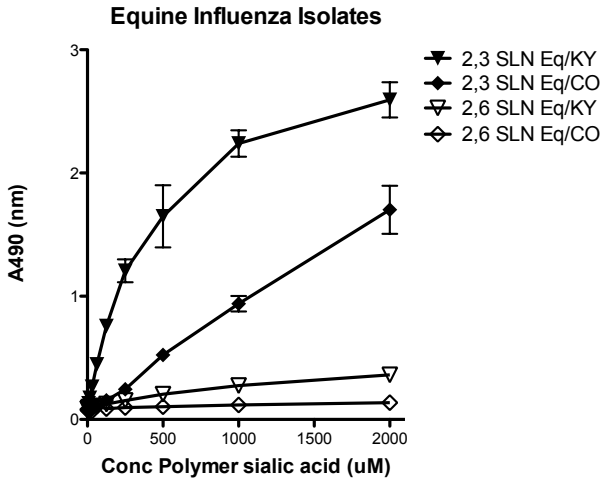
Table A-1: Statistical analyses of Ca/Wy and Eq/CO challenge dog groups compared to mock-inoculated negative controls.

	Temperature	Clinical Score	Nasal Shedding	HIA Titer
Ca/WY Inoculated	P=0.077	P=0.460	P=0.001*	P<0.001*
Ca/WY Sentinel	P=0.608	P=0.130	P=0.704	P=0.185
Eq/CO Inoculated	P=0.155	P=0.103	P=0.018*	P<0.001*
Eq/CO Sentinel	P=0.691	P=0.474	P=0.704	P=0.175

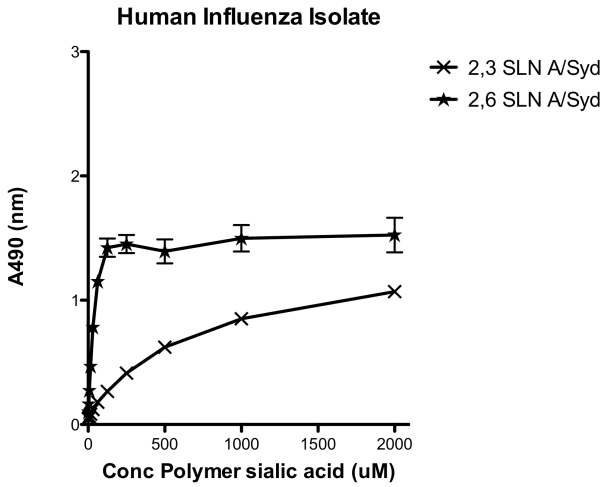
*Indicates statistical significance

With the exception of A/Syd, which bound only to fetuin-coated plates (as previously described for human influenza viruses[30,31]), all equine and canine viruses bound only to non-fetuin-coated plates. As the 2,3SL polymer demonstrated similar binding as the 2,3SLN polymer and the 2,6SL polymer is not believed to be an suitable analog for the human influenza virus receptor,[210] only the 2,3SLN and 2,6SLN data are presented here for appropriate comparisons. Based on calculated approximate K_D values (where lower values represent higher binding affinity), Eq/CO and Eq/KY demonstrated the anticipated binding preference for SA SAα2,3-gal (2,3SLN) compared to 2,6SLN (Figure A-2a). In fact, nearly no binding to SAα2,6-gal (2,6SLN) was detected. Likewise, the SAα2,6-gal control (A/Syd) preferred 2,6SLN as expected (Figure A-2b). Interestingly, Ca/CO-1, Ca/WY, and Ca/CO-2 showed the same binding preference as the equine viruses, which was characterized by higher affinity for SAα2,3-gal (2,3SLN) and only minimal binding to 2,6SLN (Figure A-2c). Table A-2 lists the K_D values for all the viruses tested with the 2,3SLN and 2,6SLN polymers. Because there was no detectable binding (NDB) to the 2,6SLN

A-2a



A-2b



A-2c

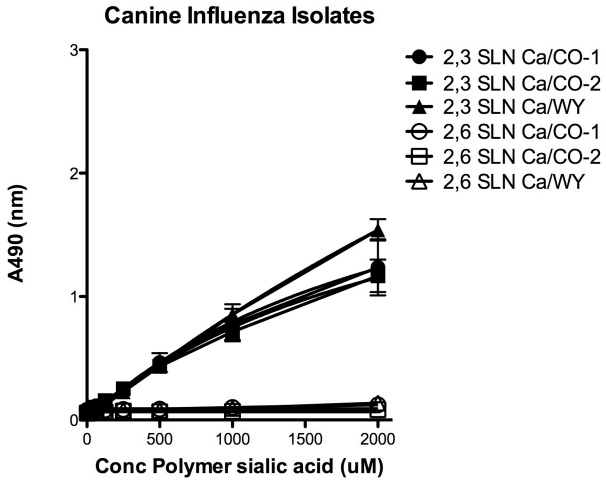


Figure A-2: α2,3SLN and α2,6SLN polymer binding by equine influenza (a), human influenza (b), and canine influenza (c) isolates were determined using a solid-phase binding assay and linear regression analysis.

Table A-2. Approximate binding affinity of equine and canine influenza viruses

	2,3 SLN Polymer		2,6 SLN Polymer	
Virus	App K_D[a]	R^2[b]	App K_D	R^2[b]
Eq/KY	11	.96	NDB[c]	-
Eq/CO	5	.99	NDB[c]	-
Ca/CO-1	5	.99	NDB[c]	-
Ca/CO-2	2	.94	NDB[c]	-
Ca/WY	2	.95	NDB[c]	-
A/Syd	139	.97	10	.99

[a] Approximate dissociation constant (K_D) values are from one representative experiment. Lower K_D values represent a higher binding affinity for that polymer (K_D is expressed in nM-1 sialic acid). Repeated experiments yielded similar results.
[b] R^2 = coefficient of determination.
[c] NDB = no detectable binding.

polymer for the canine and equine isolates upon linear regression of Scatchard plots, we were unable to determine their K_D values. However, viruses that bound to the 2,3SLN polymer demonstrated low K_D values, indicating that they had high binding affinities for SAα2,3-gal. Again, as expected, A/Syd had a human type receptor binding preference with a higher relative affinity for SAα2,6-gal than for SAα2,3-gal receptor analogues. The staining results revealed that SAα2,3-linked receptors (indicated by the red staining in Figure A-3) was the predominant receptor expressed on the airway epithelial cells of the upper respiratory tract in both horses and dogs. Furthermore, SAα2,3-gal was the primary receptor expressed on the respiratory epithelium throughout the trachea (upper, middle, and lower) and bronchus in both species. This is consistent with recently published data [9] that also found a predominance of SAα2,3-gal in the canine trachea. In contrast, alveoli demonstrated both red and green staining, suggesting that both SAα2,3-gal and SAα2,6-gal receptors are expressed deep within the respiratory tract of dogs and horses.

Review of the HA amino acid sequences indicated that the five amino acids previously described as possible dog adaptation mutations (N54K, N83S, W222L, I328T, N483T)[7,8] have been conserved in the canine viruses cultivated for use in these studies. In contrast, Eq/CO shared the previously described equine H3 consensus sequence.[7] Sequences of the Ca/WY and Eq/CO isolates used for inoculation did not differ from their respective parental virus sequences.

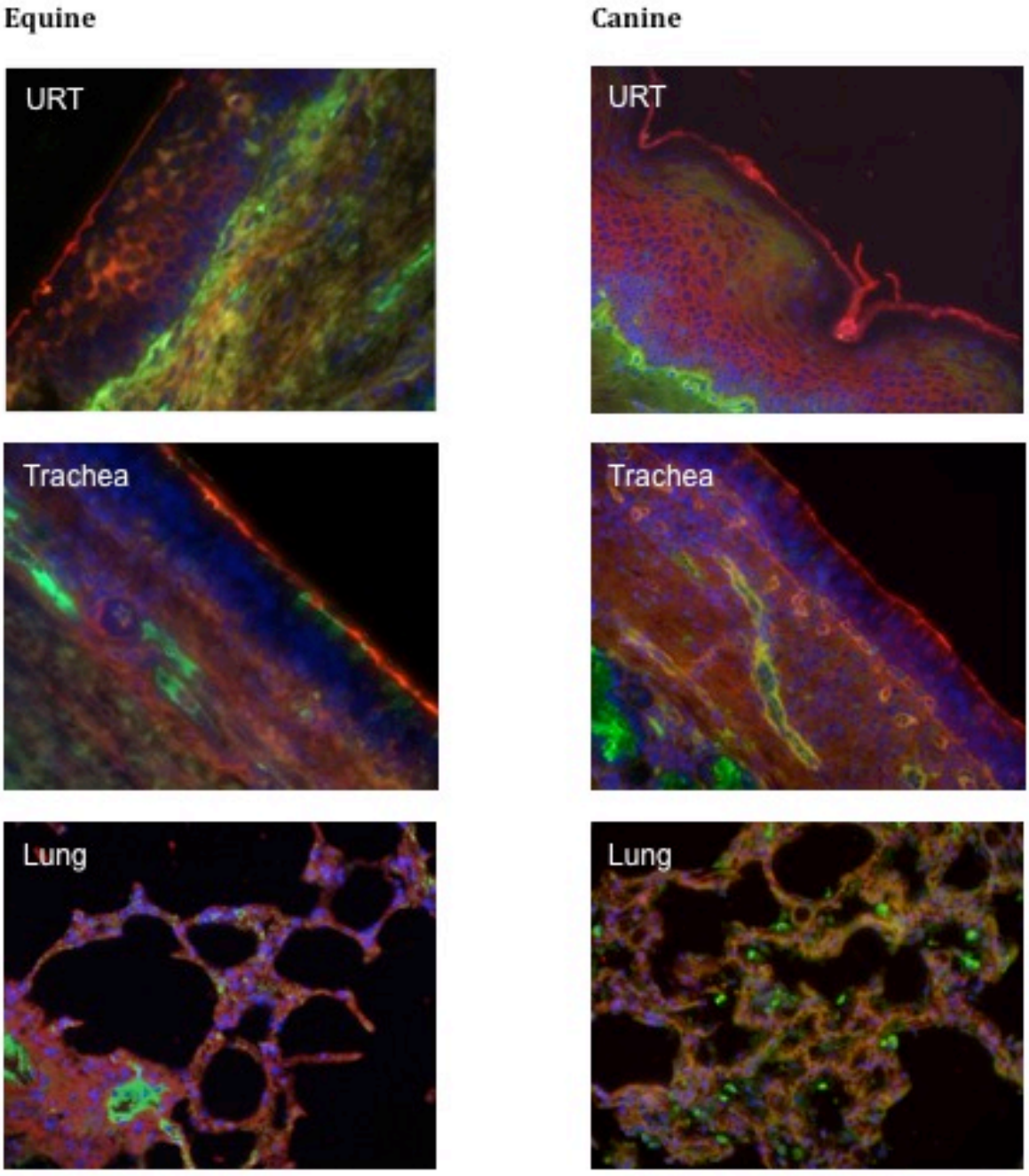


Figure A-3: Equine and canine respiratory tissues for the upper respiratory tract (URT) and lower respiratory tract (trachea, lung) stained with lectins specific for sialic acids with α2,6 - and α2,3-linkages. Green staining: reaction with fluorescein isothiocyanate (FITC)-labeled *Sambucus nigra* lectin (Vector Laboratories, Burlingame, CA) indicating the presence of sialic acids linked to galactose by an alpha2,6-linkage (SAα2,6-gal). Red staining: reaction with biotinylated *Maackia amurensis* lectin (Vector Laboratories) (detected with Alexa Fluor 594-streptavidin complex; Molecular Probes/Invitrogen, Carlsbad, CA), indicating the presence of SAα2,3-gal Tissues were counterstained with DAPI (4,6,-diamidino-2-phenylindole).

DISCUSSION

Our results demonstrate that a recent EIV isolate is able to infect and replicate in the canine host. This was evidenced by both CIV- and EIV-inoculated dogs testing positive for antibodies on HI assay and shedding detectable virus on real time RT-PCR. In contrast, recent studies conducted by members of our laboratory,[109] as well as others,[105] have revealed that CIV has virtually lost the ability to infect, replicate, and spread among susceptible horses. Interestingly, a similar pattern of infectivity and replication has been observed in primary equine and canine respiratory epithelial cells (RECs) inoculated with both CIV and EIV isolates.[109] In these experiments, CIV and EIV isolates were able to infect and subsequently replicate in canine RECs, while the EIV isolate was better able to infect and replicate in equine RECs compared to the CIV isolate. Taken together, the results from these recent studies suggest there is apparent host range restriction for CIV in horses, which is not been observed for EIV in dogs. One well-described determinant for influenza virus species-specificity that might explain CIV host range restriction, and one which we wished to examine in these studies, is receptor-binding preference. While evidence suggests that the amino acid sequence of the receptor-binding pocket (formed in part by residues 224 through 228 in H3 viruses) modulates the affinity of influenza viruses for specific SA receptors,[211] amino acid residues at other sites in the HA protein may also determine the receptor specificity of influenza viruses. For example, recent research indicates that the amino acid residue 222 in human and avian H3 viruses might serve as a key determinant for binding of human receptor analogs by the HA protein.[212] Indeed, early CIV studies postulated that, in dogs, the W222L CIV substitution might play a role in maintenance of influenza by modulating receptor-binding function.[7,8]

Interestingly, our solid-phase binding assay results demonstrate that CIV isolates, like EIV isolates, have a higher affinity for SAα2,3-gal compared to SAα2,6-gal. This preference is mirrored by a predominance of SAα2,3-linked receptors in the upper respiratory tract and trachea of dogs. The finding that CIV has a similar overall receptor-binding preference as its equine H3 ancestor might explain the natural transmission of equine influenza viruses to dogs that occurred on at least three separate occasions.[7,9,10] Our *in vivo* results confirm EIV is still capable of infecting and replicating in dogs. Transmission of EIV from infected to sentinel dogs would further indicate ability of EIV to be maintained in dogs. However, our results show no influenza infection in sentinels introduced to the EIV-inoculated

group, although a similar study found dogs could be sub-clinically infected with EIV when introduced to EIV-infected horses.[110] In spite of these conflicting reports, it still remains unclear why only one of the previous transmission events and none of the experimental studies resulted in the formation of a stable influenza virus lineage within dog populations.

This lack of dog-to-dog EIV transmission might be explained by the biochemical structure of the SA receptor itself. For example, respiratory epithelial cells in the equine trachea have been found to express mostly α2,3-linked *N*-glyconeurominic (Neu5Gc) SA receptors rather than *N*-acetylneurominic (Neu5Ac) SA receptors.[95] Another recent study done by Yamanaka et al.[105] in which solid-phase binding assays were conducted using Neu5Gc and Neu5Ac analogues, suggests there is a EIV binding preference for the Neu5Gc SA receptor compared to the Neu5Ac SA receptor moiety. However, in the same study, the CIV isolate tested did not appear to have a preference for either the Neu5Ac or Neu5Gc analogue,[105] leaving the question of CIV host range restriction in horses unanswered and, therefore, leading us to propose other determinants of CIV species-specificity.

Of these potential determinants of host range, the HA protein must still be considered as it also mediates fusion of the viral envelope with the endosomal membrane of the host cell.[22] To mediate fusion, the stalk portion of the HA has to undergo complex refolding.[22] While the specific role of the N83S substitution has yet to be determined, residue 83 is part of an extension from the central triple-stranded coiled coil (made up of H3 HA residues 76-105), which repositions and exposes the fusion protein when the HA protein is subjected to low-pH.[44] The substitution of a positively charged amino acid (asparagine) with a hydrophilic residue (serine) might change the protein structure, possibly altering HA refolding. Similarly, the substitution of threonine (a polar residue) for isoleucine (a nonpolar residue) at the HA cleavage site (residue 328)[44] may have affected host protease-viral protein interactions, thereby modulating efficacy of membrane fusion and viral entry into host cells.

Beyond the HA gene, it is possible that mutations in other RNA segments could account for virus adaptation to dogs. In this regard it is interesting that sequence alignments revealed three mutations in the PA (residues 33, 388, 675) and one mutation in the PB2 (residue 374) proteins that consistently differentiate the canine from the equine consensus sequences. As these mutations are located at previously defined functional sites of the PA (PB1 binding site, protease and cap-dependent ribonuclease

regions) and the PB2 (PB1 binding site),[213] it is possible that these substitutions are important for efficient replication of CIV particularly in the canine host. Further reverse genetics and site-directed mutagenesis studies are warranted to address the roles each gene segment plays in CIV species-specificity.

In conclusion, the results of the studies described here contribute to our overall knowledge of EIV/CIV species-specificity. We have shown that EIV is still capable of infecting dogs, although we could not show that EIV is transmitted among dogs in an experimental setting. Conversely, as reported, the opposite is true for CIV in horses.[105,109] Additionally, our *in vivo* studies detail similar findings from a previous *in vitro* experiment where both CIV and EIV were able to infect and replicate in primary canine RECs.[109] Combined with data that show a clear preference of equine RECs for EIV, we believe we have developed an *in vitro* model on which future host range studies can be based. This model might be useful in examining host-pathogen interactions that contribute to CIV transmission and maintenance within the US dog population.