DIAGNOSIS AND EPIDEMIOLOGICAL INVESTIGATION OF H3N8 CANINE INFLUENZA VIRUS

By

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To my husband and parents, thank you for all of your love and support

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TABLE OF CONTENTS

page

LIST OF TABLES

LIST OF FIGURES

LIST OF ABBREVIATIONS

CIV	Canine influenza virus
DIVA	Differentiate infected from vaccinated animals
EIV	Equine influenza virus
ELISA	Enzyme-linked immunosorbent assay
H3N8	Hemagglutinin subtype 3, Neuraminidase subtype 8
HA	Hemagglutination
HI	Hemagglutination inhibition
ILI	Influenza-like illness
MN	Microneutralization
NS1	Nonstructural protein
SEIR	Susceptible, exposed, infectious, recovered model

Abstract of Dissertation Presented to the Graduate School
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Canine influenza A virus subtype H3N8 (H3N8 CIV) was recognized in 2004 as a respiratory pathogen for dogs. The virus has since affected thousands of dogs in 34 states, and has established endemicity in several metropolitan areas of the U.S.

This PhD research encompassed two main objectives. The first objective was to develop tools for serological diagnosis of H3N8 CIV. The diagnostic performance of the H3N8 CIV hemagglutination inhibition (HI) assay was assessed and test parameter variations were evaluated. Study results promote standardization of the H3N8 CIV HI assay to ensure accurate diagnosis of infected dogs. Introduction of a H3N8 CIV vaccine confounds future diagnosis, however, because antibodies generated in response to infection or vaccination cannot be distinguished in the HI assay. Development of a test strategy that differentiates infected from vaccinated animals (DIVA) was therefore pursued. Uninfected and vaccinated dogs could not be distinguished from infected dogs in NS1 tests, however, supporting the conclusion that a NS1 immunoassay is not a viable DIVA strategy for H3N8 CIV.

The second overall objective of this research was to conduct epidemiological investigations of canine influenza to better inform prevention and control strategies. To

determine if H3N8 CIV was infecting dogs prior to 2004, a survey of archived canine sera was conducted. Antibodies to canine and equine H3 proteins were identified in dogs as early as 1999. Next, a national serosurvey for H3N8 CIV infection in dogs with influenza-like illness was conducted to identify risk factors for infection. Analyses revealed that dogs ≥1 year of age, dogs housed in boarding kennels or animal shelters, and dogs in the northeastern and western regions of the country were at highest risk for seropositivity. Based on identification of communal facilities as risk factors for infection, mathematical modeling was conducted to explore the impact of several parameters on H3N8 CIV transmission in shelters. Indirect virus transmission parameters and shelter capacity had the greatest influence on the time course of epidemics and the number of dogs affected. These factors can be targeted in future modeling studies to determine what intervention strategies would avert or mitigate epidemics.

CHAPTER 1
INTRODUCTION

Canine influenza A virus subtype H3N8 (H3N8 CIV) is a recently emerged highly contagious respiratory pathogen for dogs that originated from interspecies transmission of H3N8 equine influenza virus to dogs prior to 2004, followed by virus adaptation to facilitate dog to dog transmission.[20] Since its emergence, this novel virus has infected thousands of dogs in 34 states.[8,9,20,28,29,42,44,98,103,110,111] Dogs housed in communal settings such as racing greyhound kennels, boarding kennels, and animal shelters have been most affected. Due to efficient horizontal transmission and continued adaptation to the dog, H3N8 CIV is now considered endemic in several metropolitan areas of the country.[28,29] Risk factors for infection and the epidemiology of H3N8 CIV transmission in different dog populations, however, are not fully understood.[28,29] Past and present influenza epidemics and pandemics have demonstrated that establishment of an influenza A virus in a new host species is not only important to the health and welfare of the new host, but is also a public health concern. It is prudent to study the epidemiology of influenza A viruses in all established hosts in order to more completely understand the exposure and health risks of these pathogens, and the potential for virus transmission to new hosts.

Research Objectives

The overall objectives of this PhD research were: 1) to develop diagnostic tools for serological diagnosis of dogs infected with H3N8 CIV, and 2) to conduct epidemiological investigations of H3N8 CIV in order to better inform prevention and control strategies.

Diagnostic Test Development

To adequately study the epidemiology of canine influenza, key diagnostic tests should be developed and assessed. Although a variety of tests are available for diagnosis of H3N8 CIV infections, serological assays are often necessary to confirm diagnosis.[28,29] The hemagglutination inhibition (HI) assay is the current test of choice for diagnosis of influenza infections in animals.[134,135] HI assays are performed using techniques modified for the particular host species. There is no accepted standard HI assay for detection of CIV H3 antibodies, and variations in test parameters used by different laboratories may lead to inaccurate diagnosis. Further, the availability of an inactivated whole virus vaccine for H3N8 CIV has confounded the diagnosis of infected dogs because the HI assay cannot distinguish antibodies induced by infection vs. vaccination. Development of a test strategy for differentiation of infected from vaccinated animals (DIVA) is vital for correct diagnosis of infection, evaluation of vaccine efficacy, and for future studies on the epidemiology of H3N8 CIV. The research presented in Chapters 3 and 4 address objective 1. The specific aims and hypotheses for each chapter are as follows:

Specific aim 1 (Chapter 3): To assess the diagnostic performance of the H3N8 CIV HI assay

It was hypothesized that a standard H3N8 CIV HI assay would be a highly sensitive and specific test once a cutoff antibody titer for seropositivity was established. Further, it was hypothesized that high sensitivity and specificity would require use of red blood cells with sialic acid (SA) α2,3-Gal receptors, inactivation of nonspecific inhibitors of hemagglutination in canine serum, and use of live H3N8 CIV isolates in the HI assay.

Specific aim 2 (Chapter 4): To develop a DIVA test strategy for H3N8 CIV

Based on studies in other species, it was hypothesized that immunoassays that detect antibodies to the H3N8 CIV NS1 protein would satisfy the DIVA principle for correct identification of infected versus vaccinated dogs.

Epidemiological Investigations

It is unknown if H3N8 CIV was infecting dogs prior to 2004, causing undiagnosed or misdiagnosed respiratory disease. Risk factors for H3N8 CIV infection have also not been defined, either for individual dogs or populations of dogs. Three epidemiological studies were performed to better understand when H3N8 CIV first emerged in dogs, what dog-related (intrinsic) and exposure factors increase risk for infection, and what facility and population factors contribute to influenza epidemics in communal settings such as animal shelters. Identification of intrinsic and exposure risk factors for infection, and facility or population factors that promote virus transmission, would greatly contribute to formulation of prevention and control strategies to protect the health and welfare of dogs. The research presented in Chapters 5, 6, and 7 address objective 2. The specific aims and hypotheses for each chapter are as follows:

Specific aim 1 (Chapter 5): To investigate potential H3N8 canine influenza-like virus circulation in racing greyhounds and shelter dogs prior to 2004

It was hypothesized that a H3N8 canine influenza-like virus was circulating in racing greyhounds prior to 2004, and that this virus was associated with respiratory disease outbreaks of unknown etiology in U.S. greyhounds in the 1990s and 2003.

Specific aim 2 (Chapter 6): To determine the prevalence of and risk factors for H3N8 CIV seropositivity in a population of U.S. dogs with influenza-like illness

It was hypothesized that H3N8 CIV would be a common diagnosis in a study population of U.S. dogs with influenza-like illness (ILI), and that geographic location and

communal housing settings would be significantly associated with H3N8 CIV seropositivity.

Specific aim 3 (Chapter 7): To explore viral and/or population parameters that promote H3N8 CIV transmission in shelter facilities

It was hypothesized that H3N8 CIV transmission in shelter facilities would be driven by the high frequency of population turnover, and that indirect (fomite) modes of transmission between infected and susceptible dogs would facilitate greater viral transmission than direct modes of transmission. This was an exploratory, theoretical study using mathematical modeling.

CHAPTER 2
REVIEW OF THE LITERATURE

Influenza Viruses

Virology

Influenza viruses are enveloped viruses composed of segmented, negative-sense, single-stranded RNA.[95] Three Influenza virus genera are classified in the Family Orthyomyxoviridae. Influenza B and Influenza C viruses primarily affect humans, although reports of infections in other species have been documented in the literature.[38,73,74,93] Influenza A viruses cause epidemic and pandemic disease in a variety of avian and mammalian species. These viruses are a significant cause of morbidity and mortality in humans, domestic poultry, pigs, horses, and now dogs.[20,28,29,98,136]

Influenza A viruses are comprised of a host-derived lipid envelope and an eight segment genome, which encodes up to 12 known proteins.[19,88,95,133] The two surface proteins, the hemagglutinin (HA) and neuraminidase (NA), are used for virus subtyping. There are currently 16 HA subtypes (H1-H16) and 9 NA subtypes (N1-N9).[95] The HA protein binds sialic acid (SA) receptors on host epithelial cell surfaces, requires cleavage by host proteases to allow fusion activity, and is the major viral antigen. Through its sialidase activity the NA protein frees new virions budding from the host cell surface, which promotes virus spread. The remaining viral proteins include M2, M1, NP, PA, PB1, PB2, PB1-F2, N40, NEP (NS2), and NS1.[19,95,133] M2, a membrane protein, is responsible for ion channel activity and assembly. The M1 or matrix protein interacts with viral ribonucleoproteins and surface proteins, and is involved in nuclear export and budding. The NP or nucleoprotein is involved in RNA binding, synthesis, and nuclear

import. The PA, PB1, and PB2 proteins comprise the viral polymerase complex. PB1-F2 is a proapoptotic protein that is not expressed by all influenza viruses, and the function of the recently described N40 protein is not fully understood.[19,133,142] The NEP or nuclear export protein functions in the export of viral ribonucleoproteins. The NS1 or nonstructural protein is a multifunctional protein that inhibits the host immune response and is involved in several aspects of viral replication.[39,95]

Epidemiology

Aquatic birds are the global reservoirs of influenza A viruses, and serve as the source of novel virus subtypes for all susceptible species.[132] Previous interspecies transmissions and adaptations to the new hosts have resulted in endemic influenza virus lineages in domestic poultry, pigs, horses, and humans.[136] Although influenza A virus evolution is complex, two major mechanisms of virus evolution are typically associated with generation of epidemic and pandemic viruses. Antigenic drift occurs when progeny viruses acquire point mutations in the HA or NA proteins. In these situations unaltered epitopes can still be recognized by the immune system of previously infected individuals, however, therefore only mild disease will occur. These viruses result in influenza epidemics.[136] Antigenic shift occurs either when two influenza viruses infect the same host cell and reassort their segmented genomes, or when a virus is directly transmitted from one species to another. Antigenic shift results in major antigenic changes, such that immunity from previous infections is not protective. These viruses result in pandemics.[136]

Transmission of influenza A viruses from the avian reservoir to other birds or mammals is usually the result of reassortment events or the direct transmission of an entire influenza A virus.[67,132] It has been noted that influenza A viruses transmit

relatively frequently from the avian reservoir to other species, yet do not typically establish permanent lineages in these new hosts.[131] Other endemic influenza A virus hosts, inculding domestic poultry, pigs, horses, and humans may also serve as a source of novel virus subtypes for other birds or mammals.[136] Sporadic influenza A virus infections (of avian and mammalian origin) have been reported in recent years in several mammals, such as stone martens, felids, and dogs;[1,56,114,115] however, to date only the recently emerging H3N8 and H3N2 canine influenza viruses have established stable lineages capable of horizontal transmission in this new endemic mammalian host.[20,66,98,103,112] The complex epidemiology of influenza A viruses highlights the potential impact of these viruses on both animal and public health.

In avian species, influenza A viruses replicate in the respiratory and intestinal tracts, which results in heavy virus shedding in the saliva, nasal secretions, and feces. Although infections in wild birds are typically subclinical, fecal contamination of water allows transmission to other species primarily through the fecal-oral route.[130,132] Influenza A virus infections in domestic poultry can be mild and localized or systemic, and are also typically transmitted to other species by the fecal-oral route. Influenza infections in mammals are primarily transmitted through respiratory secretions, and result in localized or systemic disease.[136]

Endemic H1N1, H3N2, and H1N2 influenza A virus subtypes circulate seasonally in humans worldwide, and antigenic drift results in yearly influenza epidemics. Avian and swine influenza viruses are currently considered the greatest threat for generation of pandemic viruses in humans.[136] In domestic poultry the H5 and H7 influenza A virus subtypes are a significant animal health concern, since they can cause highly

pathogenic disease with mortality rates of 90-100%. Outbreaks of multiple low pathogenic subtypes, including low pathogenic H5 and H7 viruses, however also occur.[136] Whether low or highly pathogenic, avian influenza outbreaks among domestic poultry are considered a public health threat. Reassortment events between avian and human influenza A viruses resulted in the H2N2 and H3N2 pandemics of 1957 and 1968, respectively.[67,132] And direct interspecies transmissions of H5N1 avian influenza viruses to humans, first noted in 1997, have been a source of great pandemic concern and planning.[37,100,118] The role of swine in influenza A virus ecology is also complex. Multiple influenza A viruses, including H1N1, H3N2, and H1N2 subtypes currently circulate in pigs. Genetic analyses of these viruses reveal there are North American and Eurasian swine virus lineages, and that double and triple reassortants of avian, swine, and human viruses are in circulation.[136] There have been reports of sporadic swine influenza virus infections in people, however the true extent of influenza virus transmission between pigs and people is poorly understood.[81] As a timely example, the origin of the 2009 novel H1N1 pandemic virus is unknown. The virus contains swine, avian, and human genes, and therefore reasserts the hypothesized role of swine as a mixing vessel of avian and human influenza A viruses with resultant pandemic potential.[99,136]

Although there are no documented interspecies transmissions of equine influenza viruses (EIV) to people, influenza A viruses are significant respiratory pathogens of horses. Influenza-like illness (ILI) had been described in horses for centuries before the discovery of the H7N7 and H3N8 EIV in 1956 and 1963, respectively.[136] Interestingly, the H7N7 viruses have not been detected in horses since 1979, and are believed to be

extinct.[132] H3N8 EIV was first detected in horses in Miami, Florida. Over time H3N8 EIV isolates evolved into genetically and antigenically distinct, co-circulating American and Eurasian lineages; and the American lineage further evolved into the Argentina, Florida, and Kentucky sublineages.[22,61] The Florida and Kentucky sublineages were reportedly evolving in parallel in the U.S., such that these viruses alternately circulated in the equine population.[62] Currently, Florida sublineage clade 1 viruses predominate in North America and clade 2 viruses predominate in Europe.[15] Due to frequent movement of horses for competition and breeding, these viruses have resulted in sporadic EIV outbreaks worldwide.[23] Until recently there had been no documented equine influenza virus transmissions to other species, and it was suggested that the horse was an "isolated or dead-end reservoir" for influenza A viruses.[132] However, the discovery of the entirely equine-origin H3N8 CIV in the United States in 2004,[20] limited H3N8 EIV transmissions to dogs in the U.K. and Australia,[21,55,91] and the 2009 report of a sporadic H3N8 EIV transmission to pigs in China,[123] once again highlight the complex ecology of influenza A viruses.

Determinants of Interspecies Transmission

The above examples emphasize the importance of influenza A viruses to both animal and public health, and reinforce the need for greater understanding of their classical and molecular epidemiology in all affected species. Significant research efforts have focused on the molecular determinants of influenza A virus interspecies transmission, with particular effort placed on determinants of host range restriction and viral pathogenicity.[7,60,63,89,128] The requirements for interspecies transmission have been detailed, focusing on the interactions between the original and the new host, interactions between the new host and the novel virus, and host-host interactions within

the new recipient species.[60] For a successful interspecies transmission to occur, there must be contact between the two host species, the virus must be able to replicate in the new host, and the virus must be transmissible between individuals in the new host population. Methods of virus entry into the host cell, viral replication within the new host, viral evasion of the host immune response, transmission of virus to another individual within the new host population, and subsequent sustained transmission of the virus between individuals in the new host population, are all important events for interspecies transmission to occur.[60,128]

The host cell receptor-specificity of the influenza A virus HA is considered a critical determinant of host range restriction.[48,60,89,119,120,128] The HA protein binds SA receptors on host epithelial cell surfaces. The SA species present, its linkage to galactose, and its distribution in the tissues of the host species are all important factors. The primary SA species are *N*-acetyl-neuraminic acid (NeuAc) and *N*-glycolyl-neuraminic acid (NeuGc), while the primary linkages of the SA species to galactose are SAα2,3-Gal and SAα2,6-Gal.[120] Avian and equine influenza viruses bind SAα2,3-Gal receptors on the epithelial cells of the intestinal (avian) and respiratory (avian and equine) tracts; while human influenza viruses bind SAα2,6-Gal receptors on the tracheobronchial epithelium.[120] As an example of host range restriction, lectin-binding assays have shown that NeuGcα2,3-Gal receptors are dominant in the equine trachea and experimental infections in horses have further shown these receptors are essential for viral replication. This may explain why equine viruses have reportedly not been transmitted to humans, since humans do not have NeuGcα2,3-Gal receptors in the respiratory tract.[120] In contrast, it is believed that successful direct transmission of H5N1 avian influenza viruses to humans is

associated with the presence of NeuAcα2,3-Gal receptors on particular target cells of the human respiratory tract. Experiments have shown that human influenza viruses bind nonciliated tracheal epithelial cells with SAα2,6-Gal receptors, while avian viruses bind ciliated human tracheal epithelial cells with SAα2,3-Gal receptors.[77] Regardless of these findings, it is important to note that single amino acid substitutions have been shown to change viral receptor specificity from SAα2,3-Gal to SAα2,6-Gal in several HA protein subtypes, which increases potential transmission to and between species.[119]

Several other molecular determinants have been reviewed that influence viral host range restriction and/or pathogenicity.[7,60,63,89,128] It has been noted that the NA also has SAα2,3-Gal or SAα2,6-Gal receptor specificity. Since NA releases virus from the infected cell, the combination of HA and NA are therefore needed for efficient replication.[89] Regarding a specific mutation affecting pathogenicity, the glutamic acid to lysine mutation at position 627 (E627K) of the H5N1 avian influenza virus PB2 protein has been associated with increased replication and virulence in mammals.[60,89] The influenza virus NS1 protein inhibits host immunity by counteracting the innate interferon antiviral response. Another particular concern with H5N1 avian influenza viruses is that the NS1 protein also increases pathogenicity by inducing proinflammatory cytokine genes, resulting in cytokine dysregulation.[37,128] Ultimately, multiple viral and host factors are likely involved in interspecies transmission, and the specific molecular determinants required to overcome barriers to transmission are not well understood.[128]

H3N8 Canine Influenza Virus in the United States

Prior to 2004 the dog was not considered an endemic host of influenza A viruses. Previous studies demonstrated the dog was susceptible to influenza A virus infection (either naturally or experimentally), or reported serological evidence of transient natural

infection of dogs with human influenza viruses.[18,46,51,53,92,106,121] However, there was no evidence of sustained horizontal influenza A virus transmission in the canine study populations. The direct transmission of an entire H3N8 EIV from horses to dogs, and its subsequent establishment of the H3N8 CIV lineage in dogs in the U.S., was an unusual event.[20,98,103]

Molecular and Antigenic Characterization

Virologic, pathologic, and serologic evidence have demonstrated that H3N8 CIV is an emerging respiratory pathogen in dogs.[20,28,29,42,98,103,141] Molecular analyses of virus isolates indicate the interspecies transmission of an entire H3N8 EIV to the dog.[20,42,98,103] Although all eight genes of H3N8 CIV isolates share ≥96% sequence homology with 2002 and 2003 EIV isolates of the Florida sublineage clade 1, canine influenza viruses have formed a distinct monophyletic group and H3N8 CIV isolates are diverging from their equine ancestors.[20,98,103] Five signature amino acid substitutions in the CIV H3 protein distinguish all sequenced canine from equine viruses (N54K, N83S, W222L, I328T, N483T).[20] Analyses of H3N8 CIV isolates collected from 2005 through 2008 show the viruses continue to evolve, such that the original 2003 and 2004 viruses have been classified as “emerging,” while the isolates collected in and after 2005 are considered “enzootic.” Nine additional amino acid substitutions have been identified in all of the enzootic isolates including three in HA (L118V, K261N, G479E), three in PB2 (S107N, A221V, I292T), two in NA (I62L, V147I) and one in PB1 (V200I).[98,103]

The potential significance of these amino acid substitutions has been described.[20,98,103] Briefly, of the CIV H3 mutations, the change at position 54 is of unknown functional significance. The substitution of serine for asparagine at position 83 has been previously associated with antigenic drift. Position 222 is adjacent to the

receptor-binding pocket of the HA protein, therefore the substitution of leucine for tryptophan at this location could be a significant modification required for adaptation to canine SA receptors. This is further supported by a recent H3N8 CIV receptor-binding study indicating reduced binding of the virus to NeuGcα2,3-Gal receptors.[139] Position 328 is near the HA cleavage site, therefore the substitution of threonine for isoleucine could be involved in its recognition by canine proteases. The substitution of threonine for asparagine at position 483 results in the loss of a glycosylation site in the HA2 subunit that is preserved in all other HA subtypes.[20,98]

Although significant antigenic drift has not yet occurred,[8,103] continued surveillance of circulating H3N8 CIV is necessary in order to evaluate any additional adaptations that could influence pathogenicity for the dog, transmission to other species, or the efficacy of diagnostic tests or vaccines. A recent study addressing the microevolution of 2008-2009 H3N8 CIV isolates in two New York City animal shelters has supported the original conclusion that the adaptation of the virus to dogs occurred early in the process of emergence or prior to transfer, since evidence of positive selection was not detected.[42] A study focused on H3N8 CIV intrahost evolutionary dynamics has demonstrated that potentially advantageous mutations are readily generated during single virus infections, however, which could result in antigenic escape or host range variants.[43]

Epidemiology

H3N8 CIV was first isolated in January 2004 from racing greyhounds affected with severe respiratory disease at a North Florida track.[20] The outbreak was characterized by an explosive onset of coughing and nasal discharge, which affected approximately 80% of the dogs. Six dogs also died of hemorrhagic pneumonia. Thousands of greyhound dogs, at tracks in multiple states, were subsequently affected during multiple

respiratory disease outbreaks from 2004 to 2006. Additional virus isolations, as well as substantial serological evidence, indicated that H3N8 CIV was also associated with these outbreaks.[20,141]

H3N8 CIV was first detected serologically in non-greyhound dogs in a Florida shelter in August 2004, and the first virus isolation occurred in 2005.[20,98] Since that time the University of Florida and Cornell University have been conducting surveillance of dogs with ILI to determine the location of H3N8 CIV-infected dogs in the U.S. and to better define risk groups for infection. H3N8 CIV surveillance activities conducted by these institutions and others have revealed a widespread geographic distribution of infected pet and shelter dogs in 34 states.[8,9,20,28,29,42,44,98,103,110,111] The virus is sustained in the U.S. canine population by horizontal transmission, which occurs through direct transmission (direct contact between a susceptible and infected dog, or large droplet transmission through coughing and sneezing between dogs in close proximity) and indirect transmission (aerosols generated by coughing and sneezing, and fomites). Dogs in shelter facilities, boarding facilities, veterinary clinics, and other communal settings are considered at highest risk for exposure. Outbreaks have occurred in greyhound racetrack kennels, boarding kennels, shelter facilities, veterinary hospitals, and canine day care centers across the nation.[9,20,28,29,42-44,98,141] H3N8 CIV outbreaks reach epidemic proportions in these communal facilities, and have resulted in depopulation in shelters and closure of boarding kennels and canine day care centers. H3N8 CIV is considered endemic in metropolitan areas of Florida, New York, Colorado, and Pennsylvania, and infections have occurred year round.[28,29,103]

Knowledge of the prevalence of H3N8 CIV in the U.S. and risk factors associated with virus exposure in dogs is limited. In a study conducted in a university hospital in Iowa, 4 of 731 (0.5%) pet dogs with or without ILI were H3N8 CIV seropositive, indicating prior viral exposure.[110] In a study conducted in a university hospital in Colorado, 9 of 250 (3.6%) pet dogs with or without ILI were seropositive for H3N8 CIV.[8] In a study of pet dogs participating in a flyball tournament in Pennsylvania, 3 of 100 (3%) dogs without ILI were seropositive for H3N8 CIV.[111] Another study conducted in a Philadelphia shelter found that 31 of 74 (42%) tested dogs with or without ILI were H3N8 CIV seropositive.[44] In these studies, attending canine day care within the last six months, pet dogs living with other dogs, and the number of days housed in a shelter facility were associated with H3N8 CIV seropositivity.[8,44,111]

It is unknown how an H3N8 EIV was originally transmitted to dogs, although aerosol transmission or ingestion of infected horse tissues are proposed hypotheses.[20,21] Natural infection of dogs during the recent H3N8 EIV outbreak in Australia,[55] and an experimental study in Japan,[137] have demonstrated the feasibility of close-contact transmission of EIV to the dog. Experimental infections of horses with H3N8 CIV indicate horses are susceptible to infection, developing both clinical signs of respiratory disease and pathological lesions (Long MT, Gibbs EPF, Crawford PC, et al., Comparison of virus replication and clinical disease in horses inoculated with equine or canine influenza viruses. *Immunobiology of Influenza Virus Infection: Approaches for an Emerging Zoonotic Disease*, Athens, GA, July 29-31, 2007). However, a recent study in Japan demonstrated that the virus does not replicate well in horses.[139] These discrepant results could be due to differences in study methodology. Experimental infections have

demonstrated that BALB/c mice are susceptible to H3N8 CIV infection, and may serve as a useful model for some pathogenesis studies.[17] A recent study in chickens, turkeys, and Pekin ducks revealed that these species are not susceptible to infection with H3N8 CIV.[78] At this time, there is no evidence of natural H3N8 CIV transmission to any other species, including humans.

Clinical Signs and Pathogenesis

H3N8 CIV causes acute ILI in dogs. Although thousands of dogs have been infected with H3N8 CIV, the majority of the U.S. canine population has neither been exposed to nor vaccinated against the virus; therefore most dogs are susceptible to infection due to lack of immunity.[20,26,28,29,49] The hallmark of H3N8 CIV outbreaks is the explosive onset of ILI in most dogs in a facility within a short period of time.[20,28,29,98] Based on experimental infections and outbreak investigations, nearly all naïve dogs exposed to the virus become infected.[20,26,28,29,49,98] About 80% of H3N8 CIV-infected dogs exhibit some degree of clinical disease, while 20% are subclinically infected. The incubation period is 2 to 4 days, which is typical for influenza virus infection in other species.[20,26,28,29,49] H3N8 CIV-infected dogs shed virus for 7 to 10 days with peak shedding on days 2 to 4.[20,26,28,29,49] Since the incubation period overlaps with peak virus shedding, dogs can spread the virus before clinical signs appear. In addition, subclinical dogs also shed virus; therefore, all exposed dogs must be considered an infectious risk, whether or not they are showing clinical signs of respiratory infection. Once virus replication and shedding ceases, the dog is no longer contagious to other dogs. In one reported transmission study between H3N8 CIV-infected and naïve dogs, 75% of the contact dogs contracted the virus;[49] however the transmissibility of H3N8 CIV has been questioned.[28,42]

Similar to influenza in horses and humans, H3N8 CIV is a disease with high morbidity and low mortality. Two clinical syndromes have been defined: 1) a milder form consisting of cough and nasal discharge with full recovery in 2 weeks, and 2) a more severe form with pneumonia, prolonged recovery, and increased risk for mortality.[17,20] Severe infections with hemorrhagic pneumonia were noted in early outbreaks, but this appears to be unique to racing greyhounds.[20,29,98,141] H3N8 CIV replicates in epithelial cells lining the airways of the respiratory tract, causing epithelial erosion and intense inflammation (rhinitis, tracheitis, bronchitis, bronchiolitis).[17,20] The virus also replicates in canine alveolar macrophages, inducing tumor necrosis factor-alpha expression and cell death.[102] Epithelial cell necrosis and exposure of the basement membrane predisposes the dog to secondary bacterial infections. Many dogs develop purulent nasal discharge due to secondary bacterial infections, and these pathogens also contribute to the persistent cough.[17,20] Although most dogs recover in 2-3 weeks, some progress to a secondary bacterial pneumonia that can be life threatening.

Diagnosis

Virus isolation is the gold standard for diagnosis of H3N8 CIV infection, as with other species.[136] Nasal swabs collected at the onset of clinical illness can be cultured in embryonated eggs or MDCK cells to isolate virus.[20,28,29,98,103] Recovered virus is then available for molecular and antigenic characterization. False negative results are a concern with virus isolation, however, since the clinical sample must be collected during the virus-shedding period. In addition, the sample must be properly stored until testing to preserve a sufficient amount of live virus for inoculation of cultures. For these reasons virus isolation often yields false negative results.[28,29,103] A more rapid diagnosis of H3N8 CIV during the acute phase of infection can be obtained through detection of viral

nucleic acids in nasal swabs using RT-PCR (using primers for H3N8 CIV or contemporary EIV matrix and hemagglutinin genes).[20,28,29,71,98,103] RT-PCR is the most sensitive antigen detection method for H3N8 CIV, with 75% of RT-PCR positive samples yielding a virus isolate in culture.[28,29,103] Although RT-PCR does not require the presence of live virus in the sample, however, it is still prone to false negative results because sample collection must overlap with the virus-shedding period.[28,29]

Due to concerns with false negative results in other diagnostics, detection of virus-specific antibodies in the serum is often necessary for confirmation of H3N8 CIV infection.[28,29] Ideally, a blood sample is collected during the acute period of illness followed by another sample 2 to 4 weeks later. The paired serum samples are tested for antibodies that neutralize virus infection of cells (microneutralization assay, or MN) or that inhibit hemagglutination by the virus (hemagglutination inhibition assay, or HI). Seroconversion, or a four-fold rise in antibody titer, is diagnostic of recent active virus infection. Initial comparisons of MN and HI test results, based on testing of sera from SPF and H3N8 CIV experimentally infected dogs, indicated that these tests correlate well.[20] Currently the HI assay is considered the test of choice for diagnosis of influenza A viruses in animals,[134,135] and is most commonly used in diagnostic laboratories.[28,29] This test detects antibodies to the HA protein. Specific attachment of antibodies to the viral HA protein interferes with virus binding to SA receptors on red blood cells (RBC), thereby inhibiting hemagglutination. The HI assay reliably detects antibodies to the HA protein as early as 7 days post infection.[20,26,28,29]

Therefore, the ideal approach for diagnosis of H3N8 CIV infection in individual dogs is collection of nasal swabs during the acute phase of disease, along with paired

acute and convalescent serum samples.[20,28,29,98,103] Nasal swabs can be used for virus isolation and molecular antigen detection using RT-PCR, while serology serves as an ideal confirmatory test. High-throughput serological surveillance of dogs for exposure to influenza A viruses can be conducted with commercially available competitive ELISAs, which detect antibodies to the highly conserved influenza A nucleoprotein.[24,30] Positive ELISA results should then be confirmed in a second test such as the subtype specific HI assay.[24,30]

Prevention and Control

Vaccination is an important prevention and control measure for influenza A virus infection in all species,[14] and vaccination of animal populations is considered an important tool for preventing novel influenza A virus transmission from animal reservoirs to humans. Whole virus and recombinant DNA vaccines interfere with virus infection by inducing formation of antibodies against the HA protein, which mediates viral attachment to SA receptors on host epithelial cells. In addition to vaccination, biosecurity and infection control measures are useful tools for managing influenza outbreaks. In the case of H3N8 CIV, biosecurity practices (e.g., changing clothes and washing hands after handling affected dogs) in conjunction with isolation of sick and exposed dogs and effective disinfection should reduce virus transmission to other dogs.[9,28,29]

In June 2009 the USDA approved the first H3N8 CIV vaccine.[27] Similar to most influenza virus vaccines in use for other species, it contains inactivated whole virus. The H3N8 CIV vaccine is recommended for dogs at risk of exposure (i.e., not as a core vaccine) and is given in two doses, two to four weeks apart. An annual booster is currently recommended. The vaccine is intended as an aid in the control of disease

associated with H3N8 CIV and may not prevent infection; however, vaccination significantly reduces the severity and duration of clinical illness, the extent of pathology in the lungs, and the amount and duration of virus shedding.[27] Canarypox-vectored and equine herpes virus-vectored H3N8 CIV vaccines have also been described in the literature,[52,107] but have not yet received USDA approval.

Two concerns for H3N8 CIV vaccination are highlighted by observations of EIV vaccination. Although horses are routinely vaccinated for H7N7 and H3N8 influenza subtypes, the complex evolution of H3N8 EIV has resulted in multiple EIV outbreaks worldwide due to vaccine failure (i.e., antigenic mismatch between vaccine strains and circulating viruses).[75,90,138] Although significant antigenic drift has not yet been observed with H3N8 CIV,[8,98,103] this warrants further monitoring to ensure vaccine efficacy. An additional concern is that vaccination interferes with interpretation of routine serological assays such as the HI assay, since HA antibodies produced in response to natural infection cannot be distinguished from those produced due to vaccination. This has been addressed with avian,[5,31,117,124,127,143] equine,[10,94] and swine influenza[54] through development of DIVA (differentiate infected from vaccinated animals) test strategies and should be pursued for H3N8 CIV.

Influenza A Viruses in Dogs around the World

Since the discovery of H3N8 CIV in the U.S., several studies have evaluated canine populations in other countries for serological evidence of H3N8 CIV exposure. A serological survey of 225 dogs from Ontario, Canada revealed that H3N8 CIV is either not present or is currently rare in the province, despite its close proximity to affected states in the U.S.[59] The one dog that was seropositive to H3 antigen was a greyhound originally from Florida. A study from New Zealand identified no H3N8 CIV seropositive

dogs out of 251 tested.[57] A study conducted in northern Italy identified no H3N8 seropositive dogs out of 224 tested;[101] however a second study that tested sera collected from 6,858 dogs throughout Italy identified two dogs with antibodies to H3N8 CIV and EIV antigens.[24] One of these dogs moved to Italy from Florida three weeks before sample collection. The other seropositive dog was raised on a horse farm that previously experienced an equine influenza outbreak. In both cases the owners did not observe ILI in their dogs.[24]

Since the recognition of equine-origin H3N8 CIV, additional interspecies transmissions of H3N8 EIV isolates to dogs have been reported. In the United Kingdom, a retrospective study revealed that H3N8 EIV caused a respiratory outbreak among English foxhounds in 2002.[21] This was confirmed through serology, immunohistochemistry, and RT-PCR. Lectin-binding studies performed by these investigators indicated that canine tracheal epithelial cells contain primarily SAα2,3-Gal receptors. Serological evidence of a second spill-over of H3N8 EIV to dogs, in the spring of 2003, has also been reported.[91] During the 2007 H3N8 EIV outbreak in Australia, dozens of dogs in contact with infected horses developed ILI. Multiple cases were confirmed serologically and limited RT-PCR sequencing indicated the HA, M, and NA genes were identical to the circulating EIV, although no EIV isolates were obtained from infected dogs.[55] There is currently no evidence of sustained circulation of H3N8 EIV in the canine populations of either the United Kingdom or Australia.

Since 2006, interspecies transmissions of avian influenza A viruses (AIV) to dogs have also occurred. In Thailand, a fatal case of H5N1 AIV was reported in a dog that ingested an infected duck.[2,114] The virus isolate contained the multiple basic amino acid

cleavage site and the 20 amino acid NA stalk deletion characteristic of these highly pathogenic AIV isolates, and also had the E627K PB2 mutation associated with enhanced viral replication in mammals. Serological testing indicated that 160 of 629 (25%) Thai dogs had antibodies to H5N1 AIV, but sustained horizontal transmission has thus far not been reported in the country.[16] Experimental infections of dogs with H5N1 AIV have shown that dogs are susceptible to H5N1 infection and subclinically shed virus from the nose.[34,72] Virus labelling and histochemical examination, as well as lectin-binding assays performed by these investigators, revealed that dogs have SAα2,3-Gal receptors in their nasal mucosa, tracheal epithelium, bronchial epithelium, and alveoli. Although virus transmission to contact dogs or cats did not occur in one of these studies, both groups concluded that dogs may play a role in H5N1 virus adaptation to mammals and contribute to spread to other species.[34,72]

In South Korea, transmission of a H3N2 AIV to dogs was reported in 2007. Subsequent experimental and natural dog-to-dog transmission of H3N2 was reported, and serological evidence of infection has been detected in farmed and pet dogs in South Korea.[66,112,113] The H3N2 virus was isolated during outbreaks of severe respiratory disease in dogs at multiple facilities, and was reportedly traced to a dog purchased from a live animal market where chickens, ducks, pheasants, rabbits, cats, pet dogs, and farmed dogs were sold. It was hypothesized that virus transmission occurred through feeding dogs infected poultry products or by aerosol transmission. Sequencing results indicated all eight genes were ≥95.5% homologous to H3N2 AIV isolates from Asia (South Korea, China, Hong Kong, Japan). Lectin-binding assays indicated SAα2,3-Gal receptors are found in canine bronchial and bronchiolar epithelial

cells, and rarely on tracheal epithelial cells; while SAα2,6-Gal receptors were not detected in canine tracheal, bronchial, or bronchiolar epithelial cells.[112] With virological and serological evidence of sustained dog-to-dog transmission, the H3N2 subtype represents a second influenza A virus considered an emerging respiratory pathogen in dogs.

Finally, during the 2009 H1N1 pandemic several dogs were reportedly infected after contracting the virus from their owners. ProMED-mail postings reported canine cases in China and the U.S. (ProMED-mail. Influenza pandemic (H1N1) 2009, animal (30): China, canine. ProMED-mail 2009; 28 Nov: 20091128.4079. <http://www.promedmail.org>. Accessed 12 April 2011; ProMED-mail. Influenza pandemic (H1N1) 2009, animal (40) - USA (New York), canine. ProMED-mail 2009; 22 Dec: 20091222.4305. <http://www.promedmail.org>. Accessed 12 April 2011). In a recent Italian study 7 of 964 (0.7%) tested dogs had antibodies to a current H1N1 pandemic isolate in competitive ELISA, HI, and MN assays.[30]

Summary

The interspecies transmissions of H3N8 EIV and H3N2 AIV to dogs, as well as intermittent interspecies transfers of several other influenza viruses to dogs, have increased awareness regarding the potential role of canines in the ecology of influenza A viruses. The H3 subtype is now the most broadly distributed HA subtype in mammals, with lineages in humans, horses, pigs, and dogs.[98] These events raise concern, in both the animal and public health communities, regarding the impact of influenza A viruses on the health and welfare of dogs and the possible transmission of influenza A viruses from dogs to other species. Experiences in the United States, the United Kingdom,

Australia, Thailand, and South Korea demonstrate the importance of influenza A virus surveillance in canines, particularly during outbreaks in other species.[24]

CHAPTER 3
DIAGNOSTIC PERFORMANCE OF THE H3N8 CANINE INFLUENZA VIRUS HEMAGGLUTINATION INHIBITION ASSAY

Canine influenza A subtype H3N8 virus (H3N8 CIV) is a recently emerged respiratory pathogen for dogs.[20] Although this novel virus was initially identified in the racing greyhound population in the U.S., all naïve dogs are susceptible to infection, including pet dogs and dogs housed in shelters.[20,26,28,29,49] Since the initial report in 2004, H3N8 CIV infections have been documented in thousands of dogs with widespread geographic distribution in 34 states.[8,9,20,28,29,42,44,98,103,110,111] To date, there is no evidence of H3N8 CIV circulation in other countries.[24,57,59,101] Similar to other mammalian influenza A viruses, H3N8 CIV is efficiently transmitted between dogs, and can cause epidemics of influenza-like illness (ILI) in communal housing settings such as racing greyhound kennels, boarding/training facilities, and animal shelters.[9,20,28,29,42,44,98,103,141] The incubation period for H3N8 CIV is 2 to 4 days and virus shedding begins prior to the development of clinical signs, with peak shedding occurring on day 4 and total virus shedding lasting 7 to 10 days.[20,26,29,49] Serum antibodies to the CIV hemagglutinin (H3) protein are detectable as 7 to 8 days after infection.[20,26,28,29]

Molecular analyses of H3N8 CIV isolates indicate prior transmission of a Florida sublineage H3N8 equine influenza A virus (H3N8 EIV) from horses to dogs, followed by viral adaptation to the new canine host resulting in efficient dog-to-dog transmission.[20,42,98,103] H3N8 CIV isolates form a monophyletic group that is molecularly and epidemiologically distinct from currently circulating H3N8 EIV in the U.S.[103] Signature amino acid changes in the H3 protein, particularly around the receptor-binding pocket, distinguish emerging CIV isolates from their EIV ancestors, and

additional changes in endemic isolates indicate the virus continues to adapt to dogs.[20,42,43,98,103]

As for other influenza A viruses, H3N8 CIV can be diagnosed in clinical specimens by virus isolation or reverse transcription-PCR (RT-PCR) using subtype-specific primers.[20,28,29,71,98,103] RT-PCR is more sensitive than virus isolation,[28,29,103] however both assays can yield false negative results if clinical samples are collected outside the viral shedding window.[26,28,29] Therefore, serological assays are recommended for confirmation of H3N8 CIV infections, either as a diagnostic aid for current cases or as an epidemiological tool for surveillance and assessment of disease prevalence.[28,29] The hemagglutination (HA) and hemagglutination inhibition (HI) assays are classic influenza diagnostics, and are considered the tests of choice for virus surveillance and serological diagnosis of influenza A virus infections in animals.[134,135] In the HA assay, the viral hemagglutinin protein binds sialic acid (SA) receptors on the surface of red blood cells (RBC), resulting in hemagglutination. The assay is an important first step for virus hemagglutinin subtyping and virus preparation for the HI assay. Also, agglutination patterns for RBC from different species have been used to determine the SA receptor specificities of influenza A viruses.[48] In the HI assay, serum antibodies specific for the hemagglutinin protein subtype interfere with virus binding to RBC, thereby inhibiting hemagglutination and indicating previous virus exposure. A variety of factors can influence the sensitivity and specificity of the HI assay, including the matching of the SA receptor type on the RBC surface to that preferred by the viral hemagglutinin protein, the presence of nonspecific hemagglutinins and/or nonspecific inhibitors of hemagglutination in serum, and the binding specificity of serum antibodies for the

particular hemagglutinin subtype of the test virus.[134,135] Although test conditions may require modification for detection of novel viruses or viruses that have undergone antigenic drift due to point mutations in the hemagglutinin protein,[79,116] standard HI conditions have been generally established for endemic influenza viruses in avian species, pigs, horses, and humans.[134,135]

To date, the diagnostic performance of the H3N8 CIV HI assay has not been formally evaluated. Further, the effect of test parameter variations on the performance of the assay has not been described. Discrepancies exist between diagnostic laboratories and research studies in the H3N8 CIV HI cutoff antibody titer selected for seropositivity as well as the test parameters used. The first objective of this study was to assess the diagnostic performance of the "standard" H3N8 CIV HI assay as performed in our previous reports documenting H3N8 CIV infections in dogs.[20,98] The second objective was to evaluate the effect of common test parameter variations on the diagnostic performance of the H3N8 CIV HI assay; including the use of different RBC species, canine serum treatment methods, live vs. inactivated virus, and different H3N8 CIV and EIV isolates.

Materials and Methods

All studies were approved by the University of Florida Institutional Animal Care and Use Committee.

Assessing the Diagnostic Performance of the Standard H3N8 CIV HI Assay

Comparison of the H3N8 CIV HI and serum microneutralization assays. To assess the diagnostic performance of the standard H3N8 CIV HI assay, HI and serum microneutralization (MN) test results were analyzed for 879 acute and/or convalescent sera collected from 619 dogs: 529 racing greyhounds,[20,98] 50 shelter dogs,[98] and 38 pet

dogs involved in respiratory disease outbreaks in the U.S., as well as 2 beagles experimentally infected with the H3N8 A/canine/Florida/43/2004 (CN/FL/04) isolate.[20] Sera collected from specific pathogen-free (SPF) dogs[20] housed in a barrier research facility and normal ferret sera were used as negative controls for both assays, while polyclonal goat anti-EQ/Miami/63 serum served as a positive control. HI and MN testing were conducted as previously described.[20,108] For the HI assay, serial two-fold dilutions of serum pretreated with receptor-destroying enzyme (RDE) were tested with 4 hemagglutinating units (HAU) of H3N8 CN/FL/04 virus/25 µL and 0.5% turkey RBC in PBS. The endpoint antibody titer was defined as the reciprocal of the last serum dilution that completely inhibited hemagglutination. Seropositivity of a single sample was defined as a HI antibody titer ≥32 based on titers for sera from SPF dogs and dogs experimentally infected with H3N8 CN/FL/04.[20] For the MN assay, the endpoint antibody titer was defined as the reciprocal of the highest serum dilution that gave 50% neutralization of 100 $TCID_{50}$ of H3N8 CN/FL/04.[20] Seropositivity of a single sample was defined as a MN antibody titer ≥40.[20,108]

Intra-assay and inter-assay variability of the H3N8 CIV HI assay. A subset of 12 serum samples previously tested in both HI and MN assays were re-tested in the standard H3N8 CIV HI assay to assess intra-assay and inter-assay variability. This subset included 6 CIV-seronegative samples and 6 CIV-seropositive samples with HI antibody titers ranging from 32 to 1024. For intra-assay testing, each sample was tested 5 times in the same microtiter plate using the same virus and RBC preparations. For inter-assay testing, each sample was tested in duplicate on three separate days using

fresh virus and RBC preparations each day. Endpoint antibody titers were determined and defined as described above.

Data analyses. Statistical analyses comparing the H3N8 CIV HI and MN test results were conducted with standard software (XLSTAT, Addinsoft, New York, NY, USA; SigmaStat, Systat Software, San Jose, CA, USA). Spearman's rank correlation analysis was used to determine correlation of HI and MN antibody titers for 879 sera. HI and MN test results for convalescent sera from 614 dogs (n=507 H3N8 CIV-infected, n=107 uninfected) were utilized in a receiver operating characteristic (ROC) analysis to determine the sensitivity, specificity, and positive and negative predictive values of the HI assay using the MN assay as the gold standard. H3N8 CIV-seropositive samples were coded "1" based on MN titers ≥40 and seronegative samples (MN titer <40) were coded "0". The MN antibody titers for seropositive samples ranged from 40 to 15292. The recommended H3N8 CIV HI antibody titer cutoff for seropositivity was based on the optimum sensitivity and specificity values determined in the ROC analysis. Analytical results with a P-value <0.05 were considered significant. The percent coefficient of variation (% CV) was calculated for the intra-assay and inter-assay variability of the H3N8 CIV HI assay.

Evaluating the Effect of Test Parameter Variations on Diagnostic Performance of the H3N8 CIV HI Assay

Serum samples. To determine the impact of common test parameter variations on the diagnostic performance of the H3N8 CIV HI assay, a subset of seronegative and seropositive convalescent sera tested in the standard H3N8 CIV HI and MN assays was re-tested with different RBC species, serum treatment methods, and different H3N8 CIV and EIV isolates. Sera from SPF dogs were used as negative controls.

Red blood cell sources. Commercially available chicken, turkey, goose, horse, dog, sheep, and cow RBC collected with Alsevers as the anticoagulant (Lampire Biological Laboratories, Pipersville, PA, USA) were used in HA assays. The chicken, turkey, and dog RBC were also used in HI assays. All RBC were stored at 4°C and used within 2 weeks of collection.

Serum treatments. Sera were either untreated or treated with RDE (Denka Seiken, Tokyo, Japan) or periodate (Sigma-Aldrich Corp., Saint Louis, MO, USA) followed by heating at 56°C for 30 minutes to inactivate potential nonspecific inhibitors of hemagglutination in canine sera. For RDE treatment, serum samples were incubated with RDE (1 volume serum:3 volumes RDE) for 16 hours at 37°C prior to heat inactivation. Periodate treatment was conducted following a standard WHO protocol[134] that included, in a step-wise fashion, the treatment of ½ volume serum with 1 volume trypsin solution (Sigma-Aldrich Corp.), inactivation at 56°C for 30 minutes, incubation with periodate solution (3 volumes) for 15 minutes at room temperature (23°C), incubation with 3 volumes of a 1% glycerol (Sigma-Aldrich Corp.) solution for 15 minutes at 23°C, and addition of 0.85% saline for a final serum dilution of 1:10.

Viruses. H3N8 CN/FL/04 and A/canine/Jacksonville/2005 (CN/Jax/05) were kindly provided by Dr. R. Donis (Centers for Disease Control and Prevention, Atlanta, GA, USA). A/canine/FortCollins/3/2006, A/canine/Weld/2007, and A/canine/ColoradoSprings/2009 were kindly provided by Dr. G. Landolt (Colorado State University, Fort Collins, CO, USA). A/canine/Pennsylvania/96978/2009 (CN/PA/09) and A/equine/NewYork/1999 (EQ/NY/99) were kindly provided by Dr. E. Dubovi (Cornell University, Animal Health Diagnostic Center, Ithaca, NY, USA). H3N8 CN/FL/04

previously inactivated by binary ethyleneimine (BEI) treatment, an alkylating agent that inactivates virus without altering virus proteins, was kindly provided by Dr. N. Lakshmanan (Intervet Schering Plough Animal Health, Millsboro, DE, USA). A/equine/Kentucky/1991 (EQ/KY/91), A/equine/Kentucky/1995 (EQ/KY/95), and A/equine/Ohio/2003 (EQ/OH/03) were kindly provided by Dr. T. Chambers (University of Kentucky, Lexington, KY, USA). All equine viruses were cultured in embryonated chicken eggs as previously described.[20] The provided allantoic fluid aliquots were stored at -80°C until use.

All canine isolates were cultured in Madin-Darby canine kidney (MDCK) cells using a standard protocol,[20] with the described modifications. Briefly, approximately 75% confluent MDCK cells grown in 75-cm^2 flasks were inoculated with a 1:750 dilution of virus stock in PBS. The flasks were incubated for 1 hour at 35°C in a humidified atmosphere containing 5% CO_2, and then washed with sterile PBS. Dulbecco modified Eagle medium (DMEM) supplemented with 1 µg/mL TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)–treated trypsin (Sigma-Aldrich Corp.), 0.85% bovine serum albumin (BSA), HEPES buffer, and penicillin-streptomycin was added to the flasks. The inoculated cells were incubated at 35°C in a humidified atmosphere containing 5% CO_2 for 3 to 5 days, with daily evaluation for cytopathic effect. The culture supernatant was harvested, clarified by centrifugation at 2000 rpm for 10 minutes, and the amount of virus determined with the HA assay using 0.5% turkey red blood cells (Lampire Biological Laboratories) in PBS as previously described.[20] The viral culture supernatant was stored at -80°C until future use.

HA assay. Prior to evaluating the effect of using different RBC species in the HI assay, the RBC binding preferences of H3N8 CN/FL/04 were assessed in HA assays. Serial two-fold dilutions of MDCK supernatant containing live H3N8 CN/FL/04 were incubated with an equal volume (50 µL) of 0.5% chicken, 0.5% turkey, 0.5% goose, 1% horse, 1% dog, 1% sheep, and 1% cow RBC prepared in PBS or PBS containing 0.5% bovine serum albumin (PBS/BSA). Incubations were performed in V-bottom microtiter plates for 30 minutes or 1 hour at 23°C and 4°C. HAU, defined as the reciprocal of the last virus dilution that agglutinated RBC, were compared for the different RBC species and incubation conditions. The RBC binding preferences were evaluated based on the HAU and known SA receptors for each RBC species. RBC species with the highest HAU for H3N8 CN/FL/04 were selected for use in subsequent HI assays.

HI assay. Serial two-fold dilutions of untreated sera and sera treated with RDE or periodate were incubated in V-bottom microtiter plate wells with 4 HAU/25 µL of live H3N8 CIV or EIV isolates or inactivated CN/FL/04 for 30 minutes at 23°C. The serum/virus mixtures were then incubated at 23°C for 30 minutes with 0.5% chicken or turkey RBC in PBS, or for 60 minutes with 1% dog RBC in PBS/BSA. All samples were tested in duplicate. End-point antibody titers and the cutoff titer for seropositivity were defined as described above for the standard HI assay. For each serum sample, antibody titers generated by HI assays with different parameters were compared to the antibody titers determined by the standard HI assay.

Results

Assessing the Diagnostic Performance of the Standard H3N8 CIV HI Assay

Comparison of the H3N8 CIV HI and MN assays. The Spearman's rank correlation coefficient comparing the standard H3N8 CIV HI and MN assay antibody

titers for 879 sera was 0.87 (P<0.01). For the 642 seropositive samples, the geometric mean antibody titer (GMT) in the MN assay (543) was significantly (P<0.01) higher than the GMT in the HI assay (381). ROC analysis of the HI antibody titers for 614 convalescent sera (83% seropositive) using the MN titers as the gold standard resulted in an area under the curve (AUC) of 0.985 (95% CI = 0.98 to 0.99; P<0.01). The ROC-generated sensitivity, specificity, positive and negative predictive values for HI antibody titers ranging from 4 to 2048 are shown in Table 3-1. Based on a sensitivity of 99.6% and specificity of 94.4%, the optimal cutoff antibody titer for seropositivity was 32. At this cutoff, the positive predictive value (PPV) was 98.8% and the negative predictive value (NPV) was 98.1%.

Intra-assay and inter-assay variability of the H3N8 CIV HI assay. The % CV for the intra-assay H3N8 CIV HI variability testing was 4.1%. The % CV for the inter-assay variability testing was 3.0%. For all replicates, the HI titers correctly classified the dogs as H3N8 CIV-infected or uninfected using a cutoff titer of 32.

Evaluating the Effect of Test Parameter Variations on Diagnostic Performance of the H3N8 CIV HI Assay

Red blood cell binding preferences of H3N8 CIV. The HAU for H3N8 CN/FL/04 with various RBC species are summarized in Table 3-2. HAU were highest with chicken and turkey RBC, followed by goose and canine RBC. Incubation temperature and addition of BSA to the viral suspension had little effect on HAU for each RBC species.

Use of different RBC species in the H3N8 CIV HI assay. To examine the effect of RBC source on HI assay performance, RDE-treated sera from 11 H3N8 CIV-infected dogs and 14 uninfected dogs were incubated with chicken, turkey, or canine RBC. Regardless of the RBC source, all of the samples from uninfected dogs had HI antibody

titers of 4, and all of the samples from infected dogs had HI antibody titers ≥32. As shown in Table 3-3, the HI antibody titers for infected dogs were 2-to 8-fold higher when tested with chicken or turkey RBC compared to canine RBC; the GMT was significantly lower (P<0.01) when canine RBC were used.

An additional 46 serum samples from dogs infected with H3N8 CIV were RDE-treated and tested with 0.5% chicken or turkey RBC in the H3N8 CIV HI assay. The HI antibody titers ranged from 32 to 1024. In general, there was good agreement in HI antibody titers between the two RBC sources. However 30% (14/46) of the samples had a 2-fold lower titer with chicken RBC compared to turkey, resulting in one dog being misclassified as seronegative.

Use of different serum treatments in the H3N8 CIV HI assay. HI antibody titers for untreated sera from 11 H3N8 CIV-infected dogs were similar to or 2-fold higher than titers for the same samples treated with RDE (Table 3-4). Although there were no false negative results for the infected dogs, untreated sera resulted in false positive results for 87% (13/15 dogs) of the uninfected dogs when chicken RBC were used in the HI assay instead of turkey. There were no false negative or false positive results for sera treated with RDE, regardless of the RBC source. For uninfected dogs (n=4) the HI antibody titers for periodate-treated sera were similar to those obtained for the same samples treated with RDE. For H3N8 CIV-infected dogs (n=6), however, the HI antibody titers for periodate-treated sera were 2-fold lower than those obtained for the same samples treated with RDE.

Use of live or inactivated virus in the H3N8 CIV HI assay. To evaluate whether inactivated H3N8 CIV can be used instead of live virus, sera from 14 H3N8 CIV-infected

and 14 uninfected dogs were tested in the HI assay with live H3N8 CN/FL/04 or virus inactivated with BEI. Overall, the HI antibody titers for sera from infected dogs were similar when live or inactivated CIV was used (Table 3-5). However, 3 of 4 samples with HI titers near the cutoff titer for seropositivity were false negative when inactivated virus was used in the assay.

Use of different canine or equine viral isolates in the H3N8 CIV HI assay. To evaluate consistency in antibody titers when different H3N8 CIV isolates were used in the HI assay, sera from 46 dogs infected with H3N8 CIV between 2004 and 2005 were tested using 5 H3N8 CIV isolates acquired from dogs in different geographic regions from 2005 to 2009. The HI antibody titers for these samples ranged from 32 to 1024 when tested with CN/FL/04. The HI titers with the 5 isolates were equivalent to or two-fold lower than the titers with CN/FL/04 (Table 3-6). There were no false negative results for samples tested with the 5 isolates.

To determine whether H3N8 EIV isolates could be reliably substituted for canine isolates in the HI assay, sera from the 46 infected dogs were also tested with 4 H3N8 EIV isolates acquired from horses in different geographic regions from 1991 to 2003. The HI antibody titers to EQ/OH/03 were equivalent to those for the CN/FL/04 isolate (Table 3-7). The HI titers to EQ/NY/99 were 2-to 4-fold lower than those with the CN/FL/04 isolate, resulting in misclassification of 4 infected dogs (8.7%) as uninfected. The HI titers to EQ/KY/95 and EQ/KY/91 were 4-to 8-fold lower than those for the CN/FL/04 isolate, and 8 infected dogs (17.4%) were misclassified as uninfected.

Discussion

This study demonstrated a high correlation between antibody titers to H3N8 CIV determined in a serum microneutralization (MN) assay and the standard HI assay

described in our previous reports.[20,98] This standard H3N8 CIV HI assay is highly sensitive and specific when the cutoff antibody titer for seropositivity is 32. The precision of the HI assay was demonstrated by coefficients of variation <5% for intra-assay and inter-assay replications. The optimal sensitivity and specificity of the HI assay depended on treatment of serum samples with RDE, use of 0.5% turkey RBC, and use of live H3N8 CIV isolates. Use of different H3N8 CIV isolates from dogs in different geographic regions from 2005 to 2009 and contemporary H3N8 EIV isolates from horses after 2000 did not alter the sensitivity of the HI assay.

The HI assay is considered the WHO/OIE test of choice for serological diagnosis of influenza A infection in animals.[134,135] The test parameters for the standard H3N8 CIV HI assay described in previous reports included use of turkey RBC, RDE- treated sera, live CN/FL/04 virus, and a seropositivity cutoff titer of 32 based on reactivity of sera from SPF dogs and experimentally infected dogs.[20,98] Under these conditions, this study confirmed that diagnosis of H3N8 CIV-infected and uninfected dogs by the HI assay correlated well with MN assay results. ROC analysis also confirmed that the HI antibody titer cutoff of 32 resulted in the highest sensitivity (99.6%) and specificity (94.4%). Lower cutoff values increased the number of false positives and decreased specificity. Sensitivity and specificity are fixed characteristics of diagnostic tests, whereas the predictive values of a positive or negative test are affected by disease prevalence. While the PPV and NPV were high using a HI seropositivity titer cutoff of 32, these values were reflective of the high prevalence (83%) of seropositive dogs included in the tested population. However, a benefit of ROC analysis is that it allows an estimate of diagnostic accuracy that is independent of disease prevalence.[36] Therefore, in locations

where H3N8 CIV is known to circulate, the HI assay is considered a reliable and sensitive test for serological diagnosis as long as the test antigen is homologous to the circulating virus. For large-scale serological surveys seeking to estimate the prevalence of H3N8 CIV (or another influenza A virus) seropositivity in locations where the status of virus circulation is unknown, the HI assay has been recommended as a sensitive and specific confirmatory test of high-throughput screening assays such as competitive nucleoprotein ELISAs.[24,30]

Several variations in test parameters decreased the sensitivity and/or specificity of the standard H3N8 CIV HI assay, including use of RBC species other than turkey, lack of serum treatment to inactivate naturally occurring inhibitors of hemagglutination, use of periodate for serum treatment, use of inactivated virus, and use of H3N8 EIV isolates recovered from horses prior to 2000. Although there was good agreement in antibody titers for many samples when turkey or chicken RBC was used, nearly one-third of the sera had lower titers with chicken RBC. This suggests that truly infected dogs with HI antibody titers near the cutoff titer for seropositivity may be misdiagnosed when using chicken RBC.

A variety of nonspecific hemagglutinins and inhibitors of hemagglutination have been identified in animal and human sera; limited studies with few samples have shown that canine serum may have low levels of nonspecific hemagglutinins for avian red blood cells and nonspecific inhibitors of hemagglutination for some influenza viruses.[3,122] This study demonstrated that serum treatment is required to inactivate nonspecific inhibitors of agglutination in canine serum. Nonspecific inhibitors of hemagglutination were demonstrated by higher antibody titers in untreated sera from

infected dogs compared to titers obtained after RDE or periodate treatment and heat inactivation. Nonspecific inhibitors of hemagglutination were particularly evident when untreated sera were tested with chicken RBC rather than turkey RBC in the HI assay: the chicken RBC resulted in false positive reactions for nearly all of the truly uninfected dogs tested. Therefore, to decrease false positive reactions and maintain high specificity, canine sera should be treated with RDE and heat inactivation prior to testing in the H3N8 CIV HI assay.

Although use of inactivated H3N8 CIV in the HI assay would be advantageous to reduce biosafety risks to laboratory personnel and generation of biohazardous waste, the use of inactivated virus reduced the sensitivity of the HI assay when testing sera from infected dogs with antibody titers near the cutoff titer for seropositivity. In addition, RBC streaming was at times more difficult to interpret than with live virus. BEI treatment is supposed to preserve viral proteins, therefore it was unclear why BEI-inactivated virus affected the sensitivity of the assay. One possible explanation is that this alkylating agent could cause cross-linking of the H3 proteins on the viral surface that could hinder hemagglutination; however, this could not be evaluated by the investigators. Further analysis of viral inactivation methods is warranted before inactivated H3N8 CIV could be reliably substituted for live virus in the HI assay.

The use of multiple H3N8 CIV isolates recovered from dogs in 2005 to 2009 did not alter the sensitivity and specificity of the standard HI test. Molecular and serologic analyses indicate that significant antigenic change has not occurred in H3N8 CIV isolates to date.[8,98,103] If antigenic drift does occur, then use of earlier isolates in HI testing of sera from dogs exposed to drifted viruses will compromise the sensitivity of

the assay, leading to misdiagnosis of truly infected dogs. This underscores the value of continual molecular monitoring of new isolates to maintain diagnostic test accuracy. Many diagnostic laboratories have H3N8 EIV isolates for routine HI testing, and some have attempted to use these isolates for diagnosis of H3N8 CIV infection. Phylogenetic analyses of H3N8 CIV and EIV isolates have shown that the canine viruses are most closely related to contemporary EIV isolates recovered from horses since 2000.[20,42,98,103] This genetic relatedness likely explains why use of a 2003 EIV isolate in the CIV HI test did not affect sensitivity and specificity, but use of earlier EIV isolates resulted in decreased sensitivity and misdiagnosis of truly infected dogs. While avian, swine, and human H3 isolates were not evaluated in this study, it is assumed that their use in the HI assay would not identify dogs infected with H3N8 CIV based on the genetic disparity between the H3 proteins.[20] Our previous report demonstrated that HI antibody titers for dogs truly infected with H3N8 CIV were <16 when tested with human H3N2 viruses.[20]

The binding preferences of H3N8 CIV were assessed in HA assays with a variety of avian and mammalian RBC. This is important not only to selection of the best RBC for use in the HI assay to maintain diagnostic accuracy, but the agglutination patterns for RBC from different species can also provide insight into viral specificities for SA receptors.[48] This study demonstrated that H3N8 CIV preferred avian and canine RBC, which primarily have SAα2,3-Gal and SAα2,6-Gal receptors with *N*-acetyl-neuraminic acid (NeuAc) as the SA species.[41,48,140] There was less viral agglutination of horse and ruminant RBC which primarily express SAα2,3-Gal receptors with *N*-glycolyl-neuraminic acid (NeuGc) as the SA species.[48] H3N8 EIV binds both NeuAcα2,3-Gal and

NeuGcα2,3-Gal receptors, but NeuGcα2,3-Gal receptors are required for viral entry and subsequent replication in the cell.[120] The RBC agglutination patterns of H3N8 CIV suggest that the H3 protein prefers NeuAcα2,3-Gal receptors like its EIV ancestor, but not NeuGcα2,3-Gal receptors. This supports the findings of a recent report showing reduced binding of H3N8 ClV to NeuGcα2,3-Gal receptor analogues.[139] These results, coupled with identification of SAα2,3-Gal receptors on respiratory epithelial cells in dogs,[21,72,80,112] suggest that the altered SA preference of CIV compared to EIV may be an important adaptation of the horse virus to the dog. This adaptation may be associated with the predictable amino acid substitutions near the H3 receptor-binding pocket that have become the molecular signature for H3N8 CIV.[20,98,103]

In conclusion, the HI assay is a highly sensitive and specific assay for serological diagnosis of H3N8 CIV infection in dogs. Implementation of the recommended cutoff titer for seropositivity of 32 and use of “standard” test parameters including 0.5% turkey RBC, serum treatment with RDE, and use of a live H3N8 CIV isolate should minimize misdiagnosis of H3N8 CIV-infected dogs. The best diagnostic approach for recent active infection is to perform the standard HI test on both acute and convalescent serum samples to document at least a 4-fold increase in antibody titer over time. Moving forward it will be important to continue monitoring for antigenic drift in the CIV H3 protein in order to ensure that the H3N8 CIV used in the HI assay is closely related to that in circulation. In addition adequate vaccination history is now imperative to accurately interpret H3N8 CIV HI test results, since the assay cannot differentiate antibodies induced by infection from vaccination with the inactivated whole H3N8 CIV vaccine.[27]

This limits the use of the HI assay in future epidemiological studies of H3N8 CIV in the U.S. if vaccination status is unknown.

Table 3-1. ROC analysis of antibody titers determined for 614 convalescent sera in the standard H3N8 CIV HI assay using the MN antibody titers as the gold standard.

HI titer[a]	Sensitivity (%) (95% CI)[b]	Specificity (%) (95% CI)	PPV (%)[c]	NPV (%)[d]	True positives	True negatives	False positives	False negatives
4	100.0 (99.1, 100.0)	0 (0, 4.3)	82.6	---	507	0	107	0
16	99.6 (98.5, 100.0)	92.5 (85.7, 96.3)	98.4	98.0	505	99	8	2
32	99.6 (98.5, 100.0)	94.4 (88.0, 97.6)	98.8	98.1	505	101	6	2
64	97.4 (95.6, 98.5)	95.3 (89.2, 98.2)	99.0	88.7	494	102	5	13
128	90.9 (88.1, 93.1)	96.3 (90.4, 98.8)	99.1	69.1	461	103	4	46
256	66.9 (62.6, 70.8)	99.1 (94.3, 100.0)	99.7	38.7	339	106	1	168
512	39.6 (35.5, 44.0)	99.1 (94.3, 100.0)	99.5	25.7	201	106	1	306
1024	17.8 (14.7, 21.3)	100.0 (95.7, 100.0)	100.0	20.4	90	107	0	417
2048	0.6 (0.1, 1.8)	100.0 (95.7, 100.0)	100.0	17.5	3	107	0	504

[a] HI antibody titer = reciprocal of the last serum dilution that inhibited red blood cell agglutination by H3N8 CIV.
[b] 95% confidence interval.
[c] PPV = positive predictive value.
[d] NPV = negative predictive value.

Table 3-2. H3N8 CIV agglutination of avian and mammalian red blood cells under different incubation conditions.

		H3N8 CIV HAU[c]			
		23°C X 30 min		4°C X 60 min	
RBC source[a]	RBC SA[b]	PBS	PBS/BSA	PBS	PBS/BSA
Chicken	2,3/2,6 NeuAc	32	16	32	16
Turkey	2,3/2,6 NeuAc	16	16	16	16
Goose	2,3/2,6 NeuAc	8	8	8	8
Dog	2,3/2,6 NeuAc	8	16	8	16
Horse	2,3 NeuGc	2	neg[d]	2	neg
Sheep	2,3 NeuGc	2	1	2	2
Cow	2,3 NeuGc	neg	neg	1	1

[a] Species source of red blood cells (RBC) used in the HA assays. Avian RBC suspensions were 0.5%, and mammalian RBC suspensions were 1.0% in either PBS or PBS/BSA.
[b] Predominant sialic acid (SA) linkages (2,3 and/or 2,6) and species (NeuAc= *N*-acetyl-neuraminic acid; NeuGc= *N*-glycolyl-neuraminic acid) on species-specific RBC.
[c] HAU = reciprocal of the last dilution of H3N8 virus that agglutinated RBC.
[d] No RBC agglutination by undiluted H3N8 viral suspension.

Table 3-3. HI antibody titers for sera from 11 dogs infected with H3N8 CIV tested with 0.5% chicken red blood cells (chRBC), 0.5% turkey red blood cells (tRBC), or 1% canine red blood cells (cnRBC). Sera were pretreated with RDE and serial dilutions incubated with H3N8 CN/FL/04 virus prior to addition of RBC suspensions. All serum samples were tested in duplicate.

	H3N8 CIV HI antibody titer[a]		
Sample ID	chRBC	tRBC	cnRBC
1	256	64	32
2	256	128	64
3	128	128	64
4	256	256	64
5	256	512	64
6	128	128	64
7	256	256	128
8	256	512	64
9	256	512	128
10	256	512	128
11	512	512	64
GMT[b]	240	256	73[c]

[a] HI antibody titer = reciprocal of the last serum dilution that inhibited red blood cell agglutination by H3N8 CIV. The cutoff titer for seropositivity in the standard H3N8 CIV HI assay is 32.
[b] Geometric mean antibody titer for 11 dogs.
[c] The GMT for cnRBC is significantly different from chRBC and tRBC (P-value <0.01).

Table 3-4. HI antibody titers for untreated or RDE-treated sera from 11 dogs infected with H3N8 CIV and 15 uninfected dogs. Serial dilutions of the untreated or RDE-treated sera were incubated with H3N8 CN/FL/04 virus prior to addition of 0.5% turkey (tRBC) or chicken (chRBC) red blood cell suspensions. All serum samples were tested in duplicate.

		H3N8 CIV HI antibody titer[b]			
		tRBC		chRBC	
Sample ID	CIV status[a]	Untreated[c]	RDE-treated	Untreated	RDE-treated
1	uninfected	4	4	32	4
2	uninfected	4	4	32	4
3	uninfected	4	4	64	4
4	uninfected	4	4	32	4
5	uninfected	4	4	32	4
6	uninfected	4	4	32	4
7	uninfected	4	4	32	4
8	uninfected	4	4	64	4
9	uninfected	4	4	32	4
10	uninfected	4	4	4	4
11	uninfected	4	4	32	4
12	uninfected	4	4	64	4
13	uninfected	4	4	32	4
14	uninfected	4	4	32	4
15	uninfected	4	4	4	4
16	infected	512	64	256	256
17	infected	512	128	256	256
18	infected	512	128	256	128
19	infected	256	128	128	128
20	infected	512	256	512	256
21	infected	512	256	512	256
22	infected	512	512	256	256
23	infected	1024	512	1024	256
24	infected	512	512	512	256
25	infected	1024	512	1024	256
26	infected	512	512	512	256

[a] H3N8 CIV infection status determined by the MN assay.
[b] HI antibody titer = reciprocal of the last serum dilution that inhibited red blood cell agglutination by H3N8 CIV. The cutoff titer for seropositivity in the standard H3N8 CIV HI assay is 32.
[c] Sera were either untreated or treated with RDE prior to incubation with virus.

Table 3-5. HI antibody titers for sera from 14 dogs infected with H3N8 CIV tested with live or inactivated virus. Sera were pretreated with RDE and serial dilutions incubated with live or BEI-inactivated H3N8 CN/FL/04 virus prior to addition of 0.5% turkey red blood cells. All assays were conducted in duplicate.

	H3N8 CIV HI antibody titer[a]	
Sample ID	Live virus[b]	Inactivated virus[c]
1	32	4
2	32	4
3	64	4
4	64	64
5	128	128
6	128	128
7	256	128
8	256	256
9	256	256
10	512	512
11	512	512
12	512	512
13	1024	512
14	1024	512

[a] HI antibody titer = reciprocal of the last serum dilution that inhibited red blood cell agglutination by H3N8 CIV. The cutoff titer for seropositivity in the standard H3N8 CIV HI assay is 32.
[b] Live H3N8 CN/FL/04 virus harvested from MDCK cultures.
[c] H3N8 CN/FL/04 virus harvested from MDCK cultures and inactivated by BEI treatment.

Table 3-6. HI antibody titers for sera from 46 dogs infected with H3N8 CIV and tested with different H3N8 CIV isolates in the standard HI assay. Serial dilutions of RDE-treated sera were incubated with H3N8 A/canine/Florida/43/2004 (CN/FL/04), A/canine/Jacksonville/2005 (CN/Jax/05), A/canine/FortCollins/3/2006 (CN/CO/06), A/canine/Weld/2007 (CN/CO/07), A/canine/Colorado Springs/2009 (CN/CO/09), and A/canine/Pennsylvania/96978/2009 (CN/PA/09) virus prior to addition of 0.5% turkey red blood cell suspensions. All serum samples were tested in duplicate with each virus. The H3N8 CIV HI antibody titer is the reciprocal of the last serum dilution that inhibited red blood cell agglutination. The cutoff titer for seropositivity in the standard HI assay is 32.

	H3N8 CIV HI antibody titer					
Sample ID	CN/FL/04	CN/Jax/05	CN/CO/06	CN/CO/07	CN/CO/09	CN/PA/09
1	32	32	32	32	32	32
2	64	32	32	32	32	32
3	64	64	32	64	32	32
4	64	32	32	64	32	32
5	64	32	32	64	32	32
6	64	64	64	64	64	64
7	128	64	64	64	64	64
8	128	64	64	128	64	64
9	128	64	64	128	64	64
10	128	128	64	128	64	64
11	128	128	128	128	128	128
12	128	128	128	64	64	64
13	128	128	128	128	128	128
14	128	128	128	128	128	128
15	128	64	64	64	64	64
16	128	128	64	128	64	64
17	128	128	128	128	128	128
18	128	128	128	128	128	128
19	128	128	128	128	128	128
20	128	128	128	128	128	128
21	128	128	128	128	128	128
22	128	128	128	128	128	128
23	128	128	128	128	128	256
24	256	128	128	128	128	128

Table 3-6. Continued.

	H3N8 CIV HI antibody titer					
Sample ID	CN/FL/04	CN/Jax/05	CN/CO/06	CN/CO/07	CN/CO/09	CN/PA/09
25	256	256	256	128	128	128
26	256	256	256	256	256	128
27	256	256	256	256	256	256
28	256	256	256	256	128	256
29	256	256	256	128	256	256
30	512	256	512	256	256	256
31	512	512	512	512	512	512
32	512	256	256	256	256	256
33	512	256	256	256	256	256
34	512	512	512	512	256	256
35	512	256	256	256	256	256
36	512	256	256	256	256	256
37	512	512	512	512	512	512
38	512	512	512	512	512	256
39	1024	1024	1024	1024	512	512
40	1024	512	1024	512	512	512
41	1024	1024	1024	1024	1024	1024
42	1024	1024	1024	1024	1024	1024
43	1024	1024	1024	1024	1024	1024
44	1024	512	1024	512	512	1024
45	1024	512	512	512	512	512
46	1024	1024	1024	1024	1024	1024

Table 3-7. HI antibody titers for sera from 46 dogs infected with H3N8 CIV and tested with different H3N8 EIV strains in the standard HI assay. Serial dilutions of RDE-treated sera were incubated with H3N8 A/canine/Florida/43/2004 (CN/FL/04), A/equine/Ohio/2003 (EQ/OH/03), A/equine/NewYork/1999 (EQ/NY/99), A/equine/Kentucky/1995 (EQ/KY/95), and A/equine/Kentucky/1991 (EQ/KY/91) virus prior to addition of 0.5% turkey red blood cell suspensions. All serum samples were tested in duplicate with each virus. The H3N8 CIV HI antibody titer is the reciprocal of the last serum dilution that inhibited red blood cell agglutination. The cutoff titer for seropositivity in the standard HI assay is 32.

	H3N8 CIV HI antibody titer				
Sample ID	CN/FL/04	EQ/OH/03	EQ/NY/99	EQ/KY/95	EQ/KY/91
1	32	32	16	8	8
2	64	64	16	16	8
3	64	64	32	16	16
4	64	64	16	8	8
5	64	64	16	16	16
6	64	128	32	16	16
7	128	128	32	16	16
8	128	128	32	16	32
9	128	128	64	32	16
10	128	128	64	64	64
11	128	128	64	32	32
12	128	128	64	32	32
13	128	128	64	64	64
14	128	256	64	32	32
15	128	128	32	32	32
16	128	128	32	32	32
17	128	256	64	32	32
18	128	256	64	64	32
19	128	128	64	64	64
20	128	256	64	64	64
21	128	256	64	64	64
22	128	256	64	64	64
23	128	256	64	64	64

Table 3-7. Continued.

	H3N8 CIV HI antibody titer				
Sample ID	CN/FL/04	EQ/OH/03	EQ/NY/99	EQ/KY/95	EQ/KY/91
24	256	256	64	64	64
25	256	256	64	32	64
26	256	256	128	64	64
27	256	512	128	128	128
28	256	256	128	128	128
29	256	256	128	128	64
30	512	512	128	128	64
31	512	512	256	128	128
32	512	256	64	64	32
33	512	512	128	128	64
34	512	256	128	64	128
35	512	512	256	128	128
36	512	512	128	128	64
37	512	512	256	128	128
38	512	512	256	256	128
39	1024	1024	512	512	256
40	1024	1024	512	256	256
41	1024	1024	1024	1024	1024
42	1024	1024	256	256	256
43	1024	1024	512	128	256
44	1024	1024	256	128	128
45	1024	1024	256	512	512
46	1024	1024	512	256	256

CHAPTER 4
DEVELOPMENT OF A DIVA TEST STRATEGY FOR H3N8 CANINE INFLUENZA VIRUS

In June 2009, the USDA approved the first vaccine for canine influenza. The H3N8 canine influenza virus (H3N8 CIV) vaccine is intended as an aid in the control of disease, and is recommended for dogs at risk of exposure.[27] Similar to influenza vaccines in other species, the H3N8 CIV vaccine contains inactivated whole virus that primarily induces antibodies to the hemagglutinin (H3) and neuraminidase (N8) surface proteins. Although an effective vaccine is beneficial for the prevention and control of H3N8 CIV, vaccine-induced H3 antibodies interfere with the serological diagnosis of infected dogs using the hemagglutination inhibition (HI) assay. The HI assay detects antibodies to specific hemagglutinin subtypes and is currently considered the confirmatory test of choice for H3N8 CIV infection.[28,29] The HI assay however cannot distinguish H3 antibodies induced by infection vs. vaccination, thereby creating a diagnostic dilemma for differentiating infected from vaccinated dogs based on seropositivity. This diagnostic dilemma has fueled the demand for alternative testing strategies that satisfy the principle of differentiation of infected from vaccinated animals (DIVA). There are no DIVA test strategies for H3N8 CIV, and a DIVA strategy should be developed to ensure correct identification of infected dogs in the face of vaccination.

One DIVA testing strategy is based on the expected immune response to the influenza A nonstructural protein, NS1. The NS1 protein is a multifunctional protein that inhibits the host immune response and is involved in several aspects of viral replication.[39] This protein is produced in large quantities in virus-infected cells, but is not packaged into progeny virions released from infected cells.[58,64] Infected animals can mount an antibody response to the NS1 protein released from the intracellular

cytoplasm of cells dying as a result of viral replication. Since inactivated whole virus influenza vaccines contain intact virions without NS1, vaccination should not induce antibodies to the NS1 protein.[117] Therefore, infected animals can be differentiated from vaccinated animals based on the presence of NS1 antibodies, thereby satisfying the DIVA principle.

NS1 antibody tests have been developed as a DIVA strategy for avian,[5,31,124,125,127,143] equine,[10,94] and swine[54] influenza. Based on these precedents, the objective of this study was to develop a NS1 antibody ELISA to differentiate H3N8 CIV-infected from vaccinated dogs. The best DIVA strategy would be to initially test for CIV H3 antibodies using the HI assay. H3 seropositive dogs would then be tested in a NS1 antibody ELISA to differentiate between infection and vaccination. For this study, molecular techniques were used to produce recombinant H3N8 CIV NS1 protein to capture specific antibodies in an ELISA and western blot. Archived sera from known infected or vaccinated dogs were tested in the H3 HI assay and NS1 ELISA or western blot to evaluate the DIVA strategy.

Materials and Methods

Virus Preparation

The NS1 amino acid sequence in different H3N8 CIV isolates collected from 2004 to 2008 is well conserved.[20,98,103] The NS1 gene of H3N8 A/canine/Florida/43/2004 (CN/FL/04) was selected for cloning and protein expression. The NS1 gene (Gen Bank Accession #DQ124153) contains 657 nucleotides, producing a protein of 219 amino acids (MW=24.7 kDal).

The CN/FL/04 isolate was kindly provided by Dr. R. Donis (Centers for Disease Control and Prevention, Atlanta, GA, USA). The virus was grown in MDCK cells using a

standard protocol,[20] with the described modifications. Briefly, approximately 75% confluent MDCK cells grown in 75-cm^2 flasks were inoculated with a 1:750 dilution of virus stock in PBS. The flasks were incubated for 1 hour at 35°C in a humidified atmosphere containing 5% CO_2, and then washed with sterile PBS. Dulbecco modified Eagle medium (DMEM) supplemented with 1 µg/mL TPCK (L-1-tosylamido-2-phenylethyl chloromethyl ketone)–treated trypsin (Sigma-Aldrich Corp., St. Louis, MO, USA), 0.85% bovine serum albumin (BSA), HEPES buffer, and penicillin-streptomycin was added to the flasks. The inoculated cells were incubated at 35°C in a humidified atmosphere containing 5% CO_2 for 3 to 5 days, with daily evaluation for cytopathic effect. The culture supernatant was harvested, clarified by centrifugation at 2000 rpm for 10 minutes, and the amount of virus determined with the hemagglutination (HA) assay using 0.5% turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA, USA) in PBS as previously described.[20] The viral culture supernatant was stored at -80°C until future use.

Amplification and Cloning of the NS1 Gene

Total RNA was extracted from viral culture supernatant using the QIAamp® Viral RNA Mini Kit (QIAGEN Inc., Valencia, CA, USA) according to manufacturer's instructions. The RNA was reverse-transcribed using SuperScript® III Reverse Transcriptase (Invitrogen Corp., Carlsbad, CA, USA), and the cDNA amplified by PCR reactions using forward and reverse primers for the CN/FL/04 NS1 gene and Platinum® *Taq* DNA Polymerase High Fidelity (Invitrogen Corp.). The 5' end of the NS1 forward primer contained the NcoI restriction enzyme cleavage site, while the 5' end of the reverse primer contained the XhoI restriction site (Table 4-1) to facilitate insertion of the NS1 amplicon into Exon 2 of the pTriEx™ 1.1 plasmid (Novagen®, EMD Chemicals Inc.,

Gibbstown, NJ, USA) (Figure 4-1). This vector codes for the fusion of a histidine tag on the 3' end of the cloned gene to facilitate purification of the expressed protein. The PCR product was run on a 1.0% agarose gel in Tris-borate-EDTA (TBE) buffer with an exACTGene® low range plus DNA ladder (Thermo Fisher Scientific Inc., Waltham, MA, USA).

The NS1 PCR product was purified using the QIAquick® PCR Purification Kit (QIAGEN Inc.) according to manufacturer's instructions. The purified PCR product and pTriEx™-1.1 vector were double-digested separately with 20U NcoI and 40U XhoI in NE Buffer #3 (New England Biolabs, Ipswich, MA, USA) at 37ºC for 2 hours. The digested pTriEx™-1.1 vector was heat-inactivated at 65ºC for 20 minutes and dephosphorylated with 5U calf intestinal alkaline phosphatase (New England Biolabs) at 37ºC for 1 hour. These products were run on a 1.0% agarose gel in TBE buffer with a λ DNA-HindIII Digest ladder (New England Biolabs) and an exACTGene® mid range plus DNA ladder (Thermo Fisher Scientific Inc.). The NS1 insert and pTriEx™-1.1 vector were extracted from the gel using a QIAquick® Gel Extraction Kit (QIAGEN Inc.) according to manufacturer's instructions, and were run on 1.0% agarose gels in TBE. The NS1 insert and pTriEx™-1.1 vector were ligated at 1:1, 1:5, and 1:7 molar ratios with 1µL T4 DNA Ligase (New England Biolabs). The ligation reactions were incubated at room temperature (23°C) for 2 hours and then heat-inactivated at 65°C for 5 minutes. Ligation products were run on 1.0% agarose gels in TBE.

NovaBlue Singles™ Competent Cells (Novagen®) were transformed with the NS1 recombinant plasmid according to manufacturer's instructions. Transformed cells were plated on Luria Bertani (LB) agar plates containing 50 µg/mL ampicillin and incubated at

37°C for 16 hours. Twenty colonies were selected and cultured overnight in LB broth supplemented with 1 µg/mL ampicillin at 37°C in a shaking incubator at 250 rpm. Plasmid DNA mini preps were prepared from six cultures using the QIAprep® Spin Miniprep Kit (QIAGEN Inc.) according to manufacturer's instructions. Double-digestion of the mini prep products was performed using the NcoI and XhoI restriction enzymes and the products were run on 1.0% agarose gels in TBE to verify the presence of the NS1 insert.

Two of the plasmid DNA mini preps were sequenced by the University of Florida Interdisciplinary Center for Biotechnology Research (UF ICBR) sequencing core, using the NS1 forward and reverse primers. The sequences were compared to that of the CN/FL/04 NS1 gene (Gen Bank Accession #DQ124153) using the National Center for Biotechnology Information Basic Local Alignment Search Tool (NCBI/BLAST, Bethesda, MD, USA) to verify proper orientation and lack of mutations. The sequences were also entered into the Swiss Institute of Bioinformatics ExPASy translate tool (Lausanne, Switzerland) to verify that the amino acid codons and histidine tag on the carboxy terminus of the recombinant protein were in frame for proper translation.

Expression and Purification of the NS1 Protein

Rosetta™ 2(DE3)pLacI Competent Cells (Novagen®) were transformed with mini prep DNA according to manufacturer's instructions and plated on LB agar plates containing 50 µg/mL ampicillin and 34 µg/mL chloramphenicol. The bacteria were induced to express the recombinant NS1 protein using Overnight Express™ Instant TB Medium (Novagen®) supplemented with 50 µg/mL ampicillin and 34 µg/mL chloramphenicol, at 37°C in a shaking incubator at 250 rpm for 24 hours. For the first expression culture, 1 mL samples were collected at the initial time point (0) and then

every two hours from 12 to 24 hours to monitor total protein production over time. The samples were centrifuged at 13,000 rpm for 5 minutes and the pellets lysed with BugBuster® Protein Extraction Reagent supplemented with benzonase nuclease, rlysozyme, and protease inhibitors (Novagen®) according to manufacturer's instructions. The total protein was analyzed by sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE) as described below.

Expression cultures were subsequently harvested at 24 hours by centrifugation at 3500 rpm for 30 minutes and the pellets lysed with BugBuster® Protein Extraction Reagent. Attempts were made to purify recombinant NS1 protein from the soluble fraction of the lysed culture pellets using nickel-NTA (Ni-NTA) His-Bind® resin, chromatography columns, and a Ni-NTA buffer kit (Novagen®) according to manufacturer's instructions; however, insufficient quantities were obtained.

Larger quantities of recombinant NS1 protein were purified from the insoluble fraction (inclusion bodies) of lysed pellets using a large-scale purification process conducted under denaturing conditions as follows. Culture pellets were resuspended in lysis buffer containing 8M urea, 50mM Tris-HCl, 2 mM ß-mercaptoethanol, 1M NaCl, and 20% glycerol (final pH 8.0), and incubated at 4°C for 24-48 hours. The lysate was sonicated on ice using an Ultrasonics W225R sonicator set at 70% maximum output three to five times for 30-60 second intervals, then centrifuged in a J2-21 Beckman centrifuge with a JA-20 rotor at 19,000 rpm for 1.5 hours. The resultant supernatant was passed through a 0.45µm sterile filter to remove any particulate material and added dropwise to a renaturing wash buffer (wash buffer 1) containing 50mM NaH_2PO_4, 300mM NaCl, and 20mM imidazole (final pH 8.0). The renatured lysate was filter-sterilized and loaded onto

Ni-NTA resin (QIAGEN Inc.) in a Bio-Rad Laboratories Inc. (Hercules, CA, USA) column (10 by 2.5 cm) attached to an Econo-line (Bio-Rad Laboratories Inc.) gradient purification pump system set at 0.65 mL/min. After flow-through of the lysate, the column was washed with three volumes of buffer (wash buffer 2) containing 50mM NaH_2PO_4, 300mM NaCl, 20mM imidazole, and 2% Triton X-114 (final pH 8.0), followed by five volumes of wash buffer 1. The recombinant NS1 protein was eluted in fractions with an elution buffer containing 50mM NaH_2PO_4, 300mM NaCl, and 250mM imidazole (final pH 8.0). Fractions were analyzed by SDS-PAGE and western blot as described below. Fractions containing the recombinant NS1 protein were pooled, filter-sterilized, and dialyzed with wash buffer 1 to remove imidazole. The purified NS1 protein was concentrated by ultrafiltration in an Amicon stirred cell using a 10 kDal molecular weight cut off membrane (Millipore, Billerica, MA, USA), exchanging the buffer for PBS (pH 7.4). The protein concentration was determined using a Quick Start™ Bradford Protein Assay (Bio-Rad Laboratories Inc.) according to manufacturer's instructions. The protein was analyzed by western blot as described below and by mass spectrometry at the UF ICBR to assess purity.

SDS-PAGE and Western Blot Assays for NS1 Protein Analysis

Total protein from expression cultures or purified NS1 protein was denatured in Laemmli's sample buffer (Bio-Rad Laboratories Inc.) supplemented with ß-mercaptoethanol according to manufacturer's instructions and heated at 95°C for 5 minutes. Extracted total protein from untransformed Rosetta™ 2 *E. coli* (Novagen®) served as a negative control for protein expression gels. Total protein samples (5 µL/well) or purified NS1 protein (0.5 to 1 µg/well) and Precision Plus Protein™ All Blue Standards (Bio-Rad Laboratories Inc.) were run on pre-cast 12% Tris-HCl Ready Gels®

using Tris-Glycine-SDS (TGS) buffer (Bio-Rad Laboratories Inc.) at 200V for 35 minutes. Proteins were visualized by incubating gels with Bio-Safe™ Coomassie (Bio-Rad Laboratories Inc.) according to manufacturer's instructions.

Western blots were performed to evaluate the antigenicity of the purified NS1 protein. Unstained SDS-PAGE gels were transferred to Immun-Blot® PVDF membranes at 110V for 70 minutes using Tris-Glycine (TG) buffer (Bio-Rad Laboratories Inc.). Blots were blocked with 5% (wt/vol) nonfat dry milk in PBS for 2 hours at 23°C, and washed three times with wash buffer (150mM NaCl in sterile water with 0.05% Tween® 20). Blots were incubated for 1 hour at 23°C with an affinity-purified goat IgG anti-influenza A NS1 antibody (Santa Cruz Biotechnology Inc., Santa Cruz, CA, USA) diluted 1:200 in PBS. Following three washes with wash buffer, blots were incubated for 1 hour at 23°C with a mouse anti-goat IgG antibody conjugated to horseradish peroxidase (HRP) (Santa Cruz Biotechnology Inc.) diluted 1:10,000 in PBS. Following three washes with wash buffer, antigen-antibody reactions were visualized by adding 3, 3', 5, 5'-tetramethylbenzidine (TMB) peroxidase substrate with membrane enhancer (Kirkegaard & Perry Laboratories Inc., Gaithersburg, MD, USA) according to manufacturer's instructions. Blots were rinsed in water to stop color development.

Synthetic NS1 Peptide

A synthetic H3N8 CIV NS1 peptide containing 16 amino acids ($NS1^{34-49}$ = DRLRRDQKSLRGRGST) was synthesized by RS Synthesis (Louisville, KY, USA). This sequence is conserved between the H3N8 CIV isolates and is comparable to a NS1(B) avian influenza sequence determined by another study to be hydrophilic and antigenic.[124] This peptide was used in an ELISA as described below.

Serum Samples

Sera from 20 specific pathogen-free (SPF) dogs involved in a H3N8 CIV vaccine trial were kindly provided by Dr. N. Lakshmanan (Intervet Schering Plough Animal Health, Millsboro, DE, USA). These included sera from dogs that were vaccinated and subsequently challenged with H3N8 CIV (n=10), and dogs that were unvaccinated and experimentally infected with H3N8 CIV (n=10). Sera were collected from the vaccinated and challenged dogs prior to vaccination (day 0), 21 days after the first vaccination, 14 days after the second vaccination, and 10 days after challenge with H3N8 CIV; therefore, 40 samples were available for testing from these 10 dogs. Sera from unvaccinated dogs experimentally infected with H3N8 CIV were collected 14 to 18 days after infection.

Serum samples from uninfected dogs (n=9) and dogs naturally infected with H3N8 CIV (n=59) were also included in the study. Samples from infected dogs included acute phase sera (<10 days postinfection; n=18 dogs) and convalescent phase sera (>21 days postinfection; n=41 dogs).

ELISA for Detection of NS1 Antibodies in Canine Sera

The NS1 ELISA was initially tested with serial dilutions of the recombinant NS1 protein and goat anti-NS1 antibody. The wells in EIA/RIA flat bottom microtiter plates (Corning® Costar®, Cambridge, MA, USA) were coated with two-fold serial dilutions of the recombinant NS1 protein in 0.05M carbonate-bicarbonate coating buffer (pH 9.6) resulting in protein concentrations ranging from 4 µg/well to 32 ng/well. A no antigen control column was included in each plate. Plates were sealed and incubated overnight at 4°C, washed three times with wash buffer (150mM NaCl in sterile water with 0.05% Tween® 20), and blocked with PBS containing 5% nonfat dry milk for 1 hour at 23°C.

After washing three times, a 1:200 dilution of affinity-purified polyclonal goat IgG anti-influenza A NS1 antibody (Santa Cruz Biotechnology Inc.) in PBS/5% milk was added to the wells. A no antibody control row was included in the plate. Plates were incubated at 23°C for 1 hour. After washing eight times, HRP-conjugated mouse anti-goat IgG antibody (Santa Cruz Biotechnology Inc.) diluted 1:10,000 in PBS/5% milk was added to the wells. Following incubation at 23°C for 1 hour, the plates were washed eight times, then incubated in the dark with TMB peroxidase substrate according to manufacturer's instructions for approximately 30 minutes at 23°C. Reactions were stopped with TMB stop solution (Kirkegaard & Perry Laboratories Inc.). Endpoint absorbance values were read at 450 nm in an automatic microtiter plate reader.

For checkerboard titrations with canine sera, microtiter wells were coated with two-fold serial dilutions of the recombinant NS1 protein in coating buffer with NS1 protein concentrations ranging from 4 µg/well to 4 ng/well. A no antigen control column was included in each plate. Canine sera were two-fold serially diluted in PBS/5% milk in the wells, starting at a dilution of 1:200. A no serum control row was included in each plate. Affinity-purified HRP-conjugated sheep anti-dog IgG heavy chain antibody (Bethyl Laboratories Inc., Montgomery, TX, USA) was diluted 1:10,000 in PBS/5% milk and added to the wells. Plates were developed and read as previously described.

An ELISA was performed with the synthetic $NS1^{34-49}$ peptide as described above with the following modifications. Wells were coated with 1 or 2 µg of the NS1 peptide diluted in coating buffer. Canine sera were diluted 1:100 or 1:400 (tested in duplicate), and the HRP-conjugated sheep anti-dog IgG heavy chain detection antibody was diluted 1:5,000 in SuperBlock® (PBS) Blocking Buffer (Thermo Fisher Scientific Inc.).

Western Blot Assays for Detection of NS1 Antibodies in Canine Sera

The reactivity of canine sera to the H3N8 CIV NS1 protein was initially assessed in western blot format according to the general procedure described above, but optimized with the following modifications. The ideal NS1 concentration was 0.5 µg to 1 µg/well and proteins were transferred to 0.2 µm nitrocellulose membranes (Bio-Rad Laboratories Inc.). SuperBlock® (PBS) Blocking Buffer was used for the blocking buffer to eliminate nonspecific background staining. Canine sera, positive and negative control antibodies, and detection antibodies were diluted in SuperBlock® with 0.05% Tween® 20. Blots were incubated with a 1:200 dilution of canine serum, a 1:10,000 dilution of HRP-conjugated sheep anti-dog IgG heavy chain antibody or a 1:10,000 dilution of HRP-conjugated goat-anti dog IgG-Fc antibody (LifeSpan Biosciences Inc., Seattle, WA, USA). The positive control combination was goat anti-influenza NS1 antibody and HRP-conjugated mouse anti-goat IgG. The negative control combination was polyclonal goat anti-Histone H2B IgG antibody (Santa Cruz Biotechnology Inc.) and HRP-conjugated mouse anti-goat IgG. Blots were developed with TMB peroxidase substrate and membrane enhancer as previously described.

Western blots were also performed with canine sera that had been pretreated by one of two methods: 1) incubation with 1% (wt/vol) *E. coli* acetone powder (Sigma-Aldrich Inc.) at 4°C for 30 minutes, and centrifuged at 13,000 rpm for 10 minutes;[40] or 2) preadsorption with *E. coli* acetone powder as described followed by incubation with receptor destroying enzyme (RDE; Denka-Seiken, Tokyo, Japan)(1 part serum: 3 parts RDE) for 16 hours at 37°C and heat-inactivation for 30 minutes at 56°C.

To test large panels of sera by western blot, the recombinant NS1 protein (0.5 µg/well) and Precision Plus Protein™ All Blue Standard were electrophoresed on 4-12%

NuPAGE® Novex® precast gels (Invitrogen Corp.) according to manufacturer's instructions. Gels were transferred via the iBlot® Dry Blotting System to 0.2 µm nitrocellulose membranes (Invitrogen Corp.) according to manufacturer's instructions. Blots were blocked with SuperBlock® for 1 hour at 23°C and washed three times with wash buffer. The remaining incubations and wash steps were as previously described. Canine sera and positive and negative control antibodies were diluted 1:500 in SuperBlock® with 0.05% Tween® 20. Secondary detection antibodies (HRP-conjugated sheep anti-dog IgG-Fc for canine sera and HRP-conjugated mouse anti-goat IgG for controls) were diluted 1:5,000 in SuperBlock® with 0.05% Tween® 20. Blots were developed with TMB peroxidase substrate and membrane enhancer as previously described.

Results

NS1 Protein Expression and Purification

Restriction enzyme digestion of six plasmid DNA mini preps indicated that all contained the ~650bp H3N8 CIV NS1 gene insert (Figure 4-2). Sequence analysis of two plasmid DNA mini preps confirmed that the NS1 gene was in proper orientation in both samples and that no mutations had occurred. The amino acid codons and histidine tag were also in frame for proper translation.

Recombinant NS1 protein expression was detectable at 12 hours and peaked by 18 hours (Figure 4-3). Initial attempts to purify substantial amounts of recombinant NS1 protein from the soluble fraction of the protein extract were unsuccessful (Figure 4-4). Most of the recombinant protein was present in inclusion bodies in the insoluble fraction (Figures 4-4 and 4-5); therefore, large-scale protein purification was performed under denaturing conditions. Once purified and refolded the antigenicity of the NS1 protein

was verified by western blot (Figure 4-6) and ELISA (Table 4-2) using an anti-influenza A NS1 antibody. In both formats, the minimum amount of detectable NS1 protein was ~32 ng. Mass spectrometry indicated that the NS1 protein contained only one major contaminating *E. coli* protein, which had a molecular weight of 80 kDal. Since this protein was much larger than the NS1 protein (25 kDal), it was not considered a concern for immunodiagnostic testing.

ELISA to Detect NS1 Antibodies in Canine Sera

In the NS1 ELISA using goat anti-NS1 as the primary antibody, there was a linear decline in absorbance values when the recombinant NS1 protein concentration decreased from 500 ng/well to 32 ng/well (Table 4-2).

Checkerboard titrations with recombinant NS1 protein and canine sera revealed false positive reactivity with serum from uninfected/unvaccinated dogs (Table 4-3). Substitution of the secondary HRP-conjugated sheep anti-dog IgG heavy chain antibody with an alkaline phosphatase-conjugated anti-dog IgG heavy chain antibody did not resolve the false positive reactions (data not shown). A sandwich ELISA using anti-influenza NS1 antibody as the coating agent followed by addition of NS1 protein to capture NS1 antibody in canine sera also did not eliminate false positive reactions with serum from uninfected/unvaccinated dogs (data not shown).

To address potential binding of canine antibodies to contaminating *E. coli* proteins in the NS1 preparation as a cause of false positive reactions, the ELISA was performed with a synthetic 16 amino acid NS1 peptide ($NS1^{34-49}$) comparable to a NS1(B) avian influenza peptide determined by another study to be a major antibody-binding region of NS1 proteins.[124] Use of the synthetic peptide did not resolve the false positive reactions for sera from uninfected/unvaccinated dogs (Table 4-4).

Western Blot Assays to Detect NS1 Antibodies in Canine Sera

When the optimized western blot was performed with canine sera, staining of the NS1 band was consistently observed with sera from uninfected/unvaccinated dogs. Substitution of anti-dog IgG heavy chain with anti-dog IgG Fc as the secondary antibody did not eliminate false reactivity with uninfected/unvaccinated dog serum. Importantly, when the western blots were performed with only the secondary antibody (i.e., no serum present), no NS1 bands were observed, indicating that the false positive results were likely attributable to a naturally occurring IgG antibody in canine serum that cross-reacts with NS1 protein or contaminating *E. coli* protein. This was addressed by two serum pretreatment methods: preadsorption of sera with *E. coli* acetone powder and preadsorption plus treatment with RDE and heat-inactivation. Neither serum treatment method resolved the false positive results for sera from uninfected/unvaccinated dogs.

To determine if nonspecific antibody reactivity with NS1 was a variable or universal phenomenon among dogs, a large panel of serum samples (n=118) from 88 dogs with known H3N8 CIV infection or vaccination status was tested in the NS1 western blot in a blinded fashion. This panel included sera from 9 uninfected/unvaccinated SPF dogs, acute phase sera from 18 naturally infected dogs, convalescent sera from 41 naturally infected dogs, sera collected from 10 SPF dogs at different time points after H3N8 CIV vaccination and subsequent viral challenge, and sera from 10 SPF dogs 14 to 18 days after experimental H3N8 CIV infection. Regardless of infection or vaccination status, samples from all dogs appeared seropositive for NS1 antibody (Figures 4-7 and 4-8).

Discussion

The H3N8 CIV recombinant NS1 protein was successfully expressed in *E. coli* and purified for diagnostic immunoassays. The identity and purity of the recombinant NS1 protein was verified through sequencing and mass spectrometry analysis. The protein antigenicity was demonstrated in both ELISA and western blot formats using an anti-influenza A NS1 antibody. However, ELISA and western blot assays using the recombinant NS1 protein to capture specific antibodies in sera from H3N8 CIV-infected dogs lacked specificity based on false positive reactivity in sera from uninfected or unvaccinated dogs and dogs vaccinated with the H3N8 CIV whole virus vaccine. Therefore, the NS1 ELISA and western blot evaluated in this study cannot differentiate between uninfected, infected, and vaccinated dogs. This finding is similar to that of another study conducted with sera from uninfected, infected, and vaccinated dogs tested in a NS1 western blot (Dr. N. Lakshmanan, Intervet Schering Plough Animal Health, Millsboro, DE, USA, personal communication).

There are several possibilities for the lack of differentiation between uninfected or vaccinated dogs and H3N8 CIV-infected dogs by the immunoassays for NS1 antibody. The NS1 ELISA and western blots performed with secondary anti-dog IgG antibody in the absence of canine serum were negative, demonstrating the necessity for canine IgG for seropositivity. Naturally occurring antibodies to ubiquitous *E. coli* may have recognized trace *E. coli* contamination in the NS1 protein preparation. This was considered unlikely however, because preadsorption of canine sera with *E. coli* acetone powder and use of the synthetic NS1 peptide in the ELISA did not eliminate seroreactivity for uninfected or vaccinated dogs.

Cross-reactivity with avian influenza NS1 protein has been reported for sera from chickens infected with infectious bursal disease virus, avian reovirus, avian pneumovirus, and other avian pathogens.[127] Increased seroreactivity to NS1 protein was also reported for chickens in influenza-free commercial flocks compared to SPF chickens, suggesting potential cross-reactive antibodies in the commercial poultry.[127] Similar to chickens, antibodies to common canine pathogens induced by exposure or vaccination may have cross-reactivity with canine influenza NS1 protein. In addition to H3N8 CIV, other pathogens associated with acute respiratory disease in dogs include canine distemper virus, canine adenovirus type 2, canine parainfluenza virus, canine respiratory coronavirus, canine herpesvirus, and the recently described canine pneumovirus.[28] Based on a BLAST search excluding influenza A NS1 proteins, no proteins from these viruses or other pathogens (including canine parvovirus) were identified as closely related to NS1. Instead, a protein from the bay scallop (Gen Bank Accession #EU623917.1) was most closely related to influenza A NS1 protein. The significance of this similarity is unknown. It is also uncertain if some degree of cross-reactivity could still occur with the above listed canine pathogens despite their proteins not being closely related to the NS1 protein. It is important to note, however, that none of the uninfected SPF dogs had been exposed to or vaccinated against these pathogens, yet their sera contained cross-reactive antibodies to the NS1 protein in the immunoassays.

Although the histidine tag on the carboxy terminus of the NS1 recombinant protein was valuable for purification, a peptide formed by union of the histidine residues with the NS1 protein may have been recognized by ubiquitous antibodies in canine serum that

are not specific for the recombinant protein. False positive results have been noted with histidine tagged recombinant proteins in ELISA testing by others (Dr. W. Dundon, Istituto Zooprofilattico Sperimentale delle Venezie, Legnaro, Italy, personal communication). Identification of cross-reactive antibodies attributable to the histidine tag on recombinant NS1 protein would require production of recombinant protein without the tag or removal of the tag after purification. However, the possibility of cross-reactive antibodies to the histidine tag seems unlikely since use of a synthetic NS1 peptide in the ELISA did not eliminate seroreactivity in uninfected or vaccinated dogs.

The inability of the NS1 immunoassays to differentiate uninfected or vaccinated dogs from infected dogs may also be due to the possibility that infected dogs produce very low amounts of antibody to the H3N8 CIV NS1 protein that are not detectable in the face of nonspecific reactions. Variability in NS1 antibody production has been reported between species and between influenza viruses.[5,10,31,125,127] Antibodies to the NS1 protein or its peptides are not produced in all influenza-infected animals, and the emergence and persistence of NS1 antibodies can vary considerably. In studies with other species, antibodies to NS1 protein typically appeared early after infection and persisted for at least 1 to 5 weeks, but the amounts of antibody were relatively low.[5,10,31,54,94,124,125,127,143] Although the appearance and persistence of NS1 antibodies in influenza-infected dogs is unknown, sera tested in this study were collected from naturally and experimentally infected dogs at several time points post-infection, including <1 week, 14 to 18 days, and 3 to 4 weeks. The NS1 seroreactivity of these samples was indistinguishable from that obtained with samples from uninfected or

vaccinated dogs, preventing any conclusions regarding the emergence and persistence of NS1 antibodies in canine sera.

Overall, the specificity issues encountered in this study could not be resolved and supported the conclusion that an influenza A NS1 DIVA test is not a viable or reliable diagnostic strategy for differentiating dogs infected with H3N8 CIV from uninfected dogs or vaccinated dogs. Since a DIVA test strategy is necessary for correct identification of infected dogs in the face of vaccination, future work should focus on development of alternative DIVA strategies. A successful strategy will not only allow correct identification of infected dogs with unknown vaccination history, but will also enable serological evaluation of vaccine efficacy in the field as well as future epidemiological studies of canine influenza.

Table 4-1. Primers for PCR amplification and cloning of the H3N8 CIV NS1 gene. The primers contain clamp sequences for polymerase fidelity and restriction enzyme sites for gene ligation into the plasmid vector.

Primer	Target	Sequence
NS1-forward	NS1 (1-26)	5' CTCCTCCCATGGATTCCAACACTGTGTCAAGC 3' CTCCTC = clamp CCATGG = NcoI restriction site
NS1-reverse	NS1 (657-627)	5' CTCCTCCTCGAGTTTCTGCTTTGAAGGGAATGAAGG 3' CTCCTC = clamp CTCGAG = XhoI restriction site

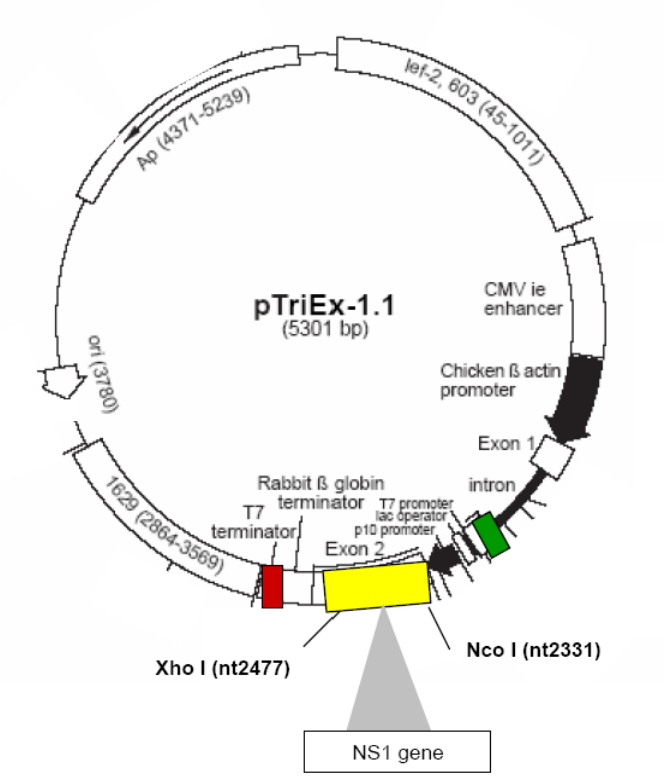


Figure 4-1. The pTriEx™ 1.1 (Novagen®) plasmid for cloning of the H3N8 CIV NS1 gene. The gene was inserted into exon 2 (yellow box) at restriction enzyme sites for NcoI and XhoI.

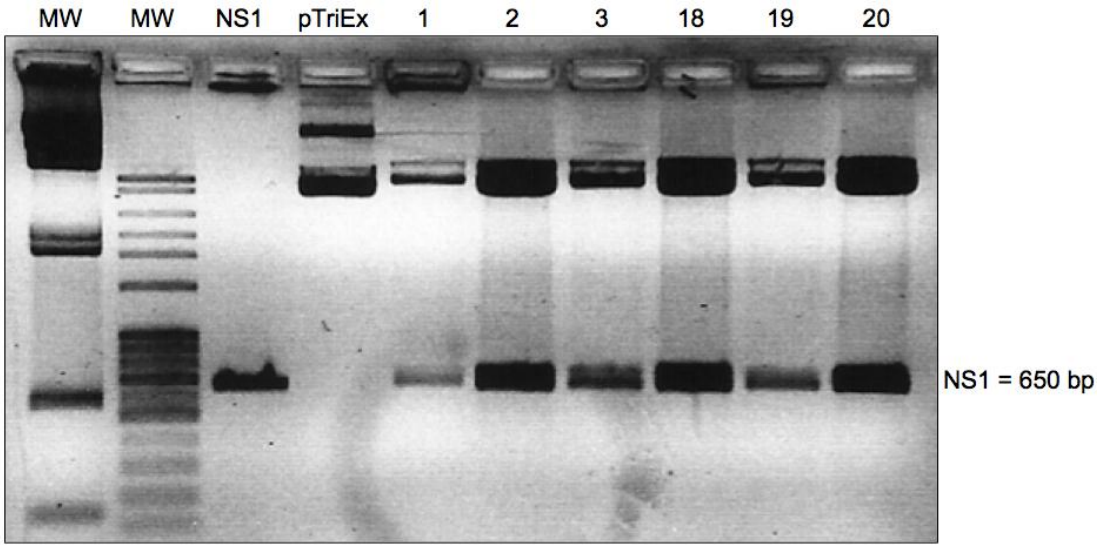


Figure 4-2. SDS-PAGE electrophoresis of plasmid preparations (1, 2, 3, 18, 19, 20) digested with NcoI and XhoI restriction enzymes to release the NS1 gene cloned into the pTriEx™ 1.1 plasmid vector. MW = molecular weight marker, NS1 = Purified NS1 PCR product, pTriEx = pTriEx™ 1.1 (Novagen®) vector DNA.

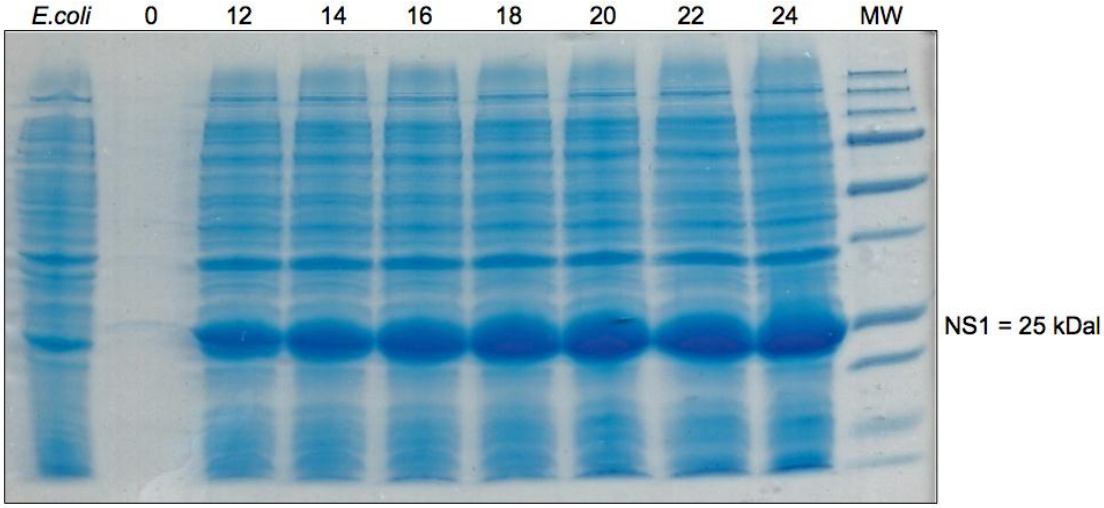


Figure 4-3. SDS-PAGE electrophoresis of total protein extracted from recombinant H3N8 CIV NS1 plasmid-transformed Rosetta™ 2 *E. coli* (Novagen®) and stained with Coomassie blue. To assess peak NS1 protein expression, samples were collected from transformed cell cultures at the time of inoculation (0) and every two hours from 12 to 24 hours after autoinduction. Expression of the recombinant NS1 protein (MW=25 kDal) was apparent at 12 hours. *E. coli* = untransformed cells. MW = molecular weight marker.

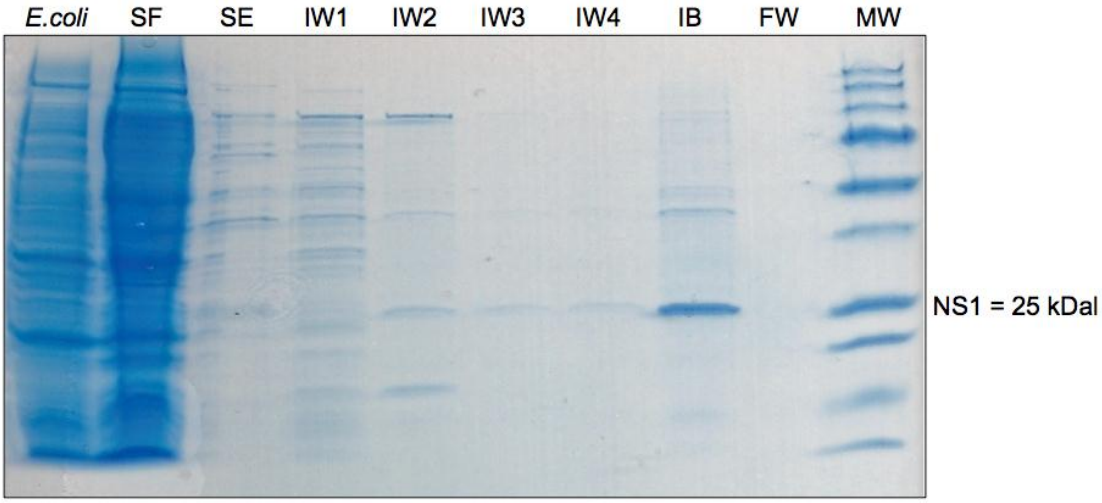


Figure 4-4. SDS-PAGE electrophoresis of soluble and insoluble fractions of a lysed recombinant NS1 plasmid-transformed Rosetta™ 2 *E. coli* (Novagen®) cell pellet and stained with Coomassie blue. *E. coli* = untransformed cells. SF = soluble fraction. SE = purified elution from soluble fraction. IW(1-4) = washes 1-4 of insoluble fraction. IB = resuspended inclusion bodies. FW = final insoluble fraction wash. MW = molecular weight marker. Small amounts of recombinant NS1 protein are detected in the insoluble fraction washes; however, the majority is in the resuspended inclusion body fraction (IB).

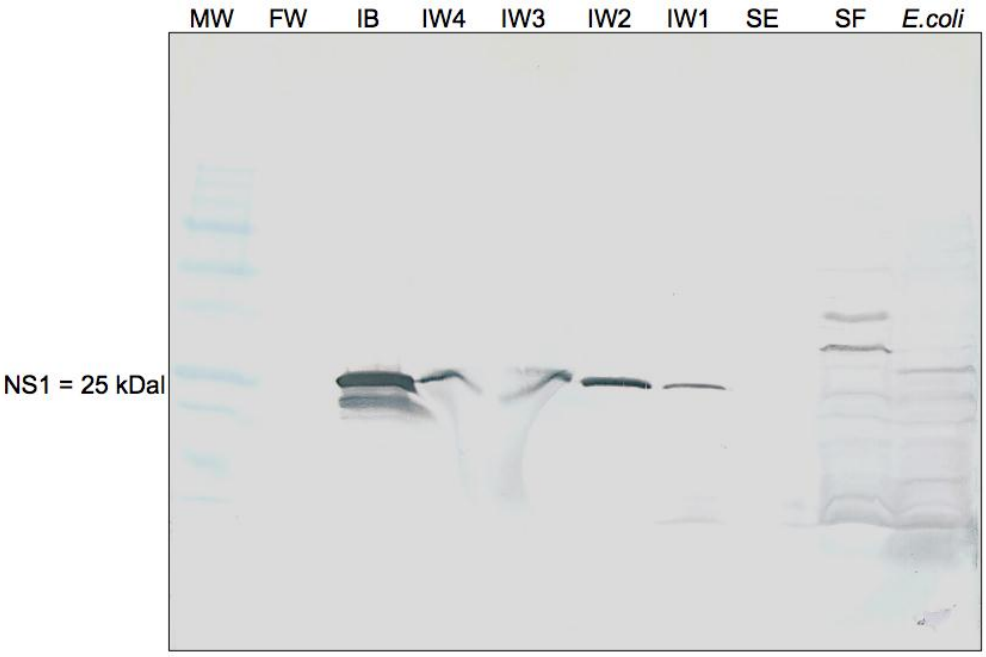


Figure 4-5. Western blot of soluble and insoluble fractions of a lysed recombinant NS1 plasmid-transformed Rosetta™ 2 *E. coli* (Novagen®) cell pellet and stained with an affinity-purified antibody to influenza NS1 protein. *E. coli* = untransformed cells. SF = soluble fraction. SE = purified elution from soluble fraction. IW(1-4) = washes 1-4 of insoluble fraction. IB = resuspended inclusion bodies. FW = final insoluble fraction wash. MW = molecular weight marker. Small amounts of recombinant NS1 protein are detected in the insoluble fraction washes; however, the majority is in the resuspended inclusion body fraction (IB).

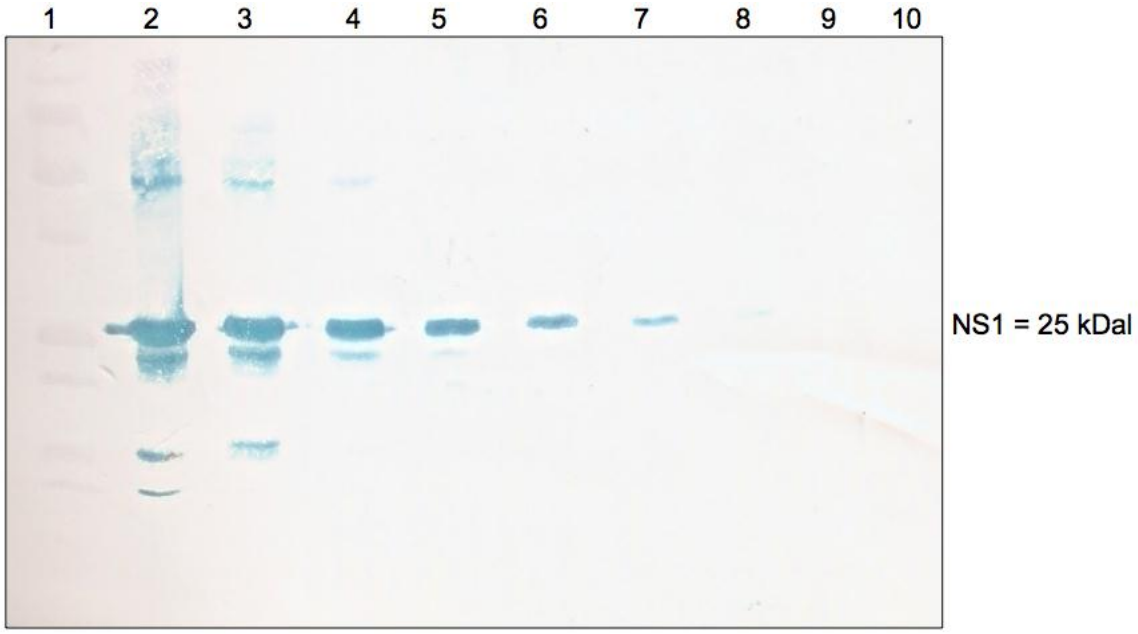


Figure 4-6. Western blot of purified H3N8 CIV NS1 protein detected using goat anti-influenza NS1 IgG antibody and a HRP-conjugated mouse anti-goat IgG antibody. Lanes 2 through 10 contain serial two-fold dilutions of the purified protein representing from 1µg in lane 2 to 4ng in lane 10. The protein is detectable at 16 ng (lane 8).

Table 4-2. Checkerboard titration results for the H3N8 NS1 antibody ELISA. Serial two-fold dilutions of recombinant NS1 protein were incubated with goat anti-influenza NS1 antibody (1:200) followed by HRP-conjugated mouse anti-goat IgG (1:10,000). Absorbance values are reported for duplicate wells for each NS1 concentration.

H3N8 CIV NS1 protein concentration								
4 µg	2 µg	1 µg	500 ng	250 ng	125 ng	62 ng	32 ng	0
3.8	3.8	3.8	3.5	2.6	1.5	0.7	0.4	0.1
3.9	3.9	3.9	3.7	3.1	1.8	1.0	0.5	0.1

Table 4-3. Checkerboard titration results for the H3N8 NS1 antibody ELISA. Serial two-fold dilutions of uninfected and H3N8 CIV-infected dog sera were incubated with varying NS1 protein concentrations followed by addition of HRP-conjugated anti-dog IgG heavy chain antibody diluted 1:10,000. Absorbance values are reported.

	H3N8 CIV NS1 protein concentration											
Serum	4 µg	2 µg	1 µg	500 ng	250 ng	125 ng	62 ng	32 ng	16 ng	8 ng	4 ng	0
Uninfected												
1:200	3.5	3.3	3.2	2.8	2.1	1.6	1.2	0.9	0.8	0.8	0.8	0.8
1:400	2.9	3.1	2.7	2.4	1.7	1.1	0.8	0.7	0.6	0.5	0.5	0.5
1:800	2.7	2.5	2.7	1.7	1.2	0.8	0.6	0.5	0.3	0.3	0.3	0.3
1:1600	2.1	1.8	2.2	1.6	1.0	0.5	0.4	0.2	0.2	0.2	0.2	0.2
0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1
H3N8 CIV-infected												
1:200	2.1	2.2	1.6	1.1	0.9	0.7	0.6	0.5	0.5	0.5	0.5	0.5
1:400	1.3	1.2	0.9	0.7	0.5	0.4	0.4	0.4	0.4	0.3	0.3	0.3
1:800	0.7	0.6	0.5	0.4	0.3	0.2	0.2	0.2	0.2	0.2	0.2	0.2
1:1600	0.4	0.4	0.3	0.2	0.2	0.2	0.1	0.1	0.1	0.1	0.1	0.1
0	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1	0.1

Table 4-4. The H3N8 NS1 antibody ELISA was performed with varying concentrations of the synthetic $NS1^{34-49}$ peptide incubated with uninfected and H3N8-CIV infected dog sera followed by addition of HRP-conjugated anti-dog IgG heavy chain antibody (1:5,000). Sera were tested in duplicate, and average absorbance values are reported.

	NS1 peptide concentration	
Serum	2 µg	1 µg
Uninfected		
1:100	1.72	1.84
1:400	1.17	1.16
H3N8 CIV-infected		
1:100	1.60	1.47
1:400	1.30	1.01

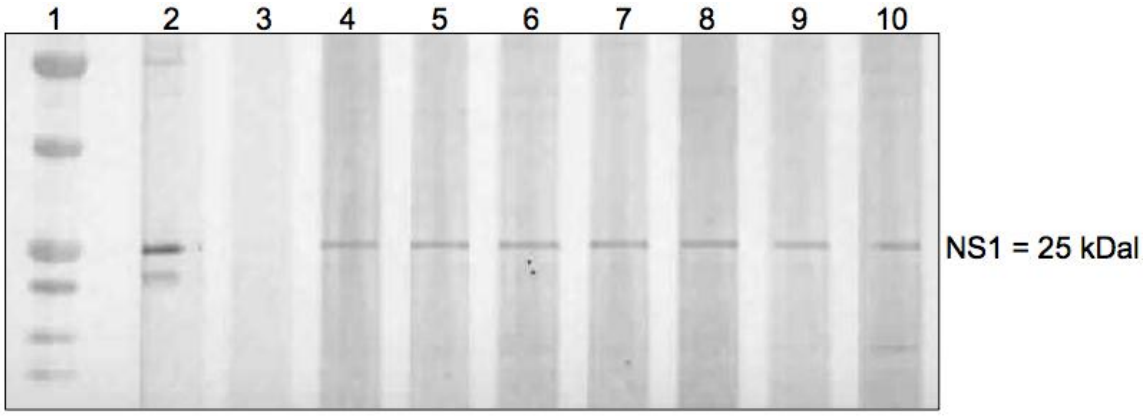


Figure 4-7. H3N8 CIV NS1 western blot with sera from uninfected SPF dogs and dogs experimentally infected with H3N8 CIV. NS1 protein concentration = 0.5 µg per well. Canine sera and controls diluted 1:500. HRP-conjugated anti-goat IgG and HRP-conjugated anti-dog IgG-Fc secondary antibodies diluted 1:5000. Lanes: 1 = molecular weight marker; 2 = goat anti-influenza NS1 antibody (positive control); 3 = goat anti-histone antibody (negative control); 4, 6, 8, 10 = sera from SPF dogs; 5, 7, 9 = sera from experimentally infected dogs collected 14 days postinfection.

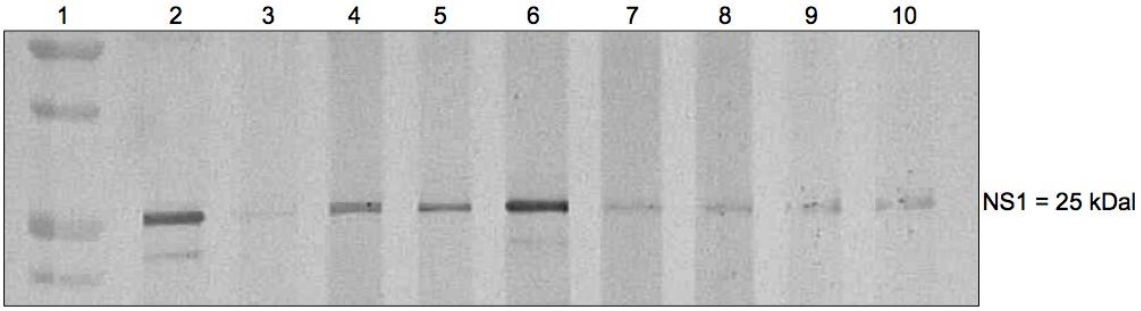


Figure 4-8. H3N8 CIV NS1 western blot with sera from uninfected, infected, and vaccinated dogs. NS1 protein concentration = 0.5 µg per well. Canine sera and controls diluted 1:500. HRP-conjugated anti-goat IgG and HRP-conjugated anti-dog IgG-Fc secondary antibodies diluted 1:5000. Lanes: 1 = molecular weight marker; 2 = goat anti-influenza NS1 antibody (positive control); 3 = goat anti-histone antibody (negative control); 4 = uninfected SPF dog; 5, 6 = acute and convalescent sera from a dog infected with H3N8 CIV; 7 = dog vaccinated with one dose of H3N8 CIV vaccine; 8 = dog vaccinated with two doses of H3N8 CIV vaccine; 9 = vaccinated dog challenged with H3N8 CIV; 10 = dog experimentally infected with H3N8 CIV.

CHAPTER 5
SEROLOGICAL EVIDENCE OF H3N8 CANINE INFLUENZA-LIKE VIRUS CIRCULATION IN U.S. DOGS PRIOR TO 2004

Canine influenza A virus subtype H3N8 (H3N8 CIV) was identified as a respiratory pathogen for dogs in 2004.[20] The virus was first isolated from racing greyhounds during a respiratory disease outbreak at a Florida track, and was subsequently identified as the cause of multiple respiratory disease outbreaks among thousands of racing greyhounds in multiple states through 2006.[20,141] In 2005, dogs in Florida shelters were the first non-greyhound cases of canine influenza to be confirmed by virus isolation, although serological evidence of canine influenza in non-greyhound dogs had been reported in 2004.[20,98] Ongoing surveillance of U.S. dogs with influenza-like illness (ILI) indicates widespread geographic distribution of H3N8 CIV infections in 34 states, and the virus has become endemic in metropolitan areas of the northeastern and western regions of the country.[8,9,20,28,29,42,44,98,103,110,111]

Molecular and phylogenetic analyses of H3N8 CIV isolates recovered from dogs from 2003 to 2008 support the interspecies transfer of an entire H3N8 equine influenza A virus (H3N8 EIV) from the horse to the dog;[20,42,98,103] however, it is unknown when this transmission occurred or what population(s) of dogs were originally affected. Prior to the discovery of H3N8 CIV in 2004, frequent respiratory disease or “kennel cough” outbreaks of unknown etiology had occurred in racing greyhounds throughout the U.S. in the 1990s and 2003.[20,28,29] Limited testing of archived tissues and serum samples from racing greyhounds has suggested that a H3N8 CIV-like virus may have circulated in this population as early as 2000.[20] Archived sera collected from 2000 to 2003 from 65 racing greyhounds were tested in the hemagglutination inhibition (HI) assay with a 2004 isolate of H3N8 CIV and 9 dogs were seropositive indicating previous virus exposure.[20]

Further, examination of archived lung tissue from a greyhound that died of hemorrhagic bronchopneumonia during a respiratory disease outbreak at a Florida track in March 2003 yielded the earliest known H3N8 canine influenza isolate (A/canine/Florida/242/2003).[20] Information on the prevalence of H3 antibodies in pet and shelter dogs prior to 2004 is also limited. To our knowledge, only one study has tested pet dog sera collected prior to 2004 for antibodies to CIV H3; and all 75 dogs from this Colorado study were seronegative.[8]

The objectives of this study were 1) to determine the prevalence of CIV and EIV H3 antibodies in racing greyhound and shelter dog sera collected from 1984 to 2004 to further investigate when H3N8 viruses first infected dogs, and 2) to analyze potential risk factors associated with seropositivity. This retrospective serosurvey provides additional information about the emergence of H3N8 CIV in the canine population.

Materials and Methods

Study Design

A retrospective serosurvey of convenience samples collected from racing greyhounds and dogs housed in animal shelters from 1984 to 2004 was conducted.

Serum Samples

Archived serum samples from 702 racing greyhounds were tested for antibodies to CIV and EIV H3 proteins. One set of samples (n=153 dogs) was collected in 1984 and 1985 from greyhounds located at tracks and farms in north Florida. These samples were originally collected for a heartworm antigen test study. Another set of samples (n=549 dogs) was collected from 1999 to 2004 from greyhounds in multiple states as they came off the tracks and entered a commercial blood-banking program (Hemopet/Pet Life-Line, Garden Grove, CA, USA). Available history for racing

greyhounds was recorded using unique ear tattoos and a corresponding database housed at www.greyhound-data.com. Serum samples from shelter dogs (n=1,568) were collected on entry into north Florida animal shelters from 1984 to 2004.

Viruses

Madin-Darby canine kidney (MDCK) cell cultures were inoculated with H3N8 A/canine/Florida/43/2004 (CN/FL/04) and the culture supernatant stored at -80°C prior to use. H3N8 A/equine/New York/1999 (EQ/NY/99), A/equine/Kentucky/1995 (EQ/KY/95), and A/equine/Kentucky/1991 (EQ/KY/91) isolates were cultured in embryonated chicken eggs and the allantoic fluids stored at -80°C prior to use. CN/FL/04 was kindly provided by Dr. R. Donis (Centers for Disease Control and Prevention, Atlanta, GA, USA), EQ/KY/91 and EQ/KY/95 were kindly provided by Dr. T. Chambers (University of Kentucky, Lexington, KY, USA), and EQ/NY/99 was kindly provided by Dr. E. Dubovi (Cornell University, Animal Health Diagnostic Center, Ithaca, NY, USA).

HI Assays

HI assays were conducted as previously described.[20] Briefly, serum samples were pretreated with receptor destroying enzyme (RDE, Denka Seiken, Tokyo, Japan) followed by heating at 56°C for 30 minutes to inactivate nonspecific inhibitors of hemagglutination. Serial two-fold dilutions of each sample were incubated in 96-well V-bottom microtiter plates with 4 hemagglutinating units/25µL of test virus for 30 minutes at room temperature (23°C), followed by an additional incubation for 30 minutes at 23°C with an equal volume of 0.5% turkey red blood cells in PBS.

All sera were tested with the CN/FL/04 isolate and one of the three equine isolates. H3N8 EIV isolates were included in the HI assays to address the possibility

that dogs were initially infected by these (or similar) viruses, prior to viral adaptation resulting in the currently circulating H3N8 CIV. This rationale is supported by molecular evidence that H3N8 EIV isolates have undergone significant antigenic drift since the 1980s, [15,22,61,62] and all H3N8 CIV isolates analyzed to date are most closely related to the Florida sublineage of EIV circulating in U.S. horses since 2000.[20,98,103] Additionally, H3 antibodies in dogs infected with CIV since 2004 have reduced reactivity with EIV isolates obtained before 1999.[98] Based on this rationale, the 1984 to 1985 greyhound samples were tested with CN/FL/04 and EQ/KY/91, and the 1999 to 2004 greyhound samples were tested with CN/FL/04 and EQ/NY/99.

To facilitate testing the large number of shelter dog samples, untreated sera were initially screened at 1:10 and 1:40 dilutions in the HI assay. Samples collected from 1984 to 1992 were screened using CN/FL/04 and EQ/KY/91, the 1993 to 1998 samples were screened with CN/FL/04 and EQ/KY/95, and the 1999 to 2004 samples were screened with CN/FL/04 and EQ/NY/99. Sera that were positive in the screening HI assays were subsequently treated with RDE and heat-inactivation and re-tested as previously described.

All greyhound and shelter dog sera were tested in duplicate. Sera collected from specific pathogen-free dogs housed in a barrier research facility served as negative controls in all assays. Convalescent serum samples from racing greyhounds naturally infected with H3N8 CIV served as positive controls. Their seropositivity was previously confirmed by both HI and serum microneutralization (MN) assays.[20,98] Endpoint H3 antibody titers were defined as the reciprocal of the last serum dilution that completely

inhibited hemagglutination. Seropositive dogs were defined as those with endpoint antibody titers ≥32 as previously described.[20]

Data Analysis

HI test results and other recorded data for the greyhounds (n=702) and shelter dogs (n=1,568) were managed in standard software (Excel®, Microsoft, Redmond, WA, USA). For the greyhounds sampled from 1999 to 2004, the prevalence of H3 seropositive dogs was calculated for each year by dividing the number of seropositive dogs by the total number of dogs tested that year X 100. In addition, geometric mean antibody titers (GMT) were calculated for both CIV and EIV for each of these years. The Mann-Whitney rank sum test was used to test for differences between CIV and EIV H3 antibody titers. Dogs that did not have HI titers ≥32 to both CIV and EIV test viruses were not included in subsequent analyses.

The following categorical variables were recorded for each racing greyhound sampled between 1999 and 2004, using information in the online database: age at time of testing (2 years or ≥3 years); prior racing history (yes or no); location of tracks where raced; number of tracks where raced (1 track or ≥2 tracks); specific years raced (1995 to 2004); and, number of months raced (1-5 months or ≥6 months). The frequencies of the categorical variables were calculated in SPSS® 18.0 (IBM®, Somers, NY, USA), and the association of each with H3 seropositivity was evaluated by logistic regression in Egret® 2.0.3 (Cytel Inc., Cambridge, MA, USA). After matching H3 seropositive and seronegative greyhounds by the year they were sampled, conditional logistic regression was used to test the null hypotheses that prior racing history, age at time of testing, number of months raced, and total number of tracks were not associated with H3 seropositivity. Unconditional logistic regression was used to test the null hypothesis that

individual race years were not associated with H3 seropositivity. Track location could not be analyzed by logistic regression. Results were reported as odds ratios (OR) with the 95% confidence interval (95% CI). Variables with P-values <0.05 were considered significant. Similar information was not available for the 1984 to 1985 greyhounds or the shelter dogs.

Results

None of the 153 racing greyhounds in Florida in 1984 and 1985 had detectable antibodies to CN/FL/04 or EQ/KY/91 H3. Of the 1,568 dogs in north Florida shelters from 1984 to 2004, one dog that entered in 2004 had H3 antibodies (HI titer = 128 for both CN/FL/04 and EQ/NY/99).

For the 549 racing greyhounds sampled from 1999 to 2004, 133 (24.2%) had antibodies to both CIV and EIV H3 proteins. The H3 seroprevalence and CIV/EIV H3 antibody GMTs with titer ranges are summarized for each test year in Table 5-1. H3 seropositive greyhounds were identified in each year from 1999 to 2004. The EQ/NY/99 H3 antibody titers in greyhounds tested from 1999 to 2001 (n=64) were significantly (P<0.01) higher than H3 antibodies to CN/FL/04, indicating the virus to which these dogs were actually exposed may have been more closely related to its equine ancestor. These seropositive greyhounds raced at tracks in AR, AZ, CO, CT, FL, IA, TX, and WI. There was no difference (P=0.94) between the H3 antibody titers to CN/FL/04 and EQ/NY/99 for seropositive greyhounds tested in 2002 to 2004 (n=69). However, the CIV and EIV H3 antibody titers in these dogs were significantly (P<0.01) higher than the titers for dogs tested in 1999 to 2001. The greyhounds that were seropositive in 2003 and 2004 raced at tracks in AR, AZ, and FL.

Associations of several variables with H3 seropositivity in greyhounds sampled from 1999 to 2004 are listed in Tables 5-2 and 5-3. For the 481 greyhounds that had accessible historical data in the online database, greyhounds that raced at some point from 1995 to 2004 were more likely (P=0.01) to be seropositive than dogs that did not race and dogs that were 3 years of age or older at the time of testing were more likely (P<0.01) to be seropositive than younger dogs. For the 303 greyhounds that had a racing history, dogs that raced 6 months or longer were more likely (P=0.01) to be seropositive than dogs that raced for less time, and dogs that raced on 2 or more different tracks were more likely (P=0.01) to be seropositive than those that raced on 1 track. Finally, although many dogs raced in multiple years, greyhounds that raced in 1998, 2002, and 2003 were more likely (P≤0.01) to be seropositive than dogs that raced in other years.

Discussion

Based on serological evidence, a H3N8 canine influenza-like virus was circulating in the racing greyhound population from 1999 to 2004. H3 antibodies were not detected in racing greyhounds in 1984 and 1985, but the tested population was small and restricted to one geographic location (north Florida). To the authors' knowledge, there are no large serum banks for greyhounds before 1999 that are available for testing; therefore, H3N8 exposure prior to 1999 cannot be ruled out. Similar to findings of a serosurvey on a small number of Colorado pet dogs,[8] H3 antibodies were not detected in Florida shelter dogs from 1984 to 2003. However it cannot be concluded that pet and shelter dogs were not infected by H3N8 viruses before 2004, since the tested populations in both serosurveys were restricted to one geographic location.

Other than in 2002, the annual prevalence estimates of H3 seropositivity among racing greyhounds tested from 1999 to 2004 ranged from 10.3% to 40.5%. The seropositive greyhounds in 1999 to 2001 had higher H3 antibody titers to a 1999 equine virus than a 2004 canine virus, suggesting the possibility that these dogs were infected by an ancestral canine influenza virus closely related to EIV. Without virus isolates for molecular analysis, however, it is not possible to determine whether these seropositive greyhounds were infected by a progenitor virus more closely related to its EIV ancestor than contemporary CIV isolates. The higher CIV H3 antibody titers in dogs tested in 2002 to 2004 suggest these dogs were infected by a contemporary virus homologous to CN/FL/04.

Based on the results of this study, greyhounds that raced were more likely to be exposed to H3N8 viruses than dogs that did not, and dogs that were 3 years of age or older at the time of testing were more likely to be seropositive for H3 antibodies than younger dogs. Further, among greyhounds that raced, dogs that raced at 2 or more tracks, dogs that raced for 6 months or longer, and dogs that raced in 1998, 2002, and 2003 were more likely to be exposed to H3N8 viruses. While the clinical histories of the seropositive greyhounds are unknown, and frequent movement of dogs prevented analysis of particular tracks or geographic locations as potential risk factors for seropositivity, the dogs raced at tracks that were involved in “kennel cough” epidemics in 1998-1999[82,84,86,87] and 2002-2003.[85] Necropsies performed on 3 dogs that died during the 1998-1999 epidemic showed hemorrhagic pneumonia possibly caused by an unknown viral infection.[83] In addition, H3N8 CIV was isolated from archived tissue from a dog that died of hemorrhagic pneumonia during an undiagnosed respiratory epidemic

in 2003.[20] However, it is unknown whether dogs tested in this study were involved in these reported outbreaks. Without additional virus isolates from archived tissues, the role of a H3N8 canine influenza-like virus in these epidemics is admittedly speculative.

Table 5-1. Annual prevalence of H3 antibodies among 520 racing greyhounds sampled between 1999 to 2004. Sera were tested in the HI assay with CN/FL/04 and EQ/NY/99 isolates. Dogs with HI titers ≥32 for both viruses were considered seropositive.

Test year	No. seropositive	No. seronegative	Seroprevalence (%)	CIV H3 GMT[a] (range)	EIV H3 GMT (range)
1999	43	116	27.0	35 (32-128)	49 (32-128)
2000	17	44	27.9	43 (32-128)	52 (32-128)
2001	4	35	10.3	38 (32-64)	45 (32-64)
2002	1	73	1.4	64	64
2003	51	75	40.5	72 (32-256)	72 (32-128)
2004	17	44	27.9	92 (32-512)	89 (32-256)

[a] GMT = geometric mean titer.

Table 5-2. Univariable conditional logistic regression analyses of potential risk factors for H3 seropositivity among 481 racing greyhounds sampled from 1999 to 2004.

Variable	No. seropositive (%) (n = 126)	No. seronegative (%) (n = 355)	OR[a]	95% CI[b]	P-value[c]
Raced					
No	30 (23.8)	148 (41.7)	1.00	Reference	NA
Yes	96 (76.2)	207 (58.3)	1.82	(1.12, 2.95)	0.01
Age at testing					
2 yrs	32 (25.4)	135 (38.0)	1.00	Reference	NA
≥3 yrs	94 (74.6)	220 (62.0)	2.15	(1.29, 3.59)	<0.01
Number months raced					
1-5	50 (52.1)	148 (71.5)	1.00	Reference	NA
≥6	46 (47.9)	59 (28.5)	1.88	(1.12, 3.16)	0.01
Total no. tracks raced					
1	41 (42.7)	128 (61.8)	1.00	Reference	NA
≥2	55 (57.3)	79 (38.2)	1.89	(1.12, 3.21)	0.01

[a] OR = odds ratio.
[b] CI = confidence interval.
[c] P-values <0.05 were considered significant.

Table 5-3. Comparison of prevalence estimates of H3 seropositive and seronegative greyhounds based on years raced.

Variable	No. seropositive (%) (n = 96)	No. seronegative (%) (n = 207)	OR[a]	95% CI[b]	P-value[c]
Race year					
1996					
No	95 (32.3)	199 (67.7)	1.00	Reference	NA
Yes	1 (11.1)	8 (88.9)	0.26	(0.03, 2.12)	0.20
1997					
No	79 (32.4)	165 (67.6)	1.00	Reference	NA
Yes	17 (28.8)	42 (71.2)	0.84	(0.45, 1.57)	0.59
1998					
No	62 (27.7)	162 (72.3)	1.00	Reference	NA
Yes	34 (43.0)	45 (57.0)	1.97	(1.15, 3.36)	0.01
1999					
No	82 (31.8)	176 (68.2)	1.00	Reference	NA
Yes	14 (31.1)	31 (68.9)	0.96	(0.48, 1.91)	0.92
2000					
No	90 (32.5)	187 (67.5)	1.00	Reference	NA
Yes	6 (23.1)	20 (76.9)	0.62	(0.24, 1.60)	0.32
2001					
No	85 (32.6)	176 (67.4)	1.00	Reference	NA
Yes	11 (26.2)	31 (73.8)	0.73	(0.35, 1.53)	0.41
2002					
No	62 (27.1)	167 (72.9)	1.00	Reference	NA
Yes	34 (45.9)	40 (54.1)	2.28	(1.33, 3.93)	<0.01
2003					
No	49 (25.1)	146 (74.9)	1.00	Reference	NA
Yes	47 (43.5)	61 (56.5)	2.29	(1.39, 3.78)	<0.01
2004					
No	85 (31.8)	182 (68.2)	1.00	Reference	NA
Yes	11 (30.6)	25 (69.4)	0.94	(0.44, 2.00)	0.87

[a] OR = odds ratio.
[b] CI = confidence interval.
[c] P-values <0.05 were considered significant.

CHAPTER 6
PREVALENCE OF AND RISK FACTORS FOR H3N8 CANINE INFLUENZA VIRUS SEROPOSITIVITY IN A POPULATION OF U.S. DOGS WITH INFLUENZA-LIKE ILLNESS

Canine influenza A virus subtype H3N8 (H3N8 CIV) was first identified in 2004 as a novel respiratory pathogen for dogs.[20] Molecular analyses of H3N8 CIV isolates indicate prior transmission of a Florida sublineage H3N8 equine influenza A virus from horses to dogs, followed by viral adaptation to the new canine host leading to efficient horizontal transmission and induction of influenza-like illness (ILI).[20,42,98,103] The virus is now considered endemic in several U.S. communities and continues to adapt to dogs, such that H3N8 CIV is genetically and epidemiologically distinct from circulating equine viruses.[28,29,42,43,98,103]

Similar to other influenza A viruses, H3N8 CIV can cause explosive epidemics of ILI upon introduction into group-housing situations. Outbreaks have occurred in greyhound racetrack kennels, boarding kennels, shelter facilities, veterinary hospitals, and canine day care centers across the nation.[9,20,28,29,42-44,98,141] The incubation period for H3N8 CIV is 2 to 4 days, and serum antibodies to the H3 protein are detectable approximately 7 days after infection.[20,26,28,29] H3N8 CIV surveillance activities have revealed a widespread geographic distribution of infected pet and shelter dogs in 34 states;[8,9,20,28,29,42,44,98,103,110,111] however, the virus is only considered endemic in metropolitan areas of Florida, New York, Colorado, and Pennsylvania.[28,29,103]

Knowledge of the prevalence of H3N8 CIV in the U.S. and risk factors associated with virus exposure in dogs is limited. In a study conducted in a university hospital in Iowa, 4 of 731 (0.5%) pet dogs with or without ILI were H3N8 CIV seropositive, indicating prior viral exposure.[110] In a study conducted in a university hospital in

Colorado, 9 of 250 (3.6%) pet dogs with or without ILI were seropositive for H3N8 CIV.[8] In a study of pet dogs participating in a flyball tournament in Pennsylvania, 3 of 100 (3%) dogs without ILI were seropositive for H3N8 CIV.[111] Another study conducted in a Philadelphia shelter found that 31 of 74 (42%) tested dogs with or without ILI were H3N8 CIV seropositive.[44] In these studies, attending canine day care within the last six months, pet dogs living with other dogs, and the number of days housed in a shelter facility were associated with H3N8 CIV seropositivity.[8,44,111]

It is currently unclear whether age, breed, sex, potential exposure settings, or geographic location are predisposing factors that can increase susceptibility to H3N8 CIV infection in dogs. Although previous studies have provided an epidemiologic framework for investigating risk factors associated with infection, these studies were limited to one geographic location and/or used small sample sizes, making the identification of risk factors associated with H3N8 CIV infection inconclusive.[8,44,111] The objectives of this study were to estimate the prevalence of H3N8 CIV seropositivity in a large population of dogs presenting with ILI in geographically diverse regions of the U.S., and to identify risk factors for seropositivity.

Materials and Methods

The Institutional Animal Care and Use Committees of both the University of Florida (UF) and Cornell University (CU) approved diagnostic testing for this study.

Animals

The study population included 1,268 pet and shelter dogs that were presented for evaluation of ILI (e.g., cough, nasal discharge, or pneumonia) in 42 states from 2005 through 2009.

Study Design

This investigation was a cross-sectional study using a convenience sample of sera collected by veterinarians from pet and shelter dogs presenting with ILI from 2005 to 2009, and voluntarily submitted to the UF College of Veterinary Medicine and the CU Animal Health Diagnostic Center for serological diagnosis of H3N8 CIV infection.

Laboratory Procedures

The HI assay was conducted as previously described.[20] Briefly, sera were pretreated either with receptor destroying enzyme or periodate, then heated to 56°C for 30 minutes to inactivate nonspecific inhibitors of hemagglutination. Sera were serially diluted two-fold in PBS and incubated with 4 hemagglutinating units/25µL H3N8 CIV (A/canine/Florida/43/2004) in 96-well V-bottom microtiter plates for 30 minutes at room temperature. An equal volume of 0.5% turkey red blood cells (Lampire Biological Laboratories, Pipersville, PA, USA) in PBS was added to each well and incubated for 30 minutes at room temperature. The endpoint antibody titer was defined as the last dilution of serum that completely inhibited hemagglutination. The cutoff titer for seropositivity in this HI assay was previously defined as 32.[20] Sera from specific pathogen-free dogs housed in a barrier research facility were used as negative controls, while sera from dogs with confirmed H3N8 CIV infection served as positive controls.[20,98] All dogs were sampled and tested prior to the introduction of the inactivated whole virus H3N8 CIV vaccine in June 2009; therefore, information on vaccination status was not requested.

Data Collection

For each dog, the following data were requested from the submitting veterinarian: clinical signs, date of illness onset, date of sample collection, age (< 1 year or ≥ 1 year),

breed (pure, mixed), sex (male, female), potential exposure setting (shelter facility, boarding kennel, other exposure settings), and geographic location at the time of sample collection [U.S. regions = Northeast (CT, DE, MA, MD, ME, NH, NJ, NY, PA, RI, VT, WV), Southeast (AL, AR, FL, GA, KY, LA, MS, NC, SC, TN, VA), Midwest (IA, IL, IN, KS, MI, MN, MO, NE, OH, ND, SD, WI), Southwest (AZ, NM, OK, TX), West (AK, CA, CO, HI, ID, MT, NV, OR, UT, WA, WY)]. The date of illness onset and date of sample collection were requested to determine if the sample was an acute (<7 days after onset of illness) or convalescent (≥7days after onset of illness) sample. Seronegative dogs with single samples collected less than 10 days after the date of illness onset were excluded from the study, since H3N8 CIV-infection status could not be confirmed.

To determine whether certain breeds were predisposed for H3N8 CIV infection, reported dog breeds were further classified according to one of 10 canine genomic groups ("Genomic 10" = ancient-spitz, herding, mastiff-like, retriever, scent hound, sight hound, small terriers, spaniel, toy, or working dog).[126] The genomic categories of pure breed dogs and mixed breeds with one predominant breed reported were coded for analysis. When a breed was not found in the genomic reference, it was classified according to the American Kennel Club category for the breed's predominant ancestry.

Potential virus exposure settings reported by veterinarians at the time of sample collection were classified into three general categories: 1) pet dogs with "multiple" potential exposures (i.e., purchased from pet stores; visited dog parks, dog shows, or grooming facilities; exposed to another dog that may or may not have had ILI; and dogs presented to a veterinary hospital for ILI but the potential exposure setting was

unknown); 2) dogs housed in animal shelter or rescue group facilities; and 3) pet dogs recently housed in commercial boarding kennels, veterinary hospital boarding kennels, training or rehabilitation kennels, or canine day care.

Statistical Analyses

A total of 1,268 pet and shelter dogs with ILI from 2005 through 2009 were included in the analyses. H3N8 CIV seroprevalence in the study population was calculated as the number of seropositive dogs divided by the total number tested X 100. The 95% CI was estimated for the seroprevalence. Conditional logistic regression was used to model the odds of dogs being classified as H3N8 CIV seropositive as a function of investigated exposure factors. Due to potential variability in H3N8 CIV circulation between years, dogs were matched in the analyses by the year they were sampled. Initial evaluation of potential risk factors for seropositivity was performed using univariable conditional logistic regression (Egret® 2.0.3, Cytel Inc., Cambridge, MA, USA).[45] Variables associated ($P \leq 0.20$) with the outcome of interest (H3N8 CIV seropositivity) were considered for inclusion in a multivariable conditional logistic regression model. A forward stepwise approach was used to identify variables associated with seropositivity using a 2-sided P-values-to-enter and P-values-to-remove of 0.05 and 0.10, respectively. Values for the final model were considered significant at $P<0.05$. In the final model, the adjusted OR and 95% CI were reported.

Results

H3N8 CIV Seroprevalence in the Study Population

The number of H3N8 CIV seropositive dogs was 618 out of 1,268 in the study population, representing an overall seroprevalence of 49% (95% CI = 46, 52%). The geometric mean antibody titer for these dogs was 302 (range = 32 to 4,096). Samples

were submitted from dogs in 42 states; no dogs from IA, MS, MT, ND, NM, NV, RI, or WV were tested. Fewer than 10 dogs were tested in 24/42 (52%) states. Colorado (101 dogs), Florida (444 dogs), and New York (205 dogs) had the highest number of tested dogs in the West, Southeast, and Northeast respectively. The frequency of seropositive dogs by geographic region was: Northeast 62% (265/427 dogs); West 57% (144/252 dogs); Southeast 30% (192/490 dogs); Southwest 26% (11/43 dogs); and Midwest 11% (6/56 dogs).

Risk Factors Associated with H3N8 CIV Seropositivity

Age, exposure setting, and geographic region were significantly (P<0.01) associated with H3N8 CIV seropositivity in univariable conditional logistic regression analyses (Table 6-1). These 3 variables were retained as significant risk factors for H3N8 CIV seropositivity in the multivariable analyses (Table 6-2). Dogs ≥1 year of age, dogs recently housed in boarding kennels or shelter facilities, and dogs in the Northeast or West regions of the U.S. were at greatest risk for H3N8 CIV seropositivity.

Discussion

This study represents the first national survey to estimate the seroprevalence of H3N8 CIV in a geographically diverse population of dogs with ILI, and to identify risk factors associated with H3N8 CIV seropositivity. The seroprevalence of H3N8 CIV in dogs with ILI was high (49%). Dogs ≥1 year of age, dogs recently housed in boarding kennels or shelter facilities, and dogs in the Northeast or West regions of the U.S. were at greatest risk for H3N8 CIV seropositivity.

The high seroprevalence of H3N8 CIV in the study population suggests that this virus is a common respiratory pathogen in dogs in the U.S. However, this was a syndromic serosurvey limited to dogs with clinical signs suggestive of influenza-like

illness, and testing was not conducted to determine other causes of illness. Another limitation of this study was the dependency on veterinarians to voluntarily collect samples from dogs with ILI for testing. The proportion of dogs with ILI that were not tested is unknown. In addition, diagnosis of H3N8 CIV was based solely on detection of antibodies and H3N8 CIV-infection status could not be determined for many dogs due to improper timing of sample collection. Exclusion of dogs with improperly timed serum samples therefore reduced the number of seronegative dogs in the study. Ultimately, the prevalence of H3N8 CIV in this study cannot be extrapolated to all dogs with ILI. Estimation of the true prevalence of H3N8 CIV in the U.S. would require random testing of large numbers of dogs independent of clinical status.

The geographic regions of the U.S. with the highest H3N8 CIV seroprevalence were the Northeast, West, and Southeast. This should be interpreted with caution since each of these regions was overrepresented by a state from which large numbers of symptomatic dogs were tested. This may have occurred because the 3 states with highest representation (New York, Colorado, and Florida respectively) had diagnostic laboratories that actively solicited samples for H3N8 CIV testing. Although dogs in 42 states were tested, more than half of the states were represented by fewer than 10 dogs. This low sampling may have resulted from lack of interest of the veterinarian or dog owner in pursuing testing and thus did not reflect the actual numbers of dogs with ILI in these states. The underrepresentation of many states and lack of testing in other states likely resulted in underestimation of H3N8 CIV prevalence in some geographic regions. Variability in H3N8 CIV seroprevalence may also reflect variability in virus circulation by year, location, and the susceptibility of exposed dogs.

The H3N8 CIV seroprevalence in dogs from Colorado was higher in this study compared to a previous study conducted in that state.[8] One explanation is the different study designs and time periods used in each. The current study tested pet and shelter dogs with ILI from 2005 to 2009, while the previous study tested only pet dogs with or without ILI seen at a university teaching hospital from March to December 2009. The overall H3N8 CIV seroprevalence in this study was actually more comparable to the reported seroprevalence in a Philadelphia shelter (42%) than in a pet dog population tested over a two-day period during a flyball tournament (3%).[44,111]

Despite the limitations in interpretation of seroprevalence, dogs with ILI in the Northeast or West regions of the U.S. were at significantly greater risk for H3N8 CIV seropositivity. This may be attributable to the frequently reported outbreaks of H3N8 CIV in boarding kennels, veterinary clinics, and shelters in these regions, particularly in New York City, Philadelphia, and the greater Denver area.[8,28,29,42-44] Although H3N8 CIV was first recognized in Florida as the causative agent for several outbreaks from 2004 to 2007,[20,98] the Southeast was not identified as a high-risk region for H3N8 CIV seropositivity. This unexpected finding may be due to lack of canine influenza virus detection in the other southeastern states, respiratory disease in Florida due to other canine respiratory pathogens, or due to the fact that less than half of the Florida dogs were H3N8 CIV seropositive. In contrast, more than two-thirds of the large numbers of dogs tested in New York (Northeast region) and Colorado (West region) were H3N8 CIV seropositive.

Breed and sex were not associated with H3N8 CIV seropositivity in this study population of dogs with ILI. Adult dogs however were significantly more likely to be

seropositive than juveniles <1 year old, similar to another study that found older dogs had higher risk (although the study's small sample size did not allow this association to be statistically significant).[8] Age was not a risk factor for seropositivity in another recent study evaluating the point prevalence of H3N8 CIV in pet dogs participating in a flyball tournament in Pennsylvania, although the sample size was small.[111] In the present study the association of seropositivity with adults was confounded in part by other factors, including high-risk exposure settings and geographic locations. However, the association of H3N8 CIV seropositivity with the adult age group in dogs with ILI persisted after adjustment for these confounding factors in the multivariable analysis. In addition, when age was evaluated as a potential risk factor for dogs in the exposure reference category ("multiple" exposures) only, dogs ≥1 year of age were still significantly more likely to be seropositive ($P<0.01$) than dogs <1 year of age. H3N8 CIV seropositivity for the majority of dogs in this study was based on testing of one serum sample, not paired acute and convalescent samples. Therefore, the actual time (or age at the time) of viral infection could not be determined for these dogs. The association of H3N8 CIV infection with age warrants further study.

Dogs housed in boarding kennels or shelters at the time of ILI were at greater risk for H3N8 CIV seropositivity than dogs in other settings (e.g., pet stores, dog parks, groomers). The opportunity for virus transmission between infected and susceptible dogs via direct and indirect contact is very high in communal settings containing high density/high turnover populations such as boarding kennels and shelters.[8,20,28,29,42,44,98] A recent study in a Philadelphia shelter determined that the risk for H3N8 CIV exposure doubled every 3 days that a dog stayed in the shelter.[44]

In summary, this syndromic survey found a high prevalence of H3N8 CIV seropositivity in 1,268 dogs with ILI in 42 states over a 5-year period. Communal housing situations such as boarding kennels or shelter facilities posed the greatest risk for H3N8 CIV seropositivity. In addition, dogs located in the Northeast or West regions of the country, where the virus has established endemicity in several metropolitan areas, were at greater risk of infection. These results justify the need for shelters, boarding kennels, and other communal dog housing facilities to formulate, implement and/or evaluate surveillance and biosecurity protocols to reduce the risk of virus transmission between dogs and avert large-scale epidemics within these facilities.

Table 6-1. Univariable conditional logistic regression analyses of potential risk factors for H3N8 CIV seropositivity in a population of 1,268 pet and shelter dogs with ILI.

Variable	Seropositive n (%)	Seronegative n (%)	OR[g]	95% CI[h]	P-value[i]
Age[a]					
<1 yr	95 (19)	210 (35)	1.00	Reference	NA
≥ 1 yr	401 (81)	396 (65)	2.40	1.80, 3.19	<0.01
Sex[b]					
F	236 (46)	215 (47)	1.00	Reference	NA
M	282 (54)	243 (53)	1.07	0.82, 1.38	0.60
Breed[c]					
Pure	348 (68)	322 (66)	1.00	Reference	NA
Mixed	165 (32)	164 (34)	0.93	0.71, 1.23	0.64
Genomic categories[d]					
Ancient-Spitz	22 (5)	26 (6)	1.00	Reference	NA
Herding	30 (7)	26 (6)	1.36	0.61, 3.03	0.44
Mastiff-like	60 (13)	60 (14)	1.08	0.54, 2.16	0.80
Retriever	142 (31)	105 (24)	1.44	0.76, 2.73	0.25
Scent hound	53 (11)	41 (9)	1.42	0.70, 2.91	0.32
Sight hound	10 (2)	16 (4)	0.98	0.35, 2.74	0.96
Small terriers	26 (6)	36 (8)	0.79	0.36, 1.71	0.55
Spaniel	22 (5)	27 (6)	0.95	0.42, 2.15	0.90
Toy	34 (7)	38 (9)	0.92	0.43, 1.95	0.84
Working dog	61 (13)	60 (14)	1.15	0.58, 2.29	0.68
Exposure setting[e]					
Multiple[f]	57 (12)	182 (40)	1.00	Reference	NA
Shelter facilities	261 (56)	199 (44)	5.11	3.54, 7.37	<0.01
Boarding kennels	147 (32)	73 (16)	5.96	3.94, 9.00	<0.01
Geographic region					
Southwest	11 (2)	32 (5)	1.00	Reference	NA
Midwest	6 (1)	50 (8)	0.31	0.10, 0.93	0.03
Southeast	192 (31)	298 (46)	1.73	0.84, 3.52	0.13
West	144 (23)	108 (16)	4.06	1.94, 8.49	<0.01
Northeast	265 (43)	162 (25)	4.67	2.26, 9.64	<0.01

[a] Age was provided for 496/618 seropositive dogs and 606/650 seronegative dogs.
[b] Sex was provided for 518/618 seropositive dogs and 458/650 seronegative dogs.
[c] Breed was provided for 513/618 seropositive dogs and 496/650 seronegative dogs.
[d] Information adequate for genomic classification was provided for 400/618 seropositive dogs and 435/650 seronegative dogs.
[e] Exposure settings were provided for 465/618 seropositive dogs and 454/650 seronegative dogs.
[f] Multiple = purchased from pet stores; visited dog parks, dog shows, or grooming facilities; exposed to another dog with or without ILI; and taken to a veterinary hospital for ILI but a potential exposure setting was unknown.
[g] OR = odds ratio.
[h] CI = confidence interval.
[i] P-values <0.05 were considered significant.

Table 6-2. Multivariable conditional logistic regression analyses of potential risk factors for H3N8 CIV seropositivity in a population of 1,268 pet and shelter dogs with ILI.

Variable	OR[a]	95% CI[b]	P-value[c]
Age			
<1 yr	1.00	Reference	NA
≥ 1 yr	1.49	1.01, 2.19	0.04
Exposure setting			
Multiple	1.00	Reference	NA
Shelter facilities	4.17	2.70, 6.44	<0.01
Boarding kennels	5.97	3.64, 9.78	<0.01
Geographic region			
Southwest	1.00	Reference	NA
Midwest	0.31	0.05, 1.84	0.19
Southeast	1.49	0.56, 3.93	0.41
West	4.19	1.49, 11.75	<0.01
Northeast	9.27	3.30, 26.07	<0.01

[a] OR = Adjusted odds ratio.
[b] CI = confidence interval.
[c] P-values <0.05 were considered significant.

CHAPTER 7
SIMULATION STUDIES OF H3N8 CANINE INFLUENZA VIRUS TRANSMISSION IN SHELTER FACILITIES

Since its recognition in 2004 H3N8 CIV has been diagnosed as a common cause of acute respiratory disease in dogs, particularly those housed in communal settings. H3N8 CIV outbreaks have occurred in racetrack kennels, boarding kennels, shelter facilities, veterinary hospitals, and canine day care centers across the nation.[9,20,28,29,42-44,98,141] The hallmark of H3N8 CIV has been explosive respiratory outbreaks, with high morbidity due to lack of prior immunity to this emerging pathogen.[20,28,29,98] However, it is currently unclear which viral and/or population parameters promote H3N8 CIV transmission in communal settings. Information is available regarding the basic biology of H3N8 CIV, however detailed epidemiological data focused on virus transmission rates and modes of transmission is limited.[20,26,27,49] Further, population parameters that may influence virus transmission in communal settings have not been defined.

H3N8 CIV has significantly impacted the health and welfare of dogs in animal shelters, where some facilities have experienced recurrent outbreaks and some have resorted to extreme control measures such as depopulation. Outbreak management is particularly challenging for government shelters with animal control responsibilities, where ongoing admission of stray dogs and legal custody cases maintains a steady flow of susceptible dogs into the population. In addition, these shelters frequently have limited housing space for segregation of dogs based on H3N8 CIV infection status. Outside of these common challenges however, other presumably important factors vary between government shelters including total shelter capacity; intake, adoption, transfer, and euthanasia rates; and level of veterinary care. Considering the significant impact of H3N8 CIV outbreaks on shelter facilities, and the unique challenges faced by these

organizations, it is valuable to explore which viral and/or population parameters promote virus transmission in order to improve prevention and control recommendations.

A variety of mathematical modeling approaches, including compartmental (deterministic and stochastic) and network models, have been used to investigate influenza virus transmission in human, avian, swine, and equine populations.[25,32,33,65,68,69,96,109] The results of these studies have typically shown that transmission rates and contact between hosts are the most important parameters for virus spread, and that intervention strategies which include a combination of methods (vaccination, antiviral therapy, isolation and quarantine) are often most effective. More recently, several avian influenza models have also highlighted the importance of environmental transmission.[13,104,105] However it has been noted that host population organization and contact patterns vary greatly between species, making it difficult to directly apply models developed for one species to another.[6,25] Among current studies, models of equine influenza virus transmission among horses in training yards are most comparable to the scenario of H3N8 CIV transmission in shelters.[25,35,96,97] However, although the overall population sizes are similar, the population structure and movements of dogs in shelters are quite different from these equine populations. By comparison, dogs in shelter facilities are highly managed populations. Factors such as high population density, high frequency of turnover in the population, potentially lower contact rates between dogs, and potentially higher contact rates between dogs and fomites (due to increased contact with handlers and objects in the environment) likely affect virus transmission.

The objective of this study was to develop a mathematical model of H3N8 CIV transmission in shelter facilities in order to address the following questions: 1) which viral and/or population parameters most significantly influence virus transmission in shelters, in terms of the time course and the numbers of dogs affected during CIV outbreaks; and 2) given the suspected importance of indirect virus transmission in the epidemiology of H3N8 CIV in shelters, what is the relative contribution of direct vs. indirect transmission? Study results should provide a valuable foundation for future work, which will focus on development of more effective prevention and control strategies.

Materials and Methods

Model Structure

A compartmental, deterministic model was developed to simulate H3N8 CIV transmission in shelter facilities. Although stochastic models are recommended when dealing with small populations, since random events can significantly impact virus transmission (e.g., leading to virus die out),[35,97] a deterministic model was selected because of the limited data available for estimation of several parameters. Deterministic models are well established in the literature and have been recommended over stochastic models in situations where parameter data is limited, in order to explore model behavior across a range of values in conjunction with sensitivity analysis.[4]

The model was initially intended to investigate the effect of multiple viral and population parameters on the occurrence of H3N8 CIV outbreaks (i.e., epidemics) in shelters, including the time course, the numbers of dogs affected, and the relative contribution of direct and indirect virus transmission. However establishment of endemic infection was recognized during the early stages of model development, therefore the

viral and population parameters that promoted establishment of an endemic state were also investigated. A full sensitivity analysis and statistical analysis was completed.

Model classes

A modified SEIR model was developed with five dog classes and one fomites (X) class. The dog classes included susceptible (S), latent (L), infectious clinical (I_C), infectious subclinical (I_{SC}), and recovered (R) dogs.

All dogs in the susceptible class were assumed to be naïve to H3N8 CIV infection. The latent period (i.e., the time from virus exposure to virus shedding) rather than the incubation period (i.e., the time from virus exposure to development of clinical signs) was specified for the model. Although dogs exposed to H3N8 CIV begin shedding virus (i.e., become infectious) prior to the development of clinical signs,[20,26,27,49] a "latent" dog class indicates that the relatively short clinically silent virus shedding period was included in the infectious classes (in order to simplify the model).

Two infectious classes, infectious clinical and infectious subclinical dogs, were included in the model because studies of natural H3N8 CIV outbreaks as well as experimental infection studies have demonstrated that approximately 20% of H3N8 CIV-infected dogs do not develop clinical signs.[20,26,27,49] It is important to separate clinical and subclinical dogs in order to explore their roles in H3N8 CIV transmission, and to provide a framework for future models focused on development of intervention strategies. It was assumed that infectious clinical and subclinical dogs are sufficiently different to track independently, since subclinical dogs would only be identified through diagnostic testing (virus isolation, RT-PCR, serology) in clinical settings. In the model subclinical dogs were assumed to be half as infectious as clinical dogs, since coughing

and sneezing promote virus transmission via direct contact and increase the amount of virus shed into the environment.

A fomites class was included in the model because one of the goals of the study was to explore the relative contribution of direct vs. indirect virus transmission on H3N8 CIV transmission in shelters. Direct transmission accounts for virus transmission from infected to susceptible dogs and indirect accounts for virus transmission via fomites, as will be further defined when describing the model parameters. Other than recent models seeking to understand the role of environmental transmission in avian influenza virus epidemics,[13,104,105] modes of transmission have not typically been separated in influenza models. Although indirect virus transmission is poorly understood, and it ultimately is not possible to determine if an individual H3N8 CIV infection is due to direct or indirect transmission, it was hypothesized that this mode of transmission would be an important factor in the epidemiology of H3N8 CIV in highly managed shelter populations. The overall model schematic is depicted in Figure 7-1.

Model parameters

Model parameters account for shelter capacity, population parameters that reflect typical dog movements into and out of model classes, and virus transmission factors. The model parameters are listed and described in Table 7-1.

Shelters were assumed to be at full capacity at the beginning of virus introduction simulations and the populations were assumed to be homogeneous. Further, it was assumed that there were no age or sex effects in dog movements. Since they were considered to be negligible over the time frame of a simulation, the per capita birth rate and the disease independent death rate were not included in the model. For simulations, a single latent dog (*initL*) was introduced into a shelter of a particular

capacity (*initS*) at the initial time point. The total shelter capacity was therefore the sum of these values, however the *initS* parameter was considered an adequate approximation of the total shelter capacity since *initL* was set at one. This shelter capacity value (*initS*) was fixed throughout simulations since it was assumed that most shelters remain at capacity on a daily basis.

Other population parameters accounted for typical dog movements into and out of model classes, including immigration (*c*) due to shelter intakes, emigration (*m*) due to adoptions and transfers, and deaths due to euthanasia (*d*). After the introduction of one latent dog, immigration was only allowed into the susceptible class. Immigration was not dependent on the number of dogs in the class, but was expressed as a percentage of the total shelter capacity (*initS*) based on typical daily shelter intakes. To achieve a fixed shelter capacity throughout the simulations, immigration (*c*) and emigration (*m*) were considered independent variables, and euthanasia was calculated from these values based on Equation 7-1.

$$d = (c(initS) - \frac{m(S + L + I_{SC} + R)}{(S + L + 1.5I_C + I_{SC} + R)}) \quad (7\text{-}1)$$

Emigration out of model classes was dependent on the number of dogs in those classes (i.e., the upper bound was the total number of dogs in the class at a given time), and was tracked in terms of percentages of dogs leaving each class per day. It was assumed that emigration and euthanasia practices would be different for infectious clinical dogs compared to dogs in the other classes, therefore separate emigration (m_{IC}) and euthanasia (d_{IC}) parameters were established for this class. Emigration was allowed out of all classes of dogs, however it was set to zero for the infectious clinical class to mimic typical shelter practices. Euthanasia from the infectious clinical class was

set at 1.5*d*, where *d* is calculated as described above, with the assumption that clinically ill dogs would be euthanized at a higher rate than dogs in the other classes.

H3N8 CIV is transmitted by direct transmission (dog-to-dog contact, droplet transmission through coughing/sneezing) and indirect transmission (aerosols and fomites). All sources of infection were grouped into one force of infection (λ), however two terms were defined: one for direct virus transmission and one for indirect virus transmission (Equation 7-2).

$$\lambda = \frac{\alpha(I_C + aI_{SC})}{N} + \frac{\beta X}{(X + k)} \quad (7\text{-}2)$$

In the direct virus transmission term, α is the proportion of susceptible dogs that become infected when contacting infectious dogs, or when contacting infectious droplets directly coughed or sneezed on them by infectious dogs. A devaluing parameter (*a*) is included to account for the reduced infectiousness of subclinical dogs, as has been applied in other studies.[68] Direct virus transmission was scaled by the total number of dogs in the shelter (*N*), following the conventional mass action mixing approach. For the indirect virus transmission term, β is the proportion of susceptible dogs that become infected when contacting fomites (*X*); this includes contacting infectious virus on any contaminated environmental object (e.g., a food bowl or a handler) or aerosols. Indirect virus transmission was scaled by ($X + k$), where *k* is a scaling parameter representing the number of noninfectious objects in the environment. This attempts to mimic the mass action mixing approach for fomites. This approach is based on recent modeling studies seeking to understand the role of indirect transmission of several pathogens, including avian influenza virus, through environmental reservoirs such as lakes.[13,50,104,105] Although shelters are obviously not aquatic environments, it was

assumed that minimal infectious doses of H3N8 CIV would be deposited throughout the shelter environment by infectious (clinical and subclinical) dogs, which would represent a substantial source of infection for susceptible dogs.

Nearly all naïve dogs exposed to H3N8 CIV become infected due to lack of pre-existing immunity,[20,26,27,49] therefore it was assumed that all dogs in the shelter would be susceptible to infection and that all dogs in the latent class would become infectious. Further, it was assumed that a proportion of latent dogs would become infectious and develop clinical signs, while the remaining dogs would become infectious but remain subclinical.[20,26,27,49] This was accounted for by multiplying the per capita rate at which dogs become infectious (*f)* by the percentage of dogs that become clinical (*q*) or the percentage of dogs that remain subclinical (*1-q*). This approach was selected since there is no known difference in the rate at which clinical vs. subclinical dogs become infectious, and it has been used in the influenza modeling literature.[47,76]

It was assumed that all infectious dogs (clinical and subclinical) serve as potential sources of virus for susceptible dogs during the course of a simulation. However devaluing parameters were introduced for the decreased infectiousness of subclinical dogs (*a*) and for the decreased virus shedding into the environment by subclinical dogs (*b*) due to lack of clinical signs such as coughing and sneezing, which enhance virus spread.[68] Once virus shedding ceases, dogs are no longer contagious to other dogs; therefore the virus shedding period was expressed as the per capita rate at which infectious dogs recover. Severity of clinical disease and deaths due to H3N8 CIV were not considered in the model.

Model equations

Differential equations, representing the change over time for each model class during a simulated H3N8 CIV introduction, were programmed into MATLAB® (MathWorks, Natick, MA, USA) computational software. Simulations were completed using the "ode45" differential equations solver.

Equation 7-3 represents the change over time in the susceptible (*S*) class. Movement into this class is affected by immigration (*c(initS)*), representing the number of shelter intakes, expressed as a percentage of the total shelter capacity. Movement out of the class depends on emigration (*m*) due to adoptions and transfers, deaths due to euthanasia (*d*), and the transition of susceptible dogs to the latent class due to the force of infection (λS) as previously described.

$$\frac{dS}{dt} = c(initS) - (m + d)S - \lambda S \tag{7-3}$$

Equation 7-4 represents the change over time in the latent (*L*) class. Movement into the class is affected by infection of susceptible dogs (λS), while movement out of the class depends on emigration (*m*), deaths due to euthanasia (*d*), and the transition of dogs to the infectious clinical (*fqL*) and infectious subclinical (*f(1-q)L*) classes (simplified to *fL*).

$$\frac{dL}{dt} = \lambda S - (m + d)L - fL \tag{7-4}$$

Equation 7-5 represents the change over time in the infectious clinical (I_C) class. Movement into the class is affected by the transition of a percentage of latent (*fqL*) dogs, while movement out of the class depends on deaths due to euthanasia (d_{IC}), and the transition of dogs to the recovered class (γI_C) based on the per capita rate at which

infectious clinical dogs recover (γ). Emigration (m_{IC}) from this class was set to zero and d_{IC} was calculated as previously described.

$$\frac{dI_C}{dt} = fqL - (m_{IC} + d_{IC})I_C - \gamma I_C \quad (7\text{-}5)$$

Equation 7-6 represents the change over time in the infectious subclinical (I_{SC}) class. Movement into the class is affected by the transition of a percentage of latent dogs ($f(1\text{-}q)L$); while movement out of the class depends on emigration (m), deaths due to euthanasia (d), and the transition of dogs to the recovered (γI_{SC}) class.

$$\frac{dI_{SC}}{dt} = f(1-q)L - (m+d)I_{SC} - \gamma I_{SC} \quad (7\text{-}6)$$

Equation 7-7 represents the change over time in the recovered (R) class. Movement into this class is affected by the transition of infectious clinical (γI_C) and infectious subclinical (γI_{SC}) dogs, while movement out of the class depends on emigration (m) and deaths due to euthanasia (d).

$$\frac{dR}{dt} = \gamma(I_C + I_{SC}) - (m+d)R \quad (7\text{-}7)$$

Equation 7-8 represents the change over time in fomites (X). The number of infectious H3N8 CIV doses (Φ) produced per infectious dog is included in the equation, where b is the devaluing parameter for decreased virus shedding of subclinical dogs and ρ represents the inactivation of virus on fomites over time (i.e., ρ is the inverse of the length of time virus remains infectious).

$$\frac{dX}{dt} = \phi(I_C + bI_{SC}) - \rho X \quad (7\text{-}8)$$

Parameter Estimation

The model parameters, values used, and sources of data are listed in Table 7-1. Parameter estimates were based on published literature and expert opinion. When applicable, rate based transitions were chosen for mathematical simplicity. Although a fixed lag is often used for the transition from the latent to infectious classes, a rate based transition was selected because there is individual variation in this value. Latin hypercube sampling (LHS) was used for the full sensitivity analysis of the model, in order to allow simultaneous variation in the parameter values;[11,70] therefore a probability distribution was selected for each independent parameter based on the available data. Triangular distributions were used when a most probable value was supported by the literature; however, the range of these parameter values was then based on the quality of available data (i.e., parameters with less supporting data were given wider ranges). When a most probable value was not available in the literature, parameters were assigned uniform distributions. The parameter's range was then based on values considered most biologically plausible by the investigators, in order to explore the full range of potential values.

Three parameters (a, m_{IC}, and b) were set at constant values, and d and d_{IC} were calculated; therefore these values were not included in the sensitivity analysis. The values of a and b were held constant at 0.5, which is the same value used in another study.[68] The value of m_{IC} was set to 0, since it was assumed that infectious clinical dogs would not be allowed to emigrate. The euthanasia parameters (d and d_{IC}) were calculated as previously described, in order to maintain shelter capacity.

The remaining 11 independent model parameters were included in the sensitivity analysis. Three parameters (f, q, and γ) are well supported in the literature, therefore

triangular distributions with maximum, minimum, and most probable values were assigned.[20,26,27,49] Virus inactivation (ρ) is also well supported,[9,12,129] however a uniform distribution was assigned due to the variability in time to virus inactivation (based on the type of surface or medium). Values and ranges for shelter capacity and dog movement parameters (*initS*, *c*, and *m*) were determined by the investigators, based on typical shelter sizes and dog movements. A range of 50 to 400 dogs was selected for shelter capacity (*initS*), and a uniform distribution was assigned since shelters vary in size across this range. The ranges for immigration (*c*) and emigration (*m*) were also assigned uniform distributions, since these percentages likely also vary across the full ranges selected. The selected values for *c* and *m* reflect the high turnover considered typical in government shelters.

H3N8 CIV epidemic curve data are not available to estimate the transmission parameters (α, β), and ultimately it is not feasible to distinguish infections due to direct or indirect virus transmission. These values were therefore based on one published experiment and expert opinion.[49] Since the published experiment was focused on dog-to-dog transmission of H3N8 CIV, where 75% of contact dogs contracted H3N8 CIV, the study findings primarily informed the values for the α parameter in the direct transmission term. Since the transmissibility of H3N8 CIV has been questioned,[28,42] however, a triangular distribution with a central value of 0.5 and a wide range (0.2 to 0.9) was selected. This would allow exploration of potential extreme values of α, but many randomly generated values would be around the central value of 0.5. Since infections due to indirect virus transmission could not be identified in the published

study, the β parameter of the indirect transmission term was given a wide range and a uniform distribution to explore the full range of possible values.

The remaining model parameters, k and Φ, were estimated by the investigators. The values and ranges were refined by running the model program with extreme proposed values, in order to gain a better understanding of their behavior in the model. As previously noted, the environmental fomites scaling parameter (k) was based on literature addressing indirect transmission of other pathogens and environmental transmission of avian influenza virus.[13,50,104,105] These studies however addressed indirect transmission in aquatic environments, therefore a reasonable range for k in a terrestrial environment was uncertain. When the model was run with a wide range of values for k, it was found that a full spectrum of behavior was captured with values ranging from 10^2 to 10^4. Through this exploration it was also apparent that the simulation length should be set at 100 days in order to capture the time course for the range of potential epidemics. A similar process was completed for Φ. Although published studies give an idea of virus excretion per dog, based on nasal swabs collected at single time points,[20,26,27,49] it is uncertain how much virus is truly shed into the environment by infectious dogs in a day. The concept of an infectious dose was adopted based on the cited indirect transmission studies, to avoid quantifying virus particles in the environment, and a range of 10 to 100 infectious doses produced per dog per day captured the full range of model behaviors. Uniform distributions were assigned to these parameters to explore the full range of possible values.

Sensitivity Analysis

A sample size of n = 500 simulation runs was selected, and the LHS scheme was programmed into MATLAB® (MathWorks). Briefly, to generate unique parameter sets,

the program divided each assigned probability distribution into n = 500 equal intervals. Within each interval a value was randomly selected and then back-transformed to the original parameter axis, therefore 500 values were generated for each independent parameter. The values for all 11 independent parameters were then randomly reassorted into 500 unique parameter sets, which were saved to an Excel file.

Data Analysis

The 500 virus introduction simulations were run in the model program using a 100-day time period and a one-day time step. Each simulation began on day zero with the introduction of a latent dog. To assess potential virus die out in the deterministic model, the model was programmed to terminate a simulation if the sum of the latent, infectious clinical, and infectious subclinical dogs fell below 0.5. If this threshold was reached the simulation was restarted with these classes set to zero. The following outcomes of interest were analyzed.

Occurrence of an epidemic. Each simulation was characterized as epidemic or not epidemic. An epidemic was defined as ≥10% of the shelter capacity (*initS*) being infected ($I_C + I_{SC}$ dogs*)* at the peak of infection*.* Respiratory disease is considered common in animal shelters, however the level of baseline (i.e., typical) respiratory disease can vary greatly from one facility to another. Given this variability, a threshold of 10% of the population being infected (with approximately 8% exhibiting clinical signs) was considered a reasonable level to trigger recognition of an epidemic (i.e., respiratory disease beyond the normal level in the shelter). The percentage of simulations that resulted in an epidemic was calculated. The original intention was to perform logistic regression analysis, assessing which of the 11 independent model parameters

contributed to the occurrence of an epidemic; however this was not performed since only two of the simulations were classified as not epidemic.

Establishment of endemic infection. Each simulation was also characterized as endemic or not endemic. Establishment of endemic infection in a simulation was defined as one or more dogs being infected (I_C + I_{SC} dogs*)* at the steady state point (i.e., the time point at which a potential epidemic was classified as over and an endemic state was established). To standardize the determination of this steady state point, a subset of runs were evaluated in MATLAB® (MathWorks) using the "lsiminfo" solver to calculate the settling time based on four potential cutoffs. These simulations were evaluated at 100-day and 200-day simulation lengths, and the time points at which the number of infectious clinical dogs and the total number of infectious dogs settled at a tolerance of 2% or 3% were calculated for each. Across the examined runs, the 100-day and 2% tolerance cutoff provided the most consistent determination of steady state points that were also considered most biologically plausible. Figure 7-2 provides examples of these settling time checks, and the outcomes of interest are identified. The percentage of simulations that resulted in an endemic infection was calculated. The original intention was to perform logistic regression analysis, assessing which of the 11 independent model parameters contributed to the establishment of endemic infection; however this was not performed since none of the simulations were classified as not endemic.

Time course of epidemics. For each simulation classified as epidemic, two time points were calculated: the total duration and time of epidemic peak. The total duration of the epidemic, in days, was calculated from day 0 (when a latent dog was introduced) until the time when the number of infectious clinical dogs reached the steady state point.

The range and mean duration of simulated epidemics were summarized. Multiple stepwise regression was conducted in MATLAB® (MathWorks) using the “stepwisefit” function, to assess which of the 11 independent model parameters significantly affected the total duration of epidemics, with entry and removal levels set at P=0.05 and P=0.10 respectively. Stepwise regression was chosen in order to prioritize the 11 independent model parameters in terms of their effect on this outcome.

The time of the epidemic peak was defined as the day at which the number of infected dogs (I_C + I_{SC} dogs*)* was at its maximum. The range and mean for the times of the epidemic peaks were summarized. Stepwise regression, assessing which of the 11 independent model parameters significantly affected this outcome, was conducted as previously described.

Numbers of dogs. The total number of dogs infected (I_C + I_{SC} dogs*)* at the peak of the epidemic was determined for each epidemic simulation. The range and mean number of infected dogs at the epidemic peaks were summarized, and stepwise regression assessing which of the 11 independent model parameters significantly affected this outcome was conducted as previously described.

The accumulation of the total number of dogs in a shelter during an epidemic included all of the dogs in the shelter at the beginning of the simulation (the initS and initL values) plus the dogs that entered the shelter during the simulation (c(initS)) until the steady state point was reached. The range and mean number of total accumulated dogs during epidemics was described.

To address the number of dogs infected during the course of an epidemic, the accumulation of the total number of infectious and subclinical dogs during the epidemic

were determined. These values were calculated by accumulating the appropriate elements of Equations 7-5 and 7-6 respectively, from day 0 to the steady state point. The range and mean number of accumulated infectious clinical and subclinical dogs were summarized, and stepwise regression was conducted as previously described to determine which of the 11 independent model parameters significantly affected each of these outcomes.

Relative contribution of direct and indirect virus transmission. The relative contribution of the direct and indirect virus transmission terms were defined as the accumulation of the number of dogs that transitioned from the susceptible class to the latent class, due to the direct and indirect components of the force of infection (Equation 7-2), from day 0 until the epidemic peak. The direct or indirect transmission term was considered the dominant driver of the epidemic if it was >55% of the force of infection, otherwise the terms were considered equal. The percentages of epidemics that were due primarily to direct or indirect virus transmission, or that were due to both, were calculated.

To visualize the full range of model behaviors, a plot was generated for each epidemic simulation depicting the epidemic threshold, the time of the epidemic peak, the steady state point, the number of infectious clinical and subclinical dogs at these time points, and the force of infection divided between direct and indirect transmission. This also allowed the epidemic threshold and the 100-day simulation length to be further evaluated.

Results

Of the 500 H3N8 CIV introduction simulations, 498 (99.6%) were classified as epidemic. In addition, all 500 simulations were classified as endemic. Examples of

epidemic simulations are shown in Figures 7-2 through 7-5. Descriptive statistics (means and ranges) for the epidemic simulation outcomes (total duration, time of the epidemic peak, and numbers of dogs affected) are summarized in Table 7-2. A large range was expected for these outcomes, since some parameters were quite wide in their distributions. It is notable that large numbers of infectious subclinical dogs accumulated during the course of epidemics, with an average of 89 (Range = 15-237) infectious subclinical dogs accumulating.

The stepwise regression results for the epidemic time outcomes (total duration and time of the epidemic peak) are summarized in Table 7-3. For total epidemic duration, 9 of the 11 independent parameters entered the model and explained 71% of the variance. For the time of the epidemic peak, 9 parameters entered the model and explained 82% of the variance. Parameters primarily associated with indirect virus transmission (Φ, k, and β) were ranked as the top three contributors to these outcomes. The Φ and β parameters were negatively associated with these outcomes, while the k parameter was positively associated.

The stepwise regression results for the number of dog outcomes (total number of infected dogs at the epidemic peak, total number of infectious clinical and infectious subclinical dogs that accumulated during the epidemics) are summarized in Table 7-4. For the total number of dogs infected at the epidemic peak, all 11 parameters entered the model and explained 97% of the variance. For the total number of infectious clinical dogs that accumulated during the course of epidemics, 9 parameters entered the model and explained 96% of the variance. For the total number of infectious subclinical dogs that accumulated during the course of epidemics, 5 parameters entered the model and

explained 94% of the variance. The top ranked contributor was the *initS* parameter, which was positively associated with each of these outcomes. For the total number of dogs infected at the peak, parameters associated with indirect virus transmission were the next two greatest contributors. The β parameter was positively associated, and the *k* parameter was negatively associated with this outcome.

Of the 498 virus introduction simulations that resulted in an epidemic, 21% were primarily due to direct virus transmission, 63% were primarily due to indirect virus transmission, and 16% were due to both direct and indirect transmission as previously described. Figures 7-3, 7-4, and 7-5 show example plots for simulations driven primarily by direct transmission, indirect transmission, or equally by both, respectively. These graphs depict the total duration of the epidemic, the time of the epidemic peak, the steady state point, the number of infectious clinical and subclinical dogs at these points, and the force of infection divided between direct and indirect transmission.

Discussion

This is the first study to mathematically model H3N8 CIV transmission in shelter facilities. A unique feature of this model (for mammalian influenza models) was the separation of the force of infection into two terms, in order to account for both direct and indirect virus transmission. This approach was pursued because indirect virus transmission was hypothesized to be an important mode of H3N8 CIV transmission in highly managed shelter populations. There is precedent for this approach in the influenza modeling literature, in studies seeking to understand the epidemiological role of environmental transmission in avian influenza epidemics.[13,104,105] Limitations to the study largely centered on the limited or unavailable data for these and other model parameters. Investigators selected biologically plausible values in these situations,

however ranges and distributions could not be narrowly defined. In addition, since only two simulations were not classified as epidemic and no simulations were classified as not endemic, it was not possible to conduct logistic regression analysis to assess which of the 11 independent model parameters were significantly associated with the occurrence of an H3N8 CIV epidemic or the establishment of endemic infection. Even if the epidemic threshold was raised to 25% (i.e., of the shelter population being H3N8 CIV-infected), only approximately 5% of the simulations would have been classified as not epidemic; therefore the selected threshold (10%) was not considered a contributor to this result.

In this study, given the model structure and parameter ranges used, H3N8 CIV epidemics occurred in 99.6% of the simulations and endemic infection was established in all 500. Simulated epidemic times, as well as the overall numbers of dogs affected, varied widely based on the parameter values used in each simulation. Stepwise regression results indicated that multiple model parameters significantly influenced these outcomes. However, several parameters consistently ranked in the top three contributors and patterns emerged. Generally, as Φ and β increased (i.e., as more infectious virus doses were shed into the environment and a greater proportion of susceptible dogs became infected after contact with fomites) the total duration of the simulated epidemics decreased and the epidemics peaked earlier. As *k* increased (i.e., more noninfectious objects were present in the environment to scale the fomites) the total duration of the epidemic increased and the epidemic peak time occurred later. The greatest contributor to outcomes involving numbers of infected dogs was the value of *initS* (i.e., as the size of the shelter increased the number of total infected dogs at the

peak and the number of accumulated infectious clinical and subclinical dogs increased). Interestingly, however, parameters primarily associated with indirect virus transmission were the next greatest contributors to the total number of dogs infected at the peak; where the total number of dogs increased as β increased, and decreased as k increased.

In this study, 63% of the epidemic simulations were dominated by indirect transmission at the epidemic peak. Based on the defined criteria, this meant that >55% of the latent dogs that accumulated from day 0 to the epidemic peak were infected by indirect transmission in 63% of the simulations. Recent modeling studies have reported that environmental transmission is a significant driving factor in avian influenza epidemics, and that it is particularly important for persistence of infection;[13,104,105] however these studies focused on a feature unique to the biology of avian influenza, where virus is shed through the feces and is able to persist long term (i.e., months) under certain conditions in aquatic environmental reservoirs. This study attempted to capture indirect virus transmission by first quantifying the number of fomites in the environment (Equation 7-8), and then scaling them in the indirect virus transmission term by a range of noninfectious objects (Equation 7-2). The study results indicate that indirect transmission may be more important than previously considered, and that separation of the transmission terms may shed light on targets for prevention and control strategies.

For all of these outcomes, it is important to note that no interventions (i.e., halting shelter intakes; isolation, sanitation, or vaccination) were applied to the model. Such interventions theoretically would have reduced the number of susceptible animals and

the amount of infectious virus in the simulations. In addition elements of the model structure (e.g., inclusion of a fomites class and separation of the force of infection into two transmission terms), and the parameter values and distributions used also influenced these results. In order to more fully explore the parameters that affect H3N8 CIV transmission in shelters, this study highlights the importance of collecting additional epidemiological and experimental data to refine the values, ranges, and distributions of several parameters. For example, additional data on the α and β components of the direct and indirect transmission terms respectively would be particularly valuable. Additional work should also focus on how to define and parameterize virus shedding into the environment and the scaling of fomites in the indirect transmission term, since indirect virus transmission parameters such as Φ and k significantly influenced several of the simulation outcomes.

Despite these limitations, the study results overall reflect what has been observed clinically during H3N8 CIV epidemics in shelters (i.e., explosive epidemics with high morbidity). The epidemic time outcomes and numbers of dogs affected were similar to what has been observed during H3N8 CIV shelter outbreak consultations conducted by the investigators.[20,98] The reported accumulation values highlighted the large number of dogs that can be infected during the course of H3N8 CIV epidemics. The accumulated number of infectious subclinical dogs further supports the current recommendation to quarantine all H3N8 CIV exposed dogs during an epidemic, since subclinical dogs can silently shed and spread virus. Although beyond the scope of this study, the establishment of endemic infection in all of the simulations raises important clinical questions regarding which parameters might ultimately promote endemicity in shelters.

In conclusion, based on the overall results of this study, indirect virus transmission and the number of dogs in a shelter (i.e., the shelter capacity) have the greatest impact on H3N8 CIV transmission dynamics in shelter facilities. Prevention and control strategies that reduce contact between dogs and fomites, decrease the amount of infectious virus in the environment, and limit the number of incoming susceptible dogs will likely reduce the impact of epidemics in shelter facilities (and may prevent the establishment of endemic infection). Studies focused on modeling the efficacy of different intervention strategies, including isolation and vaccination, are being pursued to test these hypotheses.

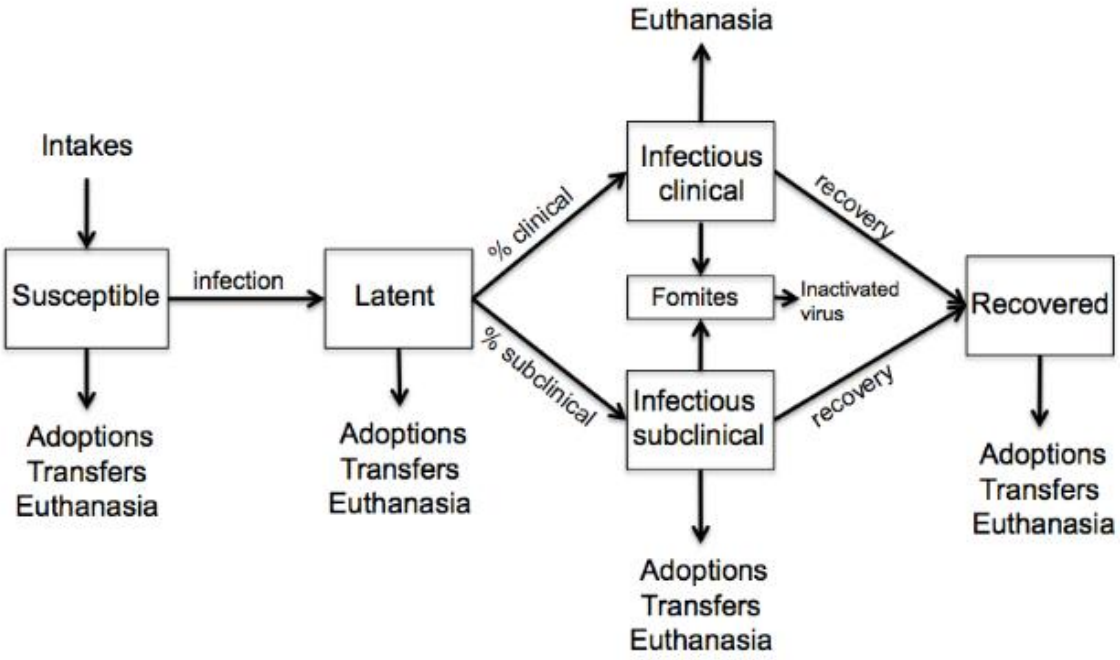


Figure 7-1. Model schematic for simulations of H3N8 CIV introduction into shelter facilities.

Table 7-1. Parameters, ranges, and distributions used for simulations of H3N8 CIV introduction into shelter facilities.

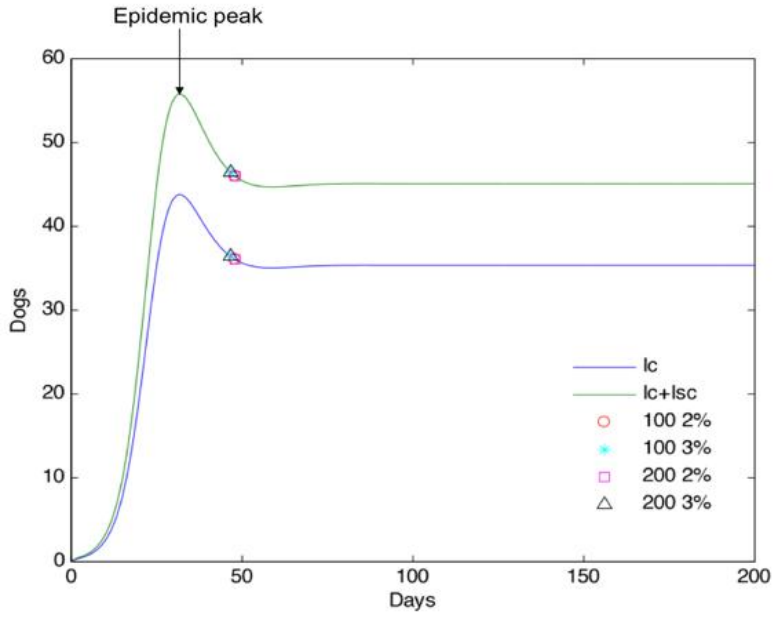
Parameters	Symbol	Distribution[a]	Range[b]	Center[c]	Sources[d]
Initial no. of susceptible dogs in the shelter population	initS	Uni	50-400		1
Initial latent dog introduced to begin simulation	initL	Con		1	1
Proportion of dogs that become infected after contact by direct transmission routes	α	Tri	0.2-0.9	0.5	1,2
Proportion of dogs that become infected after contact by indirect transmission routes	β	Uni	0.2-0.8		1
Devaluing parameter for infectiousness of subclinical dogs	a	Con		0.5	3
Scaling parameter representing the potential no. of noninfectious objects in the environment	k	Uni	10^2-10^4		1,4
Percent of shelter capacity (initS) immigrating into the susceptible class at time (t)	c	Uni	0.05-0.1		1
Percent of dogs in class (all but infectious clinical) emigrating at time (t)	m	Uni	0-0.05		1
Percent of dogs emigrating out of the infectious clinical class at time (t)	m_{IC}	Con		0	1
Percent of dogs in class (all but infectious clinical) euthanized at time (t)	d	Calc		Equation 7-1	
Percent of dogs euthanized from the infectious clinical class at time (t)	d_{IC}	Calc		1.5*d*	
Per capita rate at which latent dogs become infectious (clinical or subclinical)	f	Tri	0.33-0.95	0.5	2,5,6,7
Percent of dogs that become infectious clinical	q	Tri	0.7-0.9	0.8	2,5,6,7
Per capita rate at which infectious clinical and subclinical dogs recover	γ	Tri	0.1-0.2	0.15	2,5,6,7
No. of infectious H3N8 CIV doses produced per infectious clinical and subclinical dog	Φ	Uni	10^1-10^2		2,5,6,7

[a] Probability distribution used in LHS analysis. Tri, triangular; Uni, uniform; Calc, calculated from other parameters (not in LHS analysis); Con, constant (not in LHS analysis).
[b] Minimum-maximum values.
[c] Most probable value.
[d] Literature used: 1, Investigator opinion; 2, Jirjis et al., 2010; 3, Longini et al., 2004; 4, Rohani et al., 2009; 5, Crawford et al., 2005; 6, Deshpande et al., 2009; 7, Deshpande et al., 2009

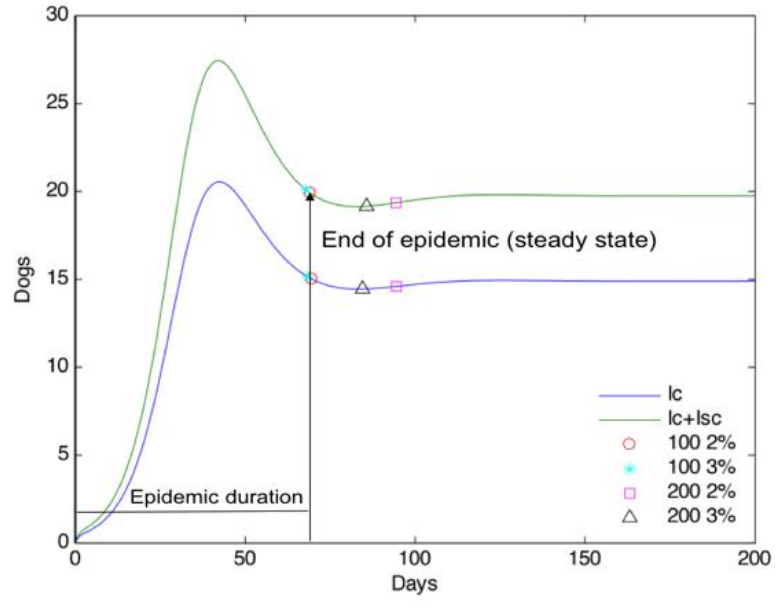
Table 7-1 continued.

Parameters	Symbol	Distribution[a]	Range[b]	Center[c]	Sources[d]
Devaluing parameter for decreased virus shedding of subclinical dogs	b	Con		0.5	3
Inactivation of infectious H3N8 CIV doses over time on fomites	ρ	Uni	0.25-0.95		8,9,10

[a] Probability distribution used in LHS analysis. Tri, triangular; Uni, uniform; Con, constant (not in LHS analysis).
[b] Minimum-maximum values.
[c] Most probable value.
[d] Literature used: 3, Longini et al., 2004; 8, Brankston et al., 2007; 9, Weber et al., 2008; Beeler, 2009.



A



B

Figure 7-2. Results of typical endemic settling time checks, used to standardize the criteria for determining the steady state point. I_C and I_{SC}= Infectious clinical and subclinical dogs, 100 2% or 100 3%= 100-day simulation where the number of infectious dogs settles to within 2 or 3% tolerance, 200 2% or 200 3%= 200-day simulation with 2 or 3% tolerance. A, Example simulation where the steady state point was comparable regardless of the criteria applied; *initS*= 220, *c*= 0.09, *m*= 0.02, α= 0.34, β= 0.40, *f*= 0.44, *q*= 0.79, γ= 0.14, Φ= 78, ρ= 0.88, *k*= 9808 (run #147). B, Example simulation where the steady state point differed between the selected criteria; initS= 367, c= 0.05, m= 0.02, α= 0.80, β= 0.36, *f*= 0.51, *q*= 0.76, γ= 0.14, Φ= 83, ρ= 0.79, *k*= 3646 (run #292).

Table 7-2. Descriptive statistics for specified outcomes of 498 H3N8 CIV introduction simulations classified as epidemic.

Outcome	Mean	Range
Epidemic times (days):		
Total duration	38	(20-93)
Epidemic peak	19	(6-59)
Total number of dogs:		
Infected (clinical and subclinical) at the epidemic peaks	88	(10-219)
Accumulated (entering shelter) during epidemics	850	(175-2226)
Accumulated infectious clinical during epidemics	358	(78-838)
Accumulated infectious subclinical during epidemics	89	(15-237)

Table 7-3. Results of stepwise regression on epidemic duration and time of epidemic peak. Parameters as defined in Table 7-1.

Outcome	Step	Entered	Removed	Coefficient	Model R^2	F	P-value[a]
Epidemic duration		Intercept		85.6246			
	1	Φ		-0.2038	0.2329	150.6229	<0.001
	2	k		0.0019	0.4546	206.3597	<0.001
	3	β		-19.4341	0.5407	193.8725	<0.001
	4	c		-201.8824	0.5836	172.7888	<0.001
	5	initS		-0.0267	0.6230	162.6221	<0.001
	6	α		-16.7487	0.6554	155.6615	<0.001
	7	f		-16.6987	0.6873	153.8814	<0.001
	8	ρ		9.9345	0.7103	149.8748	<0.001
	9	m		-77.0380	0.7180	138.1224	<0.001
	10		q	3.0075			0.6855
	11		γ	27.6981			0.0632
Time of epidemic peak		Intercept		42.9029			
	1	k		0.0014	0.3203	233.8062	<0.001
	2	Φ		-0.1300	0.5462	297.9519	<0.001
	3	β		-14.5381	0.6597	319.3555	<0.001
	4	α		-12.6420	0.7110	303.2770	<0.001
	5	f		-11.3414	0.7549	303.2277	<0.001
	6	initS		-0.0150	0.7940	315.5254	<0.001
	7	ρ		5.5029	0.8166	311.6825	<0.001
	8	c		36.3631	0.8214	281.2515	<0.001
	9	q		-7.1921	0.8229	252.0641	<0.001
	10		m	-13.1223			0.1902
	11		γ	1.8382			0.7958

[a] P-values <0.05 were considered significant.

Table 7-4. Results of stepwise regression on total number of infected dogs at the epidemic peak, as well as accumulated infectious clinical (I_C) and subclinical dogs (I_{SC}) at the steady state (SS). Parameters as defined in Table 7-1.

Outcome	Step	Entered	Removed	Coefficient	Model R^2	F	P-value[a]
Total number infected dogs at peak		Intercept		-20.5528			
	1	initS		0.4329	0.8440	2683.9579	<0.001
	2	β		46.3329	0.8767	1761.1285	<0.001
	3	k		-0.0026	0.9043	1556.3925	<0.001
	4	γ		-322.1694	0.9241	1501.8924	<0.001
	5	Φ		0.2471	0.9420	1599.8543	<0.001
	6	f		40.7496	0.9554	1757.0821	<0.001
	7	α		27.3641	0.9619	1770.7701	<0.001
	8	c		-245.3557	0.9676	1828.9364	<0.001
	9	ρ		-12.8175	0.9704	1777.8599	<0.001
	10	m		125.7297	0.9718	1678.6584	<0.001
	11	q		24.8676	0.9722	1548.6551	<0.001
Accumulated I_C dogs at SS		Intercept		-415.4966			
	1	initS		1.5538	0.9262	6230.4552	<0.001
	2	q		559.1149	0.9456	4309.0315	<0.001
	3	m		-727.2562	0.9504	3158.4911	<0.001
	4	Φ		-0.3193	0.9520	2463.5350	<0.001
	5	γ		-349.7988	0.9542	2051.7242	<0.001
	6	β		30.1928	0.9555	1758.7330	<0.001
	7	c		348.5366	0.9564	1538.8567	<0.001
	8	k		0.0016	0.9573	1372.0809	<0.001
	9	f		23.7296	0.9577	1228.0226	<0.001
	10		α	9.6745			0.3618
	11		ρ	9.4473			0.2152
Accumulated I_{SC} dogs at SS		Intercept		360.3887			
	1	initS		0.3917	0.7682	1644.0073	<0.001
	2	q		-441.9163	0.9300	3289.9189	<0.001
	3	m		-191.4053	0.9337	2320.0066	<0.001
	4	Φ		-0.0829	0.9360	1804.0266	<0.001
	5	k		0.0006	0.9378	1485.2920	<0.001
	6		c	-1.8404			0.9585
	7		α	6.4987			0.0673
	8		β	2.9971			0.3084
	9		f	5.2240			0.1777
	10		γ	-46.5237			0.0618
	11		ρ	3.2597			0.1986

[a] P-values <0.05 were considered significant.

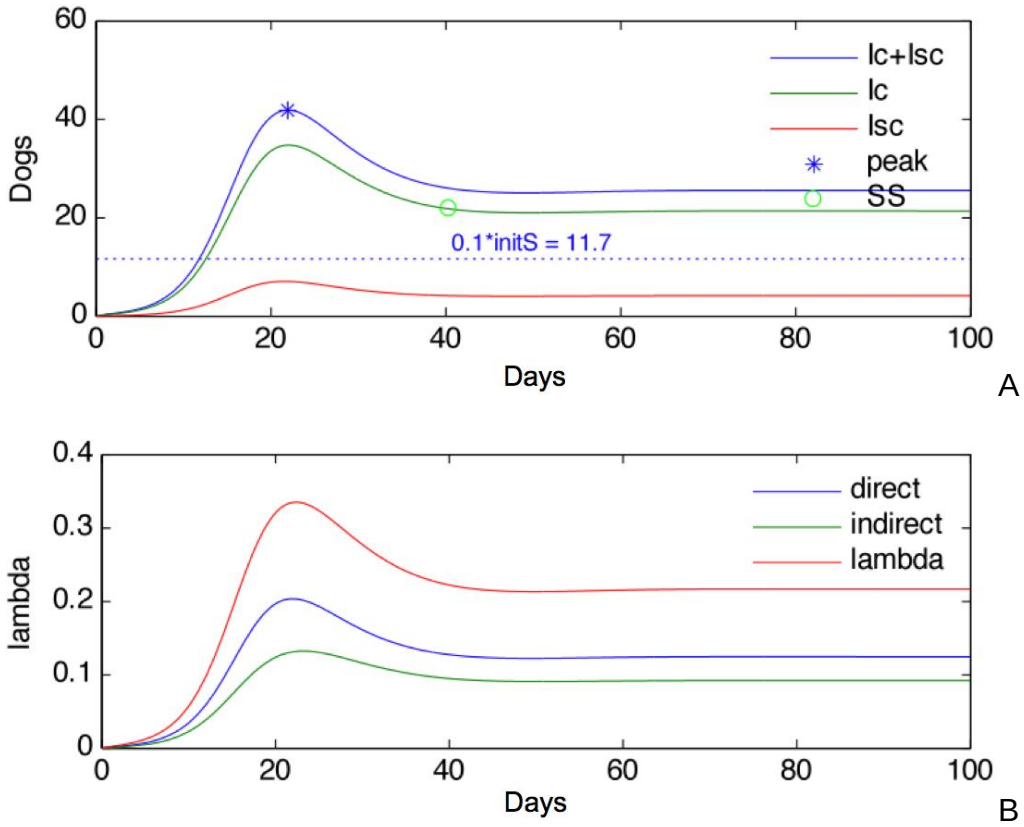


Figure 7-3. Example of a simulation dominated by direct virus transmission at the peak of the epidemic. A) Number of infected dogs (I_C= infectious clinical and I_{SC}= infectious subclinical) during the simulation, at the epidemic peak and the steady state point (SS); blue= total infectious dogs, green= clinical, red= subclinical. B) The force of infection (λ, or lambda depicted in red) separated into direct (blue) and indirect (green) transmission. Parameter values: initS= 117, c= 0.05, m= 0.04, α= 0.62, β= 0.45, f= 0.43, q= 0.81, γ= 0.14, Φ= 89, ρ= 0.89, k= 9348 (run #306).

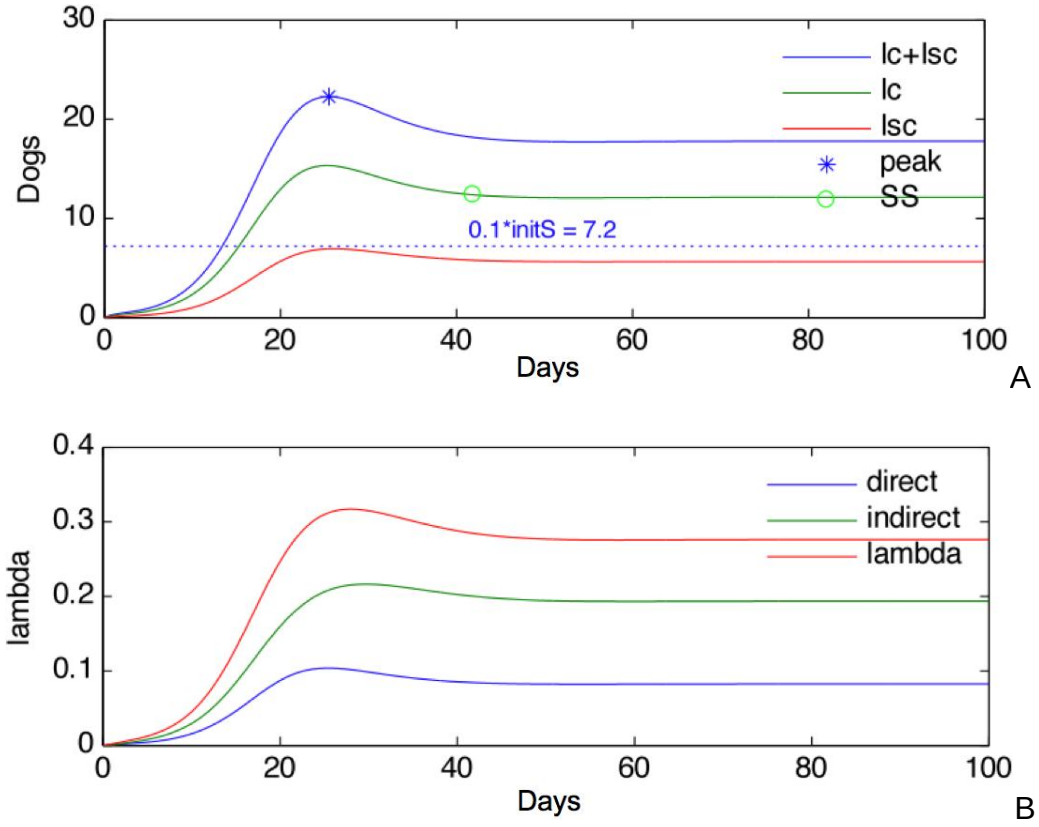


Figure 7-4. Example of a simulation dominated by indirect virus transmission at the peak of the epidemic. A) Number of infected dogs (I_C= infectious clinical and I_{SC}= infectious subclinical) during the simulation, at the epidemic peak and the steady state point (SS); blue= total infectious dogs, green= clinical, red= subclinical. B) The force of infection (λ, or lambda depicted in red) separated into direct (blue) and indirect (green) transmission. Parameter values: initS= 72, c= 0.09, m= 0.002, α= 0.40, β= 0.53, f= 0.42, q= 0.71, γ= 0.12, Φ= 70, ρ= 0.29, k= 6216 (run #391).

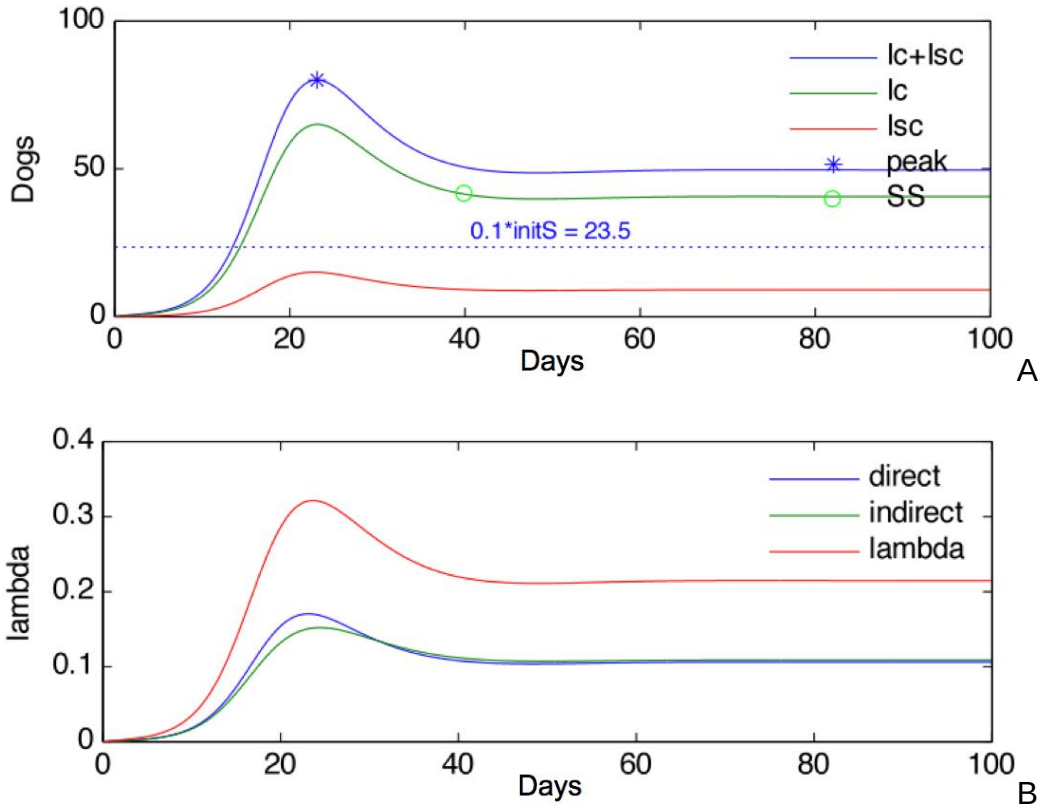


Figure 7-5. Example of a simulation equally due to direct and indirect virus transmission at the peak of the epidemic. A) Number of infected dogs (I_C= infectious clinical and I_{SC}= infectious subclinical) during the simulation, at the epidemic peak and the steady state point (SS); blue= total infectious dogs, green= clinical, red= subclinical. B) The force of infection (λ, or lambda depicted in red) separated into direct (blue) and indirect (green) transmission. Parameter values: initS= 235, c= 0.06, m= 0.04, α= 0.55, β= 0.47, f= 0.5, q= 0.79, γ= 0.15, Φ= 49, ρ= 0.84, k= 8851 (run #433).

CHAPTER 8
SUMMARY, RESEARCH CONCLUSIONS, AND ISSUES FOR FUTURE INVESTIGATORS

Summary

The overall objectives of this PhD research were to develop tools for accurate serological diagnosis of H3N8 CIV infection and to investigate the epidemiology of canine influenza in order to improve prevention and control strategies.

Serological assays are often necessary to confirm diagnosis of H3N8 CIV infection, however a standard HI assay has not been generally accepted for canine influenza. To adequately study the epidemiology of H3N8 CIV, key diagnostic tests therefore needed to be developed and assessed. The first research aim was to determine the diagnostic performance of the H3N8 CIV HI assay as conducted in our previous studies, and to evaluate the impact of common test parameter variations on diagnostic performance. The standard H3N8 CIV HI assay was then utilized in subsequent epidemiological studies. The introduction of the inactivated H3N8 CIV vaccine in June 2009 created a diagnostic dilemma, however, since the HI assay does not allow differentiation of infected from vaccinated animals (DIVA). Although epidemiological investigations conducted for this research were not confounded by vaccination, a DIVA test strategy is not currently available for H3N8 CIV; therefore, the second diagnostic research aim was to develop a DIVA test strategy for canine influenza based on the reported differential immune response to the influenza A NS1 protein.

Regarding the epidemiology of H3N8 CIV, several gaps in knowledge were addressed through this research. It is unknown if H3N8 CIV was infecting dogs prior to 2004, causing undiagnosed or misdiagnosed respiratory disease; therefore the first

epidemiological study addressed this question by testing archived sera from racing greyhounds and shelter dogs for antibodies to canine and equine H3 proteins in the standard HI assay. In addition, risk factors for H3N8 CIV infection have not been defined for individual dogs; therefore the second study utilized a syndromic serosurvey to assess potential risk factors for infection including intrinsic factors, exposure settings, and geographic location. Based on identification of communal settings as risk factors for infection, and the realization that factors which promote H3N8 CIV transmission in shelters are unknown, mathematical modeling was finally utilized to explore which factors promote virus transmission in shelter facilities. The research findings and conclusions are summarized below, followed by issues to be addressed by future investigators.

Research Conclusions

Diagnostic Test Development

Specific aim 1 (Chapter 3): Diagnostic performance of the H3N8 CIV HI assay

The results of this study indicate that the standard HI assay is a highly sensitive and specific assay for diagnosis of H3N8 CIV infection in dogs when the antibody cutoff titer for seropositivity is 32. The optimal sensitivity and specificity depends on use of "standard" test parameters including 0.5% turkey RBC, serum treatment with RDE, and use of live H3N8 CIV isolates. Variation in these parameters results in misdiagnosis of infected dogs. Standarization of this assay should ensure accurate diagnosis of H3N8 CIV-infected dogs between laboratories and research groups.

Specific aim 2 (Chapter 4): Development of a DIVA test strategy for H3N8 CIV

In this study, uninfected and vaccinated dogs could not be differentiated from H3N8 CIV-infected dogs in the NS1 immunoassays. After multiple troubleshooting efforts

the encountered specificity issues could not be resolved, supporting the conclusion that an influenza A NS1 DIVA test is not a viable or reliable diagnostic strategy for H3N8 CIV. An alternative DIVA strategy is necessary for continued accurate diagnosis of H3N8 CIV-infected dogs and for future epidemiological studies of canine influenza in the face of vaccination.

Epidemiological Investigations

Specific aim 1 (Chapter 5): Serological evidence of H3N8 canine influenza-like virus circulation in dogs prior to 2004

The evidence from this study suggests that an ancestral H3N8 canine influenza-like virus was circulating in the racing greyhound population as early as 1999. Based on the results of this study, greyhounds that raced were more likely to be exposed to H3N8 viruses. Of greyhounds that raced, dogs that raced at 2 or more tracks, dogs that raced for 6 months or longer, and dogs that raced in 1998, 2002, and 2003 were more likely to be seropositive to CIV and EIV H3 antigens. Although the clinical histories of the tested dogs are unknown, these results suggest that a H3N8 CIV-like virus may have been associated with respiratory disease epidemics that occurred at U.S. racetracks prior to 2003.

Specific aim 2 (Chapter 6): Prevalence of and risk factors for H3N8 CIV seropositivity in a population of U.S. dogs with ILI

This study identified a high prevalence of H3N8 CIV exposure in 1,268 dogs with ILI in 42 states over a 5-year period. Dogs ≥1 year of age, dogs housed in communal settings such as boarding kennels or shelter facilities, and dogs in the Northeast or West were at greatest risk for H3N8 CIV seropositivity. These results justify the need for shelters, boarding kennels, and other communal dog housing facilities to formulate, implement and/or evaluate surveillance and biosecurity protocols to reduce the risk of

virus transmission between dogs and avert or mitigate large-scale epidemics within these facilities.

Specific aim 3 (Chapter 7): Simulation studies of H3N8 CIV transmission in shelter facilities

The results of this study indicate that indirect virus transmission and the number of dogs in a shelter (i.e., the shelter capacity) have the greatest impact on H3N8 CIV transmission dynamics in shelter facilities. Prevention and control strategies that reduce contact between dogs and fomites, decrease the amount of infectious virus in the environment, and limit the number of incoming susceptible dogs will likely reduce the impact of epidemics in shelter facilities and may prevent establishment of endemic infection.

Issues for Future Investigators

Diagnostic Test Development

Moving forward it will be important to continue monitoring for antigenic drift in the CIV H3 protein in order to ensure that the H3N8 CIV used in the HI assay is closely related to that in circulation. Antigenic mismatches between circulating strains and test strains would compromise the sensitivity and specificity of the standard HI assay, resulting in misdiagnosis of infected dogs. Since the H3N8 CIV HI assay cannot differentiate antibodies induced by infection from vaccination with the inactivated H3N8 CIV vaccine, it is imperative to know whether a H3 seropositive dog was vaccinated to accurately interpret HI test results. This limits the use of the HI assay in future epidemiological studies of H3N8 CIV in the U.S. if vaccination status is unknown.

Since the H3N8 CIV NS1 immunoassays were not a viable DIVA strategy for dogs, an alternative DIVA strategy based on detection of antibodies to the influenza viral

nucleoprotein (NP) is proposed. This NP DIVA strategy, coupling the NP ELISA with use of a recombinant CIV H3 vaccine, could resolve the diagnostic dilemma created by the currently used inactivated whole virus vaccine. With this approach, H3N8 CIV-infected dogs could be differentiated from vaccinated dogs based on the presence of NP antibody: infected dogs would have antibody to both H3 and NP, while dogs vaccinated with the recombinant vaccine would only have antibodies to H3.

Epidemiological Investigations

Studies focused on modeling the efficacy of different intervention strategies, including isolation and vaccination, for averting or mitigating H3N8 CIV epidemics in shelters are being pursued in order to develop prevention and control recommendations.

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BIOGRAPHICAL SKETCH

Tara Creel Anderson graduated with a Bachelor of Science in wildlife ecology and conservation in 1999 and earned her Doctor of Veterinary Medicine (DVM) in 2003, both from the University of Florida. After graduation she practiced general small animal medicine and surgery in Gainesville, Florida for two years. She returned to graduate school at the University of Florida in the summer of 2005 to pursue a combined Master of Public Health/Doctor of Philosophy (MPH/PhD) program. During her studies she served on the joint DVM/MPH program committee and was fortunate to work with several collaborators in influenza laboratories in the U.S. and Italy. She received her MPH with a focus in epidemiology in 2007, and completed her PhD in veterinary medical sciences in 2011.