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Research Article

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Efficacy of Canine Influenza Virus (H3N8) Vaccine to Reduce Pulmonary Lesions after Co-challenge with Canine Influenza Virus and *Streptococcus equi* subsp. *zooepidemicus*

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Abstract

To examine the effectiveness of canine influenza vaccination to reduce pulmonary lesions in dogs co-challenged with CIV and *S. equi* subsp. *zooepidemicus*. In this study, four groups of 7- to 10-week-old, CIV-naive beagles (32 dogs total) were used. One group was vaccinated with CIV vaccine (group 4) and the remaining 3 groups (groups 1, 2 and 3) were not vaccinated. Group 1was challenged with CIV, group 2 was challenged with *S. equi* subsp. *zooepidemicus* and groups 3 and 4 were co-challenged with CIV and *S. equi* subsp. *zooepidemicus*. Following challenge, histopathological lung lesion scores were most severe in non-vaccinated dogs co-challenged with CIV combined with *S. equi* subsp. *zooepidemicus*. Dogs challenged with CIV alone had mild to severe histopathological lung lesion scores and dogs challenged with *S. equi* subsp. *zooepidemicus* alone developed little or no histopathological lung lesion scores. Histopathological lung lesion scores were markedly diminished in dogs vaccinated with CIV vaccine and then co-challenged. The findings confirm the results of previous studies indicating that CIV can cause respiratory disease, leading to severe bronchointerstitial pneumonia and the disease is exacerbated following secondary bacterial co-infection. Importantly, vaccination of dogs with CIV vaccine significantly reduces the severity of disease caused by this viral-bacterial co-infection.

Keywords

Canine influenza vaccine; Influenza virus; *Streptococcus equi* subsp. *zooepidemicus*

Abbreviations

CIRD: Canine Infectious Respiratory Disease; CIV: Canine Influenza Virus

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Introduction

Influenza A viruses infect and cause disease in a number of mammals and birds but with varying degrees of host adaptation [1]. In 2004, a group of racing greyhounds in Florida developed respiratory disease [2]. Some dogs died peracutely and postmortem examination revealed hemorrhages in the respiratory tract, mediastinum, and pleural cavity. Histological examination revealed tracheitis, bronchitis, bronchiolitis, and suppurative bronchopneumonia. Other affected greyhounds had a milder form of respiratory disease characterized by fever and cough and subsequently recovered. The influenza virus isolated, A/Canine/Florida/43/2004, is closely related to contemporary equine influenza A virus (H3N8) [3]. Since the isolation of the canine influenza virus (CIV) from racing greyhounds in 2004, CIV has been confirmed in pet dogs in a number of states [2-4]. Experimental challenge of dogs with CIV has induced clinical signs varying from none to severe and lung lesions consistent with influenza [5,6]. In one study, vaccination with a commercially available CIV (H3N8) vaccine resulted in reduced clinical signs and duration of viral shedding [7]. Recently, in South Korea, an avian lineage CIV (H3N2) emerged, which caused respiratory disease in naturally and experimentally infected dogs [8]. These studies confirm that influenza A viruses can infect dogs and can be transmitted from dog to dog, causing disease. CIV (H3N8) has become enzootic in the US canine population [9], whereas H3N2 has not been shown to be present in dogs in the United States.

Streptococcus equi subsp. *zooepidemicus* is associated with increased severity of respiratory disease in kenneled and sheltered dogs affected by endemic canine infectious respiratory disease (CIRD) complex [10]. Recently, *S. equi* subsp. *zooepidemicus* has been identified as a cause of disease in shelter dogs that died of a fibrinosuppurative pneumonia and hemothorax [11]. A retrospective study of a streptococcal infection in dogs found that pulmonary streptococcal lesions can present as a bronchopneumonia or a hemorrhagic form [12]. *Streptococcus equi* subsp. *zooepidemicus* is an important pathogen in canine respiratory disease. However, the ability of this bacterium to act as a primary pathogen in dogs is in question.

Influenza virus infection predisposes the animals to secondary bacterial infection resulting in increased severity of respiratory disease morbidity and mortality in many species. For example, during the 1918 influenza pandemic, secondary bacterial infection of the lungs was present in most fatal cases of the Spanish flu (H1N1) that killed 40 to 50 million people worldwide [13]. In the recent pandemic (pH1N1), severity of disease was increased in patients co-infected with *S. pneumonia* [14].

Although the interspecies transfer of mammalian influenza viruses is a relatively rare event, the establishment of CIV in the pet and racing dog populations and the ability of influenza infection to increase secondary bacterial disease warrants the examination of a CIV vaccine to decrease the incidence of influenza-associated disease. An earlier study showed that the commercially available CIV (H3N8) vaccine reduced disease severity not only when dogs were challenged with CIV alone, but, also reduced severity of disease and shedding

of both CIV and *S. equi* subsp. *zooepidemicus* following co-challenge [15]. In this study, we examined histopathologic changes in lung tissue in non-vaccinated dogs following CIV infection alone, *S. equi* subsp. *zooepidemicus* infection alone, and co-infection with CIV and *S. equi* subsp. *zooepidemicus*. We also examined histopathologic changes in lung tissue of dogs that were vaccinated with commercially available CIV vaccine and challenged with CIV and *S. equi* subsp. *zooepidemicus*.

Materials and Methods

Animals

Thirty two male and female 7 to 10 week-old beagle dogs that tested seronegative for CIV were used in this study. All dogs were housed in separate rooms by group at biosafety level 2 in the isolation unit of the University of Wisconsin, School of Veterinary Medicine, Charmany Instructional Facility, which is an IALAAC-accredited facility. Standard husbandry was practiced, and food and water were available ad libitum. All dogs used in this study were cared for in accordance with the guidelines of the Institutional Animal Care and Use Committee.

Experimental design

A summary of the experimental design is shown in Table 1. Animals were blocked by litter and randomly assigned into 4 treatment groups: group 1 was non-vaccinated, challenged with CIV alone, group 2 was non-vaccinated challenged with *S. equi* subsp. *zooepidemicus* alone, group 3 was non-vaccinated challenged with both CIV and *S. equi* subsp. *zooepidemicus* and group 4 was vaccinated with CIV (H3N8) vaccine and then challenged with both CIV and *S. equi* subsp. *zooepidemicus*. Dogs in group 4 were vaccinated subcutaneously with 1 ml of inactivated CIV H3N8 vaccine containing an aluminum-based adjuvant (Intervet/Schering-Plough Animal Health) on study days 0 and 21. The CIV challenge virus (A/Canine/Florida/14/2006 (H3N8)) used in this study was previously shown to be virulent in susceptible dogs [5,6]. The challenge was prepared to contain 7.5 $\log_{10}$ 50% tissue culture infective dose per dose. All dogs in groups 1, 3, and 4 were challenged with CIV intranasally via aerosolization on study day 35. Two isolates of *S. equi* subsp. *zooepidemicus* were used to challenge dogs and these isolates were obtained from dogs in animal shelters that died of severe CIRD complex. These two cultures were mixed in equal volumes, cultured for 24 hours, and adjusted to contain 1×10^9 CFU/ml. The dogs were challenged using an intranasal cannula, 0.5 ml of bacterial culture was instilled into each nostril of all dogs in groups 2, 3, and 4. After challenge administration, one dog in each of groups 1 and group 3 was euthanized at 8 and 9 days post-CIV challenge, due to the severity of clinical signs. Histopathologic examination was performed on a total of 30 dogs (5, 6, 9, and 10 animals in groups 1 to 4, respectively).

Table 1: Summary of the experimental groups and the study days that vaccination and challenge were administered.

Treatment Group	N	CIV[a] Vaccine (Prime)	CIV Vaccine (Boost)	CIV Challenge	*Streptococcus equi* subsp. *zooepidemicus* Challenge
1	5 (6)[b]	---	---	35	---
2	6	---	---	---	38
3	9 (10)[b]	---	---	35	38
4	10	0	21	35	38

[a]CIV: Canine Influenza Virus

[b]One dog each in group 1 and group 3 were removed from the study after challenge administration and euthanized due to the severity of clinical signs

Table 2: Scoring system used to grade histopathological lesions.

Score	Airway Epithelial Changes	Airway Inflammation	Peribronchiolar Cuffing	Interstitial Pneumonia
0	No Change	No Lesions	None	None
1	1-25%	1-25%	Mild loosely formed	Mild, focal or multifocal
2	26-50%	26-50%	Moderate well formed	Moderate, locally extensive
3	21-75%	21-75%	Prominent thick cuff	Moderate, multifocal to coalescing
4	76-100%	76-100%	----	Severe, coalescing to diffuse

Necropsy and histopathology

One specimen of lung tissue with representative lesions from each dog was fixed by immersion in 10% neutral buffered formalin, routinely processed, and embedded in paraffin wax. Sections were made at 5 µm and routinely processed and stained with hematoxylin and eosin. Pulmonary histopathology lesions were scored by 2 board-certified pathologists (JNH and MEP) who did not have knowledge of animal treatment group assignments; scores from the second board-certified pathologist (MEP) confirmed original scores from the first board-certified pathologist (JNH). Airway lesions were scored on percentage of airways in a section with epithelial changes and inflammation. Epithelial changes included loss of cilia and flattening, necrosis and proliferation of epithelium. Peribronchiolar cuffing and degree of interstitial pneumonia were also scored. Scales for scoring lesions are summarized in Table 2. A total score was determined for all dogs and then the mean was calculated and used in the comparison of severity of lesions across the groups.

Statistical analysis

Mean histopathological lung scores among different treatment groups were compared using one-way analysis of variance. Pairwise comparisons were made by using Wilcoxon exact rank sum tests, and $P<0.05$ was considered significant. Scores by the second board-certified pathologist were compared to the first board-certified pathologist by Spearman correlation. Statistical analyses were performed using SAS version 9.2.[a]

Results

Post challenge clinical observations and necropsy

Clinical observations, including viral and bacterial shedding, and necropsy findings have been described in detail by Larson et al. [15].

Histopathology

Histological examination revealed that eighty percent of all dogs showed some degree of interstitial pneumonia and pulmonary airway changes.

Group 1 - non-vaccinated CIV challenge group (n=5): Histopathological examination revealed varying degrees of interstitial pneumonia and bronchiolar changes in these dogs. Three dogs showed mild lesions, means of 0.5, 0.75, 1.5 and two with more severe lesions, scoring 3.5 and 3.75, giving a mean score for the group of 2. In

the 2 higher-scoring dogs a more severe pneumonia with the majority of bronchioles showing suppurative bronchitis and bronchiolitis (Figure 1) with epithelial proliferation and rare degeneration and necrosis. Histiocytes and type II pneumocyte hyperplasia expanded alveolar septa and alveolar lumina contained neutrophils and alveolar macrophages (Figure 2) were seen in the two dogs with higher scores. Lymphocytic and plasma cell cuffing of bronchi, bronchioles, and pulmonary vessels were observed in these animals and it was also present surrounding submucosal glands. One dog with mild lesions had some lymphocytic bronchial and bronchiolar cuffing. In addition, 4 dogs had a single to a few small-to moderate-sized areas of intra-alveolar hemorrhage.

Group 2 - non-vaccinated, *S. equi* subsp. *zooepidemicus* challenge group (*n*=6): Histopathological examination revealed that three dogs showed mild lesions scored as 0.25, 0.5 and 2.0 with 3 remaining dogs with no observed lesions, giving a mean score for the group of 0.5. The 2 lowest-scoring dogs had small numbers of neutrophils in a few bronchi and bronchioles. The dog with a mean score of 2.0 had multifocal areas of suppurative bronchopneumonia with moderate numbers of neutrophils in bronchioles and alveolar lumina (Figure 3). Alveolar lumina often contained edema and mild hemorrhage; alveolar septa were occasionally thickened by edema and fibrin. No bacterial colonies were seen. Two dogs had mild to moderate multifocal interstitial hemorrhage and 1 dog had a moderate-sized area of extensive hemorrhage. In one animal, the tunica adventitia of occasional vessels was expanded by edema and hemorrhage.

Group 3 - non-vaccinated, co-challenged with CIV and *S. equi* subsp. *zooepidemicus* (*n*=9): Histopathological examination revealed that all dogs had severe pneumonia with mean scores between 2.5 and 3.5, with 6 animals scoring greater than 3.0. Sections were diffusely consolidated with severe bronchointerstitial pneumonia with a large number of neutrophils and macrophages in airways and alveolar lumina (Figures 4 and 5). There was hyperplasia (Figure 4) of the bronchiolar epithelium and rare necrosis (Figure 4), with prominent neutrophil exocytosis. In addition, bronchioles had prominent lymphocytic cuffing (Figure 4), and all showed multifocal to diffuse perivascular cuffs of lymphocytes and plasma cells. In all dogs, peribronchial submucosal glands were often surrounded by a moderate number of lymphocytes and plasma cells. Occasional suppurative adenitis was observed. The interstitium was thickened by histiocytes and type II pneumocyte hyperplasia with alveolar macrophages and neutrophils present in alveolar lumina (Figure 5) in all sections. Four dogs had mild to moderate multifocal hemorrhage.

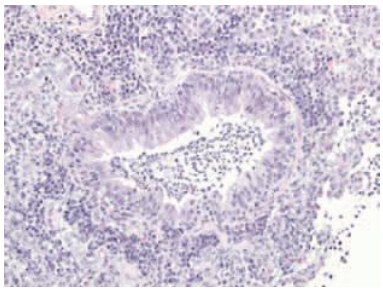

Figure 1: Lung; dog challenged with CIV (group 1). Bronchiolar lumen contains neutrophils, sloughed epithelial cells and macrophages and leukocytes are present in the bronchiole epithelium. The bronchiole is surrounded by lymphocytes and plasma cells and the adjacent alveoli infiltrated by neutrophils and histiocytes with increased alveolar macrophages. Type II pneumocyte hyperplasia is present in adjacent alveolar septa. H & E stain.

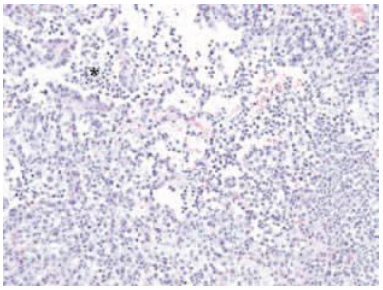

Figure 2: Lung; dog challenged with CIV (group 1). A bronchointerstitial pneumonia with alveolar septa thickened by histiocytes and lymphocytes and alveolar lumina infiltrated by neutrophils and alveolar macrophages are present. Type II pneumocyte hyperplasia is present. A respiratory bronchiole (asterisk) contains neutrophils and macrophages. Mild hemorrhage is present in an alveolar lumen. H & E stain.

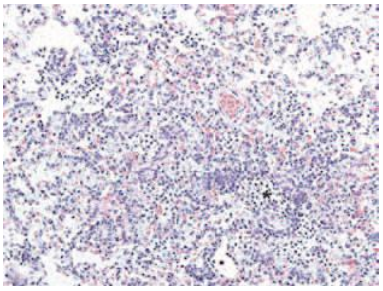

Figure 3: Lung; dog challenged with *Streptococcus equi* subsp. *zooepidemicus* (group 2). A focal suppurative bronchopneumonia characterized by neutrophils present in a respiratory bronchiole (asterisk) and alveolar lumina is present. Alveolar macrophages are increased. Edema and congestion are present in alveolar septa and there is mild hemorrhage in alveolar lumina. H & E stain.

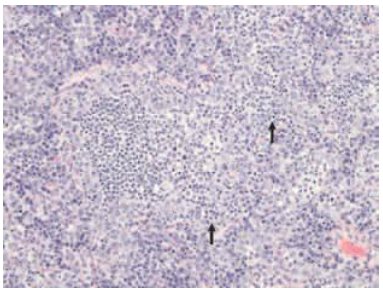

Figure 4: Lung; dog co-challenged with CIV and *Streptococcus equi* subsp. *zooepidemicus* (group 3). There is discontinuity of the bronchiole epithelium due to loss of epithelium (arrows). The bronchiole is filled with neutrophils and macrophages. Lymphocytes and plasma cells cuff the bronchiole and vessel. H & E stain.

Group 4 - vaccinated, co-challenged with CIV and *S. equi* subsp. *zooepidemicus* (*n*=10): Histopathological examination revealed that three dogs had no lesions, 5 dogs had a mean score ranging from 0.25-0.75 and 2 dogs had a mean score of 1.25. Dogs

with mild lesions occasionally had small numbers of neutrophils in occasional bronchi and bronchioles with increased numbers of lymphocytes and plasma cells surrounding bronchial glands. Small focal areas of pneumonia composed of neutrophils, histiocytes and lymphocytes in alveolar septa and lumina were present. Some blood vessels were cuffed by lymphocytes and plasma cells. One dog had moderate multifocal suppurative bronchointerstitial pneumonia with neutrophils, edema and hemorrhage in airways and alveolar lumina (Figure 6). In these areas, alveolar macrophages were increased and histiocytes were present in the septa. Blood vessels were cuffed with lymphocytes and plasma cells. However, airways were not cuffed by lymphocytes and plasma cells. In one animal, with a mean score of 1.25, a multifocal suppurative and histiocytic bronchointerstitial pneumonia was observed. Alveolar septa were thickened by histiocytes and lymphocyte and plasma cells cuffing of airways and vessels was evident. Neutrophils were present in bronchiolar and alveolar lumina.

The mean histopathology scores were significantly different between group 1 and group 4 (P=0.038) and between group 3 and group 4 (P<0.0001); however, differences were not significant when comparing group 2 and group 4 (Figure 7). There was a strong correlation (r=0.92 by Spearman correlation) between the scoring done by the 2 pathologists. Major histopathology findings are summarized in Table 3.

Discussion

Since CIV was first documented in 2004, it has become enzootic in the US canine population, causing respiratory disease that is typically mild but occasionally may be severe [2-4]. In addition, another pathogen, *S. equi* subsp. *zooepidemicus*, has been recently suggested as a primary cause of severe respiratory disease in dogs in kennels and shelters [11]. In this study, we examined the ability of CIV and *S. equi* subsp. *zooepidemicus* to cause disease both as single, primary pathogens and together as a co-infection. Importantly, we also examined the effect of previous CIV vaccination in dogs co-challenged with CIV and *S. equi* subsp. *zooepidemicus*. Most animals in all treatment groups had varying degrees of respiratory disease and interstitial pneumonia. The dogs co-challenged with CIV and *S. equi* subsp. *zooepidemicus* had the most severe disease, which was characterized by severe bronchointerstitial pneumonia.

Influenza A infection of the respiratory tract targets the respiratory epithelium, leading to tracheitis, bronchitis, and bronchiolitis and infection can extend into the interstitium causing interstitial pneumonia [16]. In our study, 3 dogs had mild lesions and 2 dogs had severe pneumonia. The dogs with mild pulmonary lesions are likely similar to dogs naturally infected with CIV that exhibited mild clinical disease and subsequently recovered [3]. At the time necropsy took place in our study (day 10 post-challenge), lesions were beginning

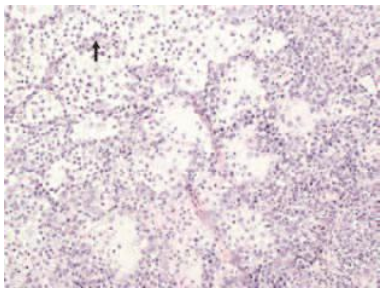

Figure 5: Lung; dog co-challenged with CIV and *Streptococcus equi* subsp. *zooepidemicus* (group 3). Alveolar septa are infiltrated by histiocytes and there is type II pneumocyte hyperplasia (arrow). Alveolar lumina are filled with edema, neutrophils and alveolar macrophages. H & E stain.

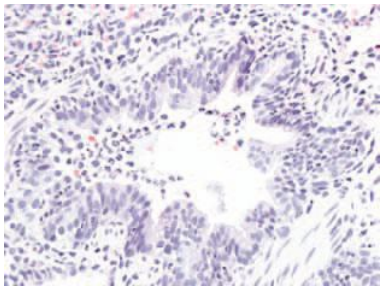

Figure 6: Lung; dog vaccinated with CIV prior to co-challenge with CIV and *Streptococcus equi* subsp. *zooepidemicus* (group 4). Suppurative bronchiolitis; the lumen is filled with neutrophils and intraepithelial neutrophils are common. H & E stain

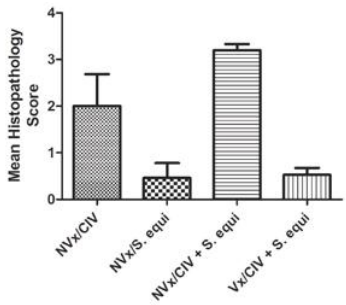

Figure 7: Mean histopathology scores for pneumonia in the 4 experimental groups. Mean histopathology scores were statistically significant between group 3 and group 4 (P<0.0001; asterisks) and between group 1 and 4 (P=0.038). NVx: Non-Vaccinated; Vx: Vaccinated; CIV: Canine Influenza Virus.

Table 3: Histopathology lesions detected in lung of four groups of dogs challenged with Canine Influenza virus (CIV) and/or *Streptococcus equi* subsp. *Zooepidemicus*.

CIV only	S. equi only	CIV + S. equi	Vx CIV/CIV + S. equi
Bronchointerstitial pneumonia	Suppurative bronchopneumonia	Diffuse bronchointerstitial pneumonia	Multifocal bronchointerstitial pneumonia
Suppurative bronchitis and bronchiolitis	Mild to moderate hemorrhage	Diffuse suppurative bronchitis and bronchiolitis	Multifocal suppurative bronchitis and bronchiolitis
Type II pneumocyte hyperplasia		Type II pneumocyte hyperplasia	
Lymphocytic and plasmacytic airway cuffing		Lymphocytic and plasmacytic airway cuffing	
Bronchiole epithelial proliferation		Bronchiole epithelial proliferation	
		Mild to moderate hemorrhage	

to resolve and were likely more severe at an earlier time point. In a separate experimental study, lesions at 4 to 6 days post-infection were described as consisting of tracheitis, bronchitis, and thickening of alveolar septa with mild hyperplasia of type II pneumocytes [17]. Macrophages were the predominant inflammatory cell type [17], which is similar to the findings in our study. Severe suppurative bronchopneumonia was present in most naturally infected racing greyhounds [3], which is similar to the 2 dogs in this study infected with CIV alone that developed severe pneumonia. *Streptococcus equi* subsp. *zooepidemicus* is a respiratory pathogen that is believed by some to cause severe disease and mortality [11]. However, *S. equi* subsp. *zooepidemicus* was not shown to be a primary pathogen in this controlled experimental study and in previous unpublished studies. Three dogs inoculated with *S. equi* subsp. *Zooepidemicus* in our study had lesions indicative of mild suppurative bronchopneumonia and differed from those reported in a natural infection in shelter dogs with respiratory disease caused by *S. equi* subsp. *zooepidemicus*, which had lesions of severe, acute, fibrinosuppurative bronchopneumonia with numerous bacteria and often hemothorax [11]. A retrospective study found that clinical streptococcal infection in dogs was most often associated with bronchopneumonia with or without hemorrhage [12], which is similar to this study.

Influenza viruses interfere with the immune system, resulting in impaired innate physical and cellular respiratory barriers allowing for the proliferation of secondary invaders, such as in humans during the 1918 Spanish flu pandemic [14]. In the present study, all dogs in the group infected with CIV and later challenged with *S. equi* subsp. *zooepidemicus* developed severe bronchointerstitial pneumonia. These findings are consistent with those of studies of various species examining the synergism between influenza and bacteria, which all describe exacerbated respiratory disease with co-pathogen involvement [14,18-20]. In addition, in natural CIV infection in greyhounds, bacterial co-pathogens were common and identified by special stains or culture. *S. equi* subsp. *zooepidemicus* was the most commonly isolated co-pathogen in natural CIV infection, confirming its importance as part of the CIRD complex as it occurred in greyhounds [17].

Vaccination for influenza is a common practice in many species to reduce the severity of disease. In our study, dogs in group 4, which were vaccinated for CIV before challenge with CIV and *S. equi* subsp. *zooepidemicus*, had a significant reduction in lesions as compared with non-vaccinated dogs in group 3, which were also co-challenged with CIV and *S. equi* subsp. *zooepidemicus*. Vaccination for CIV suppressed viral replication and, thus infection, resulting in normal to minimally reduced pulmonary defense mechanisms [15]. Therefore, adequate immune responses controlled *S. equi* subsp. *zooepidemicus* infection as shown by reduced bacterial shedding [20].

In conclusion, we examined the pathological changes associated with CIV and *S. equi* subsp. *zooepidemicus* as solitary pathogens or as co-pathogens in the development of respiratory disease in experimentally infected dogs. Severe suppurative and histiocytic bronchointerstitial pneumonia with epithelial changes in the airway was present in dogs infected with both CIV and *S. equi* subsp. *zooepidemicus* (group 3), which is consistent with synergism between the 2 pathogens. Significant reduction in disease and lesions was seen in dogs vaccinated for CIV prior to challenge with CIV and *S. equi* subsp. *zooepidemicus* (group 4). Results of this study are consistent with previous reports of natural and experimental infections describing CIV as an important pathogen in CIRD complex. The present study also confirms that, like other influenza infections, CIV replication can result in impaired pulmonary defenses and the development of significant disease associated with bacterial co-pathogens, such as *S. equi* subsp. *zooepidemicus* in this study. The spread of CIV across the United States warrants the consideration of vaccination for CIV, especially in dogs housed in groups (e.g., kennels, doggie daycare) or involved in racing or showing. Any dog that is housed for hours to days with other dogs that are potentially infected with CIV should receive two doses of CIV H3N8 vaccine (2–4 weeks apart); these high-risk environments should be avoided until at least 7 days after the second dose of vaccine is administered.

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Conflicts of Interest

The authors declare no conflicts of interest with respect to the research, authorship, and/or the publication of this article.

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