Introduction: Canine influenza is an emerging disease around the world that was first identified in 2004. Two known strains of canine influenza virus (CIV) circulate presently and large scale outbreaks continue. Viral reassortants have been identified over the last decade and canines have shown susceptibility to human, avian and swine influenzas. Canine influenza poses health risks to beloved pets and working dogs, and could potentially become a zoonotic threat. The aim of this paper is to summarize the evolution of CIV; describe its clinical significance; and to evaluate its effects on the canine immune system, comparing and contrasting this to human influenza infections. By understanding the immune response to CIV, we gain an appreciation for how disease
pathogenesis results in clinical signs and how comorbidities could impact disease presentation. This understanding may assist veterinarians in determining the importance of building herd immunity with widespread vaccination and helping to identify at-risk canine patients. A literature review was conducted covering the years 2005-2018 with search terms including ‘canine influenza’ and ‘immune response’.

**Evolution of CIV**

There are two known circulating strains of CIV in the United States, CIV H3N8 and CIV H3N2. These viruses are capable of dog to dog transmission which distinguishes them as canine. CIV H3N8-induced disease was first identified in Florida racing greyhounds in 2004 and is of equine origin, stemming from a mutation at the hemagglutinin receptor binding site which allowed the virus to adapt its host species from horses to dogs. CIV H3N8 spread rapidly throughout the racing greyhound community, typically causing fever, cough, and nasal discharge in infected dogs; however some dogs died peracutely due to pulmonary hemorrhage associated with suppurative bronchopneumonia. CIV H3N8 was first identified in a New York pet dog in 2005 and serologic evidence showed a spread to 25 states over 3 years. Currently, CIV H3N8 seropositivity rates vary widely by geography in 39 states and range from 2.3-15%. Of avian origin, CIV H3N2 was first identified in pet dogs in Chicago in 2015, although it has been circulating in SE Asia since 2005. Sequence analysis of the virus demonstrated that the Chicago strain was most similar to CIV H3N2 circulating in South Korea during 2015. Widespread outbreaks continue across the United States. Further sequence analysis has linked some U.S. CIV H3N2 outbreaks to ongoing importation of infected dogs from Korea and China.

**Clinical Significance of CIV**

Transmission dynamics of CIV H3N2 differ from CIV H3N8. CIV H3N8 has a short viral shed window from 1-7 days, with peak viral shedding occurring between days 2-4, whereas CIV H3N2 has been shown to shed intermittently at low levels by some dogs for at least 21 days. Viral shedding kinetics underpin the timing of diagnostic testing and have implications for biosecurity. For both CIV H3N8 and CIV H3N2, dogs shed virus prior to the onset of clinical signs and influenza A viruses survive on surfaces for 24-48 hours. These factors facilitate the continued spread of the disease, especially in pet dogs living highly social lifestyles and dogs kept in multi-dog housing, such as boarding kennels and shelters. Approximately 80% of dogs exposed to CIV develop clinical signs including fever, nasal and ocular discharges, and a cough that may persist for up to 3 weeks. Estimated mortality rates vary from 5-10%, but have been as high as 36% in some CIV H3N8 outbreaks. CIV H3N8 has not been shown to transmit to other species. Experimental challenge of horses with CIV H3N8 shows seroconversion but lack of disease or viral shedding. CIV H3N2 has been shown to be contagious to cats, with one Korean shelter reporting 100% morbidity and 40% mortality. Experimental challenge confirms that cats are susceptible to CIV H3N2 and can transmit infection to other cats, resulting in clinical disease.

**Influenza A Susceptibility and Reassortants**

Influenza A viruses are single stranded RNA viruses in the Orthomyxoviridae family with 8 gene segments encoding for structural and nonstructural proteins. These viruses are divided into subtypes based on the hemagglutinin (H) protein, which is responsible for attachment and infection of cells; and the neuraminidase (N) protein which is responsible for cleavage of sialic acid and release of viral particles from infected cells. There are 18 known H types and 11 known N types. The ability of the hemagglutinin to bind is host-specific. However, given the ability of RNA replication to develop mutations, antigenic drift can occur, allowing infectivity into new host ranges. In addition, co-infection with more than one influenza A virus may allow for gene reassortment, resulting in an antigenic shift and the ability to infect new host species. Host viral receptors vary by species and play a role in species-specific susceptibility to influenza. Tracheal viral receptors in birds, horses, and dogs consist of α2,3 sialic acid whereas human tracheal viral receptors consist of α2,6 sialic acid. Swine express both receptors and serve as the “mixing vessel” when co-infected with avian and human influenzas. Although dogs predominantly express α2,3 sialic acid receptors, some foci of α2,6 sialic acid receptors have been identified in the respiratory tract, which may allow the dog, like the pig, to serve as a “mixing vessel”. This potential zoonotic risk is one of the greatest challenges that has emerged from the establishment of CIV in the pet population. It has been demonstrated that dogs may be infected not only with CIV H3N8 and CIV H3N2, but also with pandemic H1N1, highly pathogenic
avian influenza (HPAI) H5N1, avian influenza H9N2, human H3N2, as well as reassortant H3N1 and H5N2. CIV H3N2 reassorted with pandemic H1N1 to create H3N1 in South Korea. This virus is capable of causing nasal shedding and mild pulmonary histopathologic changes in dogs. A CIV H3N2 isolate recovered from a naturally infected dog revealed reassortment with pandemic H1N1 and expressed the Matrix gene (M) segment from H1N1. Challenge studies in dogs using this virus demonstrated its ability to cause clinical disease by direct and indirect exposures and also to induce seroconversion and viral shedding. Similar recombinations with swine influenza and pandemic H1N1 have also been reported. Additional samples from CIV H3N2-infected dogs showed 23 different genotypic patterns of recombination with CIV H3N2 and pandemic H1N1, with canine H and human M proteins most commonly expressed. Some of these genotypes caused severe clinical disease in experimentally infected mice. Surveillance of influenza viruses in China over a 2-year period identified swine, human and canine influenza reassortants, with two genotypes capable of further infecting swine, humans, and dogs. The ability of CIV to reassort with other influenza A viruses, the demonstrated susceptibility of dogs to a variety of influenza viruses, and the close relationship between pet dogs and their owners, poses risks for the development of zoonotic influenza.

The Innate Immune Response to CIV

The difference between dogs that develop mild clinical disease and those that progress to severe disease or mortality has often been attributed to co-infection with secondary bacterial pathogens. A deeper understanding of the immune response and the impact of comorbidities may shed light on additional factors that contribute to disease severity, mortality, and chronic sequelae. A key difference in the canine response to influenza compared to that in humans is the secretion of interleukin (IL)-8. CIV H3N2 infection stimulates marked increases in IL-8 in the serum on days 3 and 6 after intranasal experimental challenge. The role of chemokine IL-8 is to recruit neutrophils. CIV H3N2 has been shown to induce neutrophilic infiltration into the lungs without secondary bacterial co-infection. There are also elevated levels of monocyte chemotactic protein (MCP-1) in the lungs post-infection. The role of chemokine MCP-1 is to recruit monocytes. (Figure 2) Histopathology of CIV H3N2 infected lungs shows mild to severe pneumonia with neutrophilic and lymphocytic infiltrates, pulmonary hemorrhage, and neutrophils and macrophages in the alveoli. These results are consistent with the known chemokine response to CIV. In general, human and swine chemokine responses to influenza show elevations in IL-6, which is responsible for the development of certain acute phase proteins.

CIV H3N2 infection also generates elevated levels of interferon (IFN)-γ and tumor necrosis factor (TNF)-α. IFN-γ and TNF-α have antiviral activities and have been identified as the pro-inflammatory cytokines in human influenza infection. TNF-α demonstrates species- and virus-specificity, but may not be released in all influenza infections. There is evidence that CIV generates macrophage activation through the classical IFN-mediated pathway resulting in TNF-α production, but innate activation of macrophages also occurs, as evidenced by increases in macrophage receptors with collagenous structure (MARCO) during infection. CIV H3N8 also has been shown to replicate in alveolar macrophages and stimulate high levels of TNF-α. These immune responses are important in limiting viral infection.

In a mouse model of human influenza, alternatively activated macrophages (AAM) contributed to increasing the susceptibility to bacterial pneumonia during the recovery phase after influenza infection. The late appearance of these macrophages represents a change in the cytokine milieu from inflammatory to maintenance. The transition to a Th helper 2 (Th2) response and the secretion of IL-4 and IL-13 decreases the bactericidal activity of macrophages. AAMs secrete arginase-1, which competes with inducible nitric oxide synthase, which known for its antibacterial effects. Although the role of AAMs is unknown in CIV, it is possible that altered cytokine expression increases the likelihood of secondary bacterial infection, which could contribute to severity of disease. The presence of parasitic infection or pulmonary migrating larval stages are common in dogs and may set the stage for a predominant Th2 response. This could then lead to the development of a suboptimal antiviral immune response, thereby complicating the course of disease.

It has been shown that CIV H3N2 infects tissues from the upper and lower respiratory tract, the bronchiolar epithelium and type I pneumocytes. The ability of CIV H3N2 to penetrate the lower respiratory tract and cause tissue destruction likely contributes to the long-term sequelae of CIV infection. Apoptosis may also be a factor in tissue damage related to CIV infection. MicroRNAs are small molecules that regulate many cellular functions and pathways, including response to viral infection. Differential
upregulation and downregulation of microRNAs have been demonstrated in dogs experimentally infected with CIV H3N2 and H5N1. Additional evaluation of dogs experimentally infected with CIV H3N2 showed an increase in microRNA in lung tissue early in infection, and specifically identified cfa-miR-143. MicroRNA 143 is known for its role in apoptosis in cancer and the apoptotic pathways upregulated by cfa-MiR-143 act via p53.

The Adaptive Immune Response to CIV
Antibodies generated after infection with influenza virus are critical to the protective response. While secretory IgA is important for immune exclusion at the mucosal surface, IgM and IgG are capable of pathogen elimination. Additionally, antibody-dependent cell mediated cytotoxicity (ADCC) can be induced by influenza A infections. Upregulation of gene expression for IgG Fc fragments in natural killer (NK) cells have been identified in dogs infected with CIV H3N2, suggesting that ADCC plays a role in the immune response to CIV. T cell differentiation to CD8+ cytotoxic T cells (CTLs) in response to virally infected cells is an essential immune response. CD4+ T cell differentiation to T helper 1 cells drives this response through the secretion of IFN, IL-2 and IL-12 and differentiation to T helper 2 cells promotes B cell-mediated antibody production. Controlled flow cytometry studies of T cell subsets from the peripheral blood of CIV H3N2-infected dogs failed to demonstrate differences between infected and uninfected dogs. However, analysis of lung tissue from CIV H3N2-infected dogs showed gene upregulation in T helper 1 and T helper 2 cells. This suggests a balance between cell mediated and humoral responses in the immune response to CIV.

Viral Interference with Immunity
Viral infections stimulate a cascade of responses from both the innate and adaptive immune system. The recognition of viral RNA by pathogen recognition receptors, specifically RIG-I, results in downstream activation of transcription factors NF-κB and interferon response factors (IRF), that ultimately produce type I IFN, activate the adaptive immune system, and produce cytokines. However, pathogens come armed with virulence factors to circumvent the host immune system. In human influenza, the NS1 viral protein blocks several steps in the immune response. CIV H3N2 has been shown to block IFN-β production by blocking NF-κB and IRF3. (Figure 3) Influenza A accessory protein PB1-F2 has been shown to decrease IFN production by blocking RIG-I, but increase pro-inflammatory cytokine production through the NF-κB pathway. PB1-F2 has also been implicated in apoptosis. PB1-F2 inflammatory residues have been identified and contribute to lung inflammation and pulmonary neutrophil infiltrates. A combination of delaying the initial immune response through decreased IFN-β, and recruitment of neutrophils and inflammatory cytokines, leads to severe clinical disease. This has occurred in avian H5N1 and pandemic human influenzas in 1918, 1957 and 1968. The sequence length of PB1-F2 varies and contributes to its virulence, with the full length variant causing more severe clinical disease and increasing susceptibility to secondary infection. Evaluation of CIV H3N8 isolates identified 65% with a full length PB1-F2 accessory protein and 22% with inflammatory residues. CIV H3N2 isolates contained 95% full length variants and 100% inflammatory residues. By contrast, human seasonal influenza had <1% with full length PB1-F2 proteins and no inflammatory residues.

Summary
CIV immune responses promote pulmonary neutrophilic infiltration. The virus is capable of deep penetration into the lower airways where pro-inflammatory cytokines and apoptosis can cause pathology. Additionally, viral interference with innate immunity contributes to successful infection and CIV expresses virulence factors that may contribute to increased morbidity and mortality. Dogs are commonly exposed to multiple pathogens, co-morbidities such as parasitic infection, and concurrent therapies such as steroid use, which could negatively impact the immune response to infection. Multiple reassortants of CIV, canine receptor capability to serve as a “mixing vessel”, and the susceptibility of the dog to multiple influenza A viruses could potentially lead to the development of canine-human zoonotic influenza. Vaccination against CIV can decrease viral circulation through canine populations, and reduce the risk of clinical disease.
Reflection

This review describes how the immune responses to CIV can be directly linked to clinical pathology. The canine chemokine immune response to influenza virus is different than human responses and explains the severity of clinical signs that can occur, even in the absence of secondary bacterial infection. The presence of CIV virulence factors that are identical to those in the most severe influenzas increases the risk of severe disease in dogs. These factors, combined with the large number of influenza reassortants identified over the last decade, generates a sense of urgency to prevent the emergence of canine to human zoonotic influenza. Widespread vaccination against CIV to prevent transmission and clinical disease is an important place to start.

Figure 1. Influenza A virus structure. Enveloped negative sense, single-stranded RNA virus containing 8 gene segments encoding for structural and non-structural proteins.
Figure 2. Innate and adaptive immune responses to CIV infection. Viral antigen presentation through endogenous pathway MHC I stimulates CD8+ cytotoxic T cells (CTL). Antigen presentation through exogenous pathway MHC II stimulates CD4+ T cell differentiation to T helper 1 (Th1) and T helper 2 (Th2) cells. Th1 cytokine expression enhances CD8+ activity. Th2 cytokine expression enhances B cell antibody production. Natural killer cell antibody dependent cell mediated cytotoxicity (ADCC) assists in viral clearance. CIV induces chemokine IL-8 and MCP-1 recruiting neutrophils and monocytes to the lung.

Figure 3. Recognition of viral RNA by cytosolic pathogen recognition receptor retinoic acid-inducible gene I (RIG-I), induces conformational change exposing a critical domain and with mitochondrial antiviral signaling proteins (MAVS) activates downstream signaling, ultimately activating transcription factors NF-κB/IRF inducing IFN genes resulting in Type I IFN production and inducing genes for pro-inflammatory cytokines. Canine influenza virus nonstructural protein NS1 blocks activation of NF-κB/IRF.
References:


