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Efficacy of the Canine Influenza Virus H3N8 Vaccine To Decrease Severity of Clinical Disease after Cochallenge with Canine Influenza Virus and *Streptococcus equi* subsp. *zooepidemicus*▽

Laurie J. Larson,[1] Jamie Henningson,[1] Patricia Sharp,[1] Bliss Thiel,[1] Muralidhar S. Deshpande,[2] Tamara Davis,[2] Huchappa Jayappa,[2] Terri Wasmoen,[2] Nallakannu Lakshmanan,[2]* and Ronald D. Schultz[1]

Department of Pathobiological Sciences, School of Veterinary Medicine, University of Wisconsin—Madison, 2015 Linden Drive West, Madison, Wisconsin 53706,[1] and Intervet/Schering-Plough Animal Health, 21401 West Center Road, Elkhorn, Nebraska 68022[2]

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Since first emerging in the North American canine population in 2004, canine influenza virus (CIV) subtype H3N8 has shown horizontal transmission among dogs, with a high level of adaptation to this species. The severity of disease is variable, and coinfection by other respiratory pathogens is an important factor in the degree of morbidity and mortality. The first influenza vaccine for dogs, an inactivated vaccine containing CIV subtype H3N8, was conditionally approved by the U.S. Department of Agriculture (USDA) for licensure in May 2009 and fully licensed in June 2010. This study evaluates the efficacy of this vaccine to reduce the severity of illness in dogs cochallenged with virulent CIV and *Streptococcus equi* subsp. *zooepidemicus*.

Canine influenza virus (CIV) was first observed in racing greyhounds in Florida in 2004. These dogs exhibited signs of respiratory disease, ranging from fever and cough to peracute death due to hemorrhagic pneumonia. The virus isolate, A/Canine/Florida/43/2004, shared >96% sequence identity with contemporary equine influenza A virus (H3N8) (5). Sustained dog-to-dog transmission of the virus has led to the widespread distribution of CIV in the United States (13). Dogs housed in groups or exposed to transient populations of other dogs, such as dogs at the racetrack, shelter, or dog daycare environments, are at the greatest risk of exposure to CIV. These dogs are also at great risk of exposure to other respiratory pathogens, such as *Streptococcus equi* subsp. *zooepidemicus*, which has been isolated from shelter dogs with acute fatal hemorrhagic pneumonia (12, 14). A vaccine against CIV (H3N8) has been developed and was licensed in 2009 (Intervet/Schering-Plough Animal Health, Elkhorn, NE) (7). In the present study, coinfection with both CIV and *S. equi* subsp. *zooepidemicus* did lead to higher morbidity in canine infectious respiratory disease (CIRD) complex caused by these pathogens. On the basis of comparative clinical signs and pathology, vaccination with canine influenza H3N8 provided protection from disease in the face of dual challenges.

MATERIALS AND METHODS

Animals. Approval from the institutional animal care and use committee was obtained before the study began. Thirty-two male and female dogs aged 7 to 10 weeks and seronegative for CIV were used in this study. Animals were grouped by litter and randomly assigned to 1 of 4 treatment groups. Group 1 contained 6 unvaccinated dogs to be challenged with CIV alone. Group 2 contained 6 unvaccinated dogs to be challenged with *S. equi* subsp. *zooepidemicus* alone. Group 3 contained 10 unvaccinated dogs to be challenged with both CIV and *S. equi* subsp. *zooepidemicus*. Group 4 contained 10 dogs that were vaccinated against CIV and then challenged with both CIV and *S. equi* subsp. *zooepidemicus*. 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 AAALAC-accredited facility. Standard animal husbandry was practiced, and food and water were available *ad libitum*.

Vaccination. One group of 10 dogs (group 4) was vaccinated subcutaneously with 1 ml of inactivated H3N8 CIV vaccine containing an aluminum-based adjuvant (Intervet/Schering-Plough Animal Health) on study days 0 and 21. There was no placebo group in this study; however, the remaining 22 dogs were kept unvaccinated throughout the study.

Samples. Blood samples for serology were collected from all dogs on study days 0, 34, and 49. Nasal swabs for bacterial isolation were collected in tryptose phosphate broth from dogs in groups 2, 3, and 4 on study days 34, 38, 41, 44, 47, and 49. Nasal swabs for viral isolation were collected in Dulbecco's modified Eagle medium containing gentamicin and amphotericin B from dogs in groups 1, 3, and 4 on study day 34 and then daily on study days 36 to 45. Swabs for bacterial isolation were always collected prior to swabs for viral isolation on the days when they coincided. On study day 49, all dogs were humanely euthanized, lungs were scored for consolidation (pneumonia), and samples of lung tissue were collected into formalin for histopathologic examination. Sterile saline was infused into large bronchi and collected for bacterial isolation at this time as well. Individuals handling samples and scoring lung lesions were not aware of the study groups.

Viral challenge. Canine influenza virus isolate A/Canine/Florida/14/2006 (H3N8) previously shown to be virulent in susceptible dogs (6, 11) was prepared to contain 7.5 $\log_{10}$ 50% tissue culture infective dose ($TCID_{50}$) per dose. All dogs in groups 1, 3, and 4 were challenged with virus given by aerosol on study day 35.

Bacterial challenge. Two isolates of *S. equi* subsp. *zooepidemicus* were obtained from dogs that died of severe CIRD complex in humane animal shelters in Nevada and Wisconsin. Primary culture was made on blood agar plates and typed. The resulting colonies were frozen at −80°C for storage. Before inoculation into dogs, these isolates were restreaked onto blood agar to ensure purity and viability. The resulting *S. equi* subsp. *zooepidemicus* colonies were mixed in equal volumes, cultured for 24 h, and prepared to contain 1×10^9 CFU per ml. Using an intranasal cannula, 0.5 ml of bacterial culture was instilled into each nostril of all dogs in groups 2, 3, and 4 on study day 38, which was 3 days after the CIV challenge.

* Corresponding author. Mailing address: Intervet/Schering-Plough Animal Health, 29160 Intervet Lane, P.O. Box 318, Millsboro, DE 19966. Phone: (302) 934-4222. Fax: (302) 934-4204. E-mail: nallakannu.lakshmanan@sp.intervet.com.

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TABLE 1. Seroconversion in four groups of dogs

Treatment group	CIV vaccination[a]	Challenge	HI antibody titer (GMT)[b]		
			Prevaccination	Prechallenge	14 days postchallenge
1	No	CIV	<10	<10	226 ± 200
2	No	*S. equi* subsp. *zooepidemicus*	<10	<10	<10
3	No	CIV + *S. equi* subsp. *zooepidemicus*	<10	<10	278 ± 339
4	Yes	CIV + *S. equi* subsp. *zooepidemicus*	<10	73 ± 65	6,378 ± 3,032

[a] CIV, canine influenza virus.
[b] The hemagglutination inhibition (HI) antibody titer (geometric mean titer [GMT]) is shown. The mean ± standard deviation are shown for some values.

Clinical observations. Beginning on study day 33 and continuing until study day 49, all dogs were observed daily for clinical signs of respiratory disease and abnormal body temperature by a veterinarian who was not aware of treatment group assignments. Each clinical sign was assigned a score based on severity. Ocular and nasal discharge was scored as follows: 0 for no discharge, 0.5 for serous discharge, 1.0 for mild mucopurulent discharge, and 2.0 for severe mucopurulent discharge. Coughs was scored as follows: 0 for no cough; 0.5 for mild cough; 1.0 for moderate, persistent cough; and 2.0 for severe cough accompanied by choking or retching sounds. Sneezing, dyspnea, and depression were scored as follows: 0 for absent and 2 for present. Body temperature was scored as follows: 0 for a temperature of <39.5°C and 2 for a temperature of ≥39.5°C.

Sample processing. Serum samples collected on study days 0, 34, and 49 were tested for antibodies against CIV by hemagglutination inhibition (HI) assay as described previously (11). Live CIV titers in nasal swabs and challenge material were determined by virus isolation and titration on Madin-Darby canine kidney (MDCK) cells as described previously (11). Bacteria were isolated from nasal swabs and lung washes via plating on blood agar, and identification to the species level was done by Gram staining and metabolic assays (16).

Pathology. On study day 49, all remaining dogs were euthanized, and lungs were evaluated for gross lesions. Each lung lobe was scored for percent consolidation, and weighted lung lesion scores and total score for each dog were calculated as described previously (6, 7). Fresh lung tissues and lung lavage samples were collected for bacterial isolation, and lung tissues in buffered formalin were collected for histopathology.

Statistical analysis. Median lung scores, summed clinical scores, area under the curve for virus shedding, the number of days of virus shedding, and the number of days of bacterial shedding were compared between treatment groups by Wilcoxon rank sum tests. Statistical analysis was performed using SAS version 9.1.3 (SAS Institute, Cary, NC). P values of <0.05 were declared statistically significant.

RESULTS

Serology. All vaccinated dogs in group 4 seroconverted to CIV following vaccination (Table 1). The hemagglutination inhibition (HI) antibody titers ranged from 10 to 160, with a geometric mean titer (GMT) of 73 just before challenge. The majority (80%) of vaccinates developed an HI titer of 40 or greater (data not shown). All nonvaccinated dogs in groups 1, 2, and 3 were CIV seronegative at the time of CIV challenge (HI titer of <10). Following challenge, antibody titers in vaccinated dogs increased significantly (GMT > 6,378) compared with unvaccinated cohorts, demonstrating the efficacy of the vaccine in priming the immune system against virulent CIV. The minimum HI antibody titer after challenge was 2,560 in vaccinated dogs (data not shown). Nonvaccinated dogs challenged with CIV in groups 1 and 3 seroconverted following CIV challenge, with a GMT of 226 and 278, respectively. All dogs in group 2 remained seronegative for CIV at the time of necropsy, confirming that they were not exposed to CIV and biosecurity procedures were efficient.

Clinical signs. All dogs were monitored and scored for body temperature and clinical signs of respiratory disease. In group 1 (CIV alone), 2 of the 6 dogs developed clinical fever (≥39.5°C) for only 1 day. This was in contrast to previous experimental challenge studies (6, 7), where none of the dogs challenged with CIV developed fever. One of the 2 dogs also developed severe depression. In group 2 (*S. equi* subsp. *zooepidemicus* alone), 1 of the 6 dogs developed fever for only 1 day. In group 3 challenged with both CIV and *S. equi* subsp. *zooepidemicus*, 90% (9 of 10) of the dogs developed fever and a majority (70%) exhibited fever for at least 2 days. Fifty percent (5 of 10) of the vaccinated dogs (group 4) also developed fever following dual challenge; however, the fever lasted for only 1 day.

All dogs challenged with CIV and CIV plus *S. equi* subsp. *zooepidemicus* (groups 1, 3, and 4) exhibited a range of clinical signs starting from 2 days postchallenge (Fig. 1). Coughing and dyspnea were the predominant clinical signs. All dogs in group 1 (6 dogs) and group 3 (10 dogs) exhibited various degrees of coughing, which lasted an average of 2.7 days and 4.5 days, respectively. On the other hand, only 60% (6 of 10) of the vaccinated dogs in group 4 exhibited a mild cough, which lasted an average of 1.3 days. One dog in group 1 was euthanized at 9 days postchallenge due to respiratory distress and pneumonia. One dog in group 3 died at 8 days postchallenge due to severe pneumonia. None of the 6 dogs challenged with *S. equi* subsp. *zooepidemicus* alone exhibited any clinical signs. Dogs challenged with CIV plus *S. equi* subsp. *zooepidemicus* showed significantly higher clinical scores than dogs challenged with CIV alone (median score of 17.8 versus 7.0, respectively; $P = 0.012$). The CIV vaccine significantly reduced the clinical scores induced by CIV plus *S. equi* subsp. *zooepidemicus* (median score, 2.5; $P < 0.0001$). The results suggest that CIV causes severe respiratory disease in dogs that could lead to mortality. *S. equi* subsp. *zooepidemicus* acts as a secondary pathogen and enhances CIV-induced disease. In this study, *S. equi* subsp. *zooepidemicus* did not induce any clinical disease on its own.

Virus shedding. Nasal virus shedding was monitored in all CIV-challenged dogs (groups 1, 3, and 4) on the day before challenge and then every day from day 1 through day 10 postchallenge. The average virus titer for each group, expressed as $\log_{10}$ $TCID_{50}$/ml, was plotted against days postchallenge (Fig. 2). Fifty percent of the dogs in groups 1 and 4 and 100% of dogs in group 3 started shedding CIV in nasal secretions from day 1 postchallenge. By day 2 postchallenge, all dogs in groups 1, 3, and 4 were positive for nasal virus shedding. Viral shedding in groups 1 and 3 reached its peak between day 4 and day 5 after challenge with CIV, followed by a precipitous drop on day 6 (Fig. 2). Peak viral shedding in group 4 was on day 2 post-CIV challenge, followed by a precipitous drop by day 3.

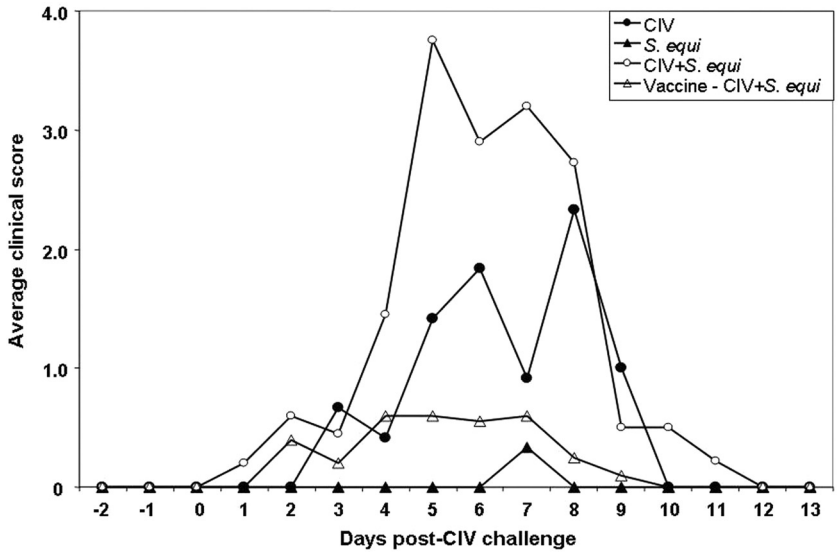

FIG. 1. Clinical disease. All dogs were challenged with CIV on study day 35 and monitored daily from day −2 through 13 days postchallenge for clinical signs. One dog in group 1 was euthanized at 9 days postchallenge, and one dog in group 3 died at 8 days postchallenge. The average clinical scores for each treatment group were plotted against time (days after CIV challenge).

Dogs in group 4 continued to shed virus at low levels until day 7 postchallenge. Both nonvaccinates and vaccinates stopped shedding CIV in their nasal secretions by day 8 postchallenge. The mean estimates for the area under the curve were significantly higher for groups 1 and 3 than for group 4 ($P = 0.0075$ and $P < 0.0001$, respectively). The mean number of days of viral shedding in groups 1 and 3 was also significantly higher than for group 4 (5.7 days and 5.8 days versus 4 days; $P = 0.0356$ and $P = 0.0033$, respectively). The results demonstrate that the CIV vaccine significantly reduced nasal virus shedding in vaccinated dogs as reported previously (7).

Bacterial shedding. All dogs in all groups were negative for *S. equi* subsp. *zooepidemicus* shedding at the time of challenge with CIV or *S. equi* subsp. *zooepidemicus* (Fig. 3). Interestingly, none of the 6 dogs in group 2 challenged with *S. equi* subsp. *zooepidemicus* alone was positive for nasal shedding of *S. equi* subsp. *zooepidemicus* at any time point after challenge. However, *S. equi* subsp. *zooepidemicus* was isolated from the lung lavage sample from 1 dog (17%) in group 2. In contrast, 100% of the dogs in group 3, challenged with both CIV plus *S. equi* subsp. *zooepidemicus*, were positive for *S. equi* subsp. *zooepidemicus* in their nasal secretions at 3 and 6 days after *S. equi* subsp. *zooepidemicus* challenge; the majority (67%) continued to shed bacteria for at least 9 days after challenge. The majority (67%) of dogs in group 3 were also positive for *S. equi* subsp. *zooepidemicus* in their lung washes (11 days after *S. equi* subsp. *zooepidemicus* challenge), suggesting that the bacteria colonized and multiplied in the lower respiratory tract. The majority (60%) of CIV-vaccinated dogs challenged with CIV plus *S. equi* subsp. *zooepidemicus* also shed *S. equi* subsp. *zooepidemicus* in their nasal secretions 3 days after *S. equi* subsp. *zooepidemicus* challenge. From postchallenge day 6 onwards, the number of dogs shedding *S. equi* subsp. *zooepidemicus* was substantially less in group 4 compared to group 3. Only 20% of the vaccinated dogs were positive for *S. equi* subsp. *zooepidemicus* in their lung lavage samples (Fig. 3).

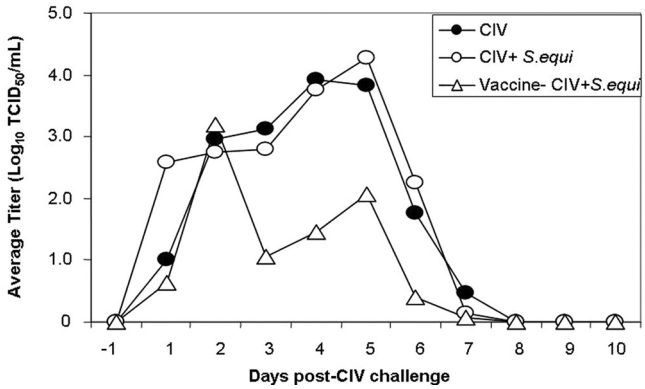

FIG. 2. Virus shedding. All dogs in groups 1, 3, and 4 were challenged with CIV on study day 35 (day 0 after CIV challenge). Nasal swabs were collected on the day before challenge (day −1) to confirm that the dogs were free of CIV shedding. Nasal virus shedding was monitored in challenged dogs daily for 10 days (days 1 to 10 postchallenge). One dog in group 1 was euthanized at 9 days postchallenge, and one dog in group 3 died at 8 days postchallenge. The average virus titers for each treatment group, expressed as $\log_{10}$ $TCID_{50}$/ml, were calculated and plotted against time (days after CIV challenge).

Lung pathology. Lung consolidation/pneumonia is the major pathological lesion in all influenza infections. All dogs (100%) in the CIV and the CIV plus *S. equi* subsp. *zooepidemicus* challenge groups (groups 1 and 3) exhibited various degrees of lung consolidation, whereas only 3 dogs (30%) in the CIV-vaccinated group (group 4) developed lung consolidation, 2 of which were very mild (Fig. 4). Only 1 dog (17%) in the group challenged with *S. equi* subsp. *zooepidemicus* alone (group 2) exhibited lung lesions, and they were mild. In group 1, lung lesions were predominantly observed in cranial lobes, whereas the lesions appeared to be present in all lung lobes in group 3. Lung lesions were characterized by hemorrhages and reddish consolidation and hepatization. Lung lesion scores in groups 1,

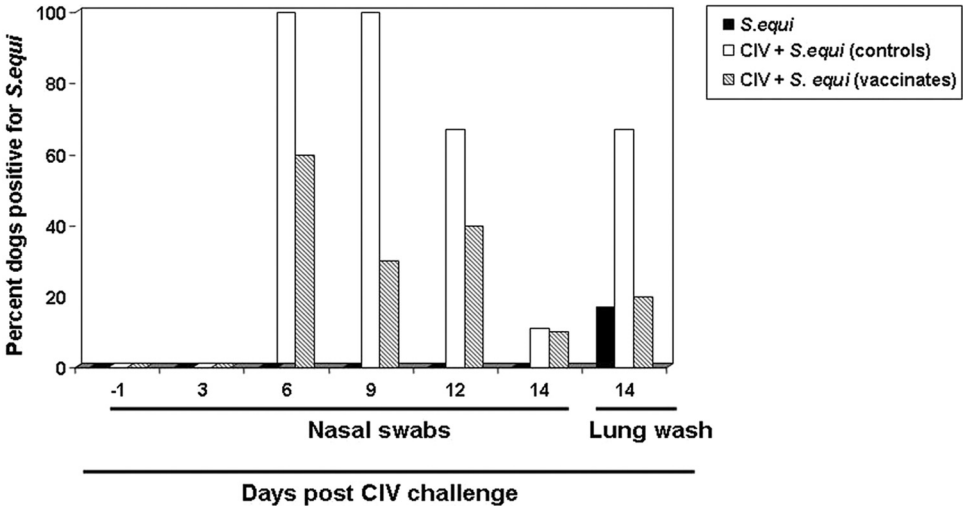

FIG. 3. Bacterial isolation from nasal swabs and lung washes. All dogs in groups 2, 3, and 4 were challenged with CIV on study day 35 (day 0) and with *S. equi* subsp. *zooepidemicus* on study day 38 (day 3 after CIV challenge). Nasal swabs for bacterial isolation were collected on the day before CIV challenge (day −1) and 3, 6, 9, 12, and 14 days postchallenge. At the time of necropsy, lung wash samples were also collected. The results are expressed as the percentage of dogs positive for *S. equi* subsp. *zooepidemicus.*

3, and 4 ranged from 0.5 to 32.97 (median score, 17.27), 5.53 to 39.74 (median lung score, 23.29), and 0 to 25.54 (median score, 0), respectively. Lung scores in group 4 were significantly lower than in group 1 ($P = 0.0019$) and group 3 ($P = 0.0002$). Although the lung scores in group 3 were higher than group 1, they were not significantly different ($P = 0.5622$). Data demonstrate that the CIV vaccine aids in the prevention of lung lesions induced by CIV alone and CIV plus *S. equi* subsp. *zooepidemicus*.

Histopathologic changes were most severe and extensive in the dual-challenged, unvaccinated group. Examples of histopathologic lesions are shown in Fig. 5. Bronchioles showed signs of necrosis and in many instances were filled with neutrophils (Fig. 5D). Alveolar septa were thickened, and alveolar lumens were filled with inflammatory cells. Signs of hemorrhage were noted. In contrast, lung sections of dual-challenged, vaccinated dogs showed mild pathology, with mild edema and slight thickening of alveolar septa in some instances (Fig. 5E). The group of dogs challenged with CIV only showed moderate pathology, including some evidence of hemorrhage and thickened alveolar septa (Fig. 5B); however, changes were generally less severe than those found in the unvaccinated dogs challenged with both CIV and *S. equi* subsp. *zooepidemicus*. Single challenge with *S. equi* subsp. *zooepidemicus* produced only mild pathological changes (Fig. 5C), if any.

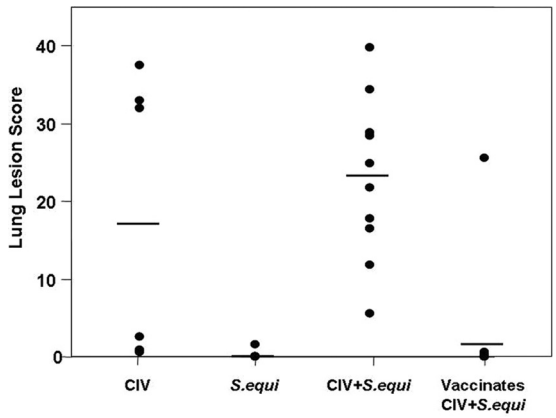

FIG. 4. Lung lesions. The gross lung lesion scores are shown. One dog in group 1 was euthanized at 9 days postchallenge, and one dog in group 3 died at 8 days postchallenge. All remaining dogs were euthanized at the end of the study (study day 49). Lung lesions were scored for consolidation, and the total lesion score for each dog was calculated.

DISCUSSION

Canine infectious respiratory disease (CIRD) complex is an important disease in dogs that is caused by a number of viral and bacterial pathogens (1, 3, 4, 8, 9). In recent years, CIV and *S. equi* subsp. *zooepidemicus* have been isolated frequently from cases of canine respiratory disease, particularly in kennels and in shelter dogs (2, 3, 5, 12, 14). This study was performed to investigate the pathogenesis of dual infection with CIV and *S. equi* subsp. *zooepidemicus* in dogs. Additionally, an inactivated-CIV H3N8 vaccine, the only U.S. Department of Agriculture (USDA)-licensed vaccine in the United States (manufactured and marketed by Intervet/Schering-Plough Animal Health), was evaluated for efficacy in reducing the severity of the disease caused by experimental challenge with CIV plus *S. equi* subsp. *zooepidemicus.*

S. equi subsp. *zooepidemicus* is a normal inhabitant of the upper respiratory tracts of many mammals (15, 17) and has been isolated from dogs with wound infections, septicemia (15), and acute necrotizing hemorrhagic pneumonia (10). Additionally, *S. equi* subsp. *zooepidemicus* has been isolated frequently from the lower respiratory tracts of clinically healthy dogs and dogs with CIRD (3). In the current study, the dogs experimentally infected with *S. equi* subsp. *zooepidemicus* alone did not exhibit any clinical signs of respiratory disease. *S.*

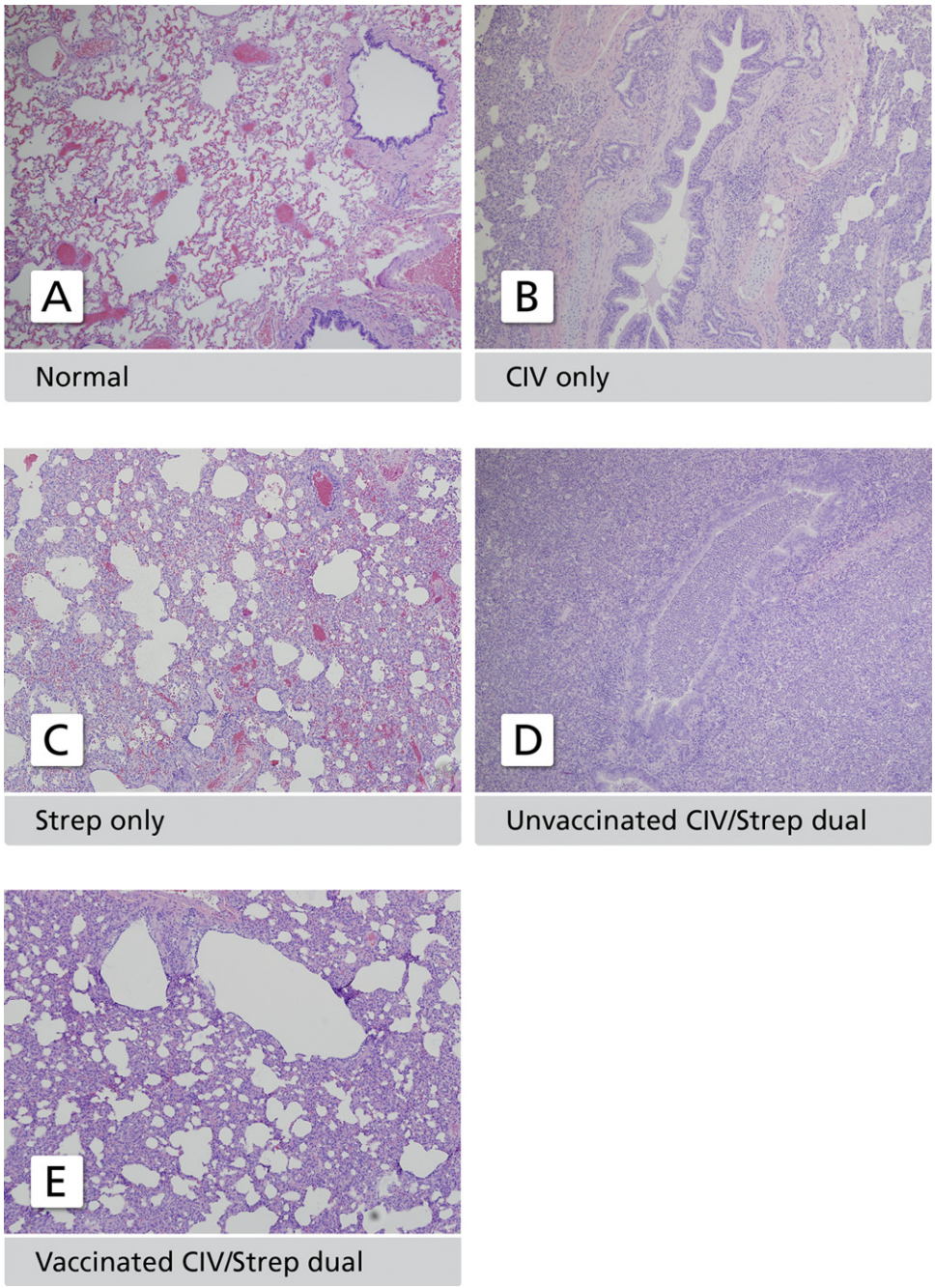

FIG. 5. Examples of histopathologic lung lesions. Lung sections from a healthy dog (A), from a dog challenged with CIV only (B), from a dog challenged with *S. equi* subsp. *zooepidemicus* (C), from a dog challenged with CIV and *S. equi* subsp. *zooepidemicus* (D), and from a CIV-vaccinated dog challenged with CIV and *S. equi* subsp. *zooepidemicus* (E) are shown.

equi subsp. *zooepidemicus* could be isolated from the lung of only 1 dog (17%) infected with *S. equi* subsp. *zooepidemicus* alone, suggesting that *S. equi* subsp. *zooepidemicus* does not cause respiratory disease in healthy dogs.

However, severity of disease caused by CIV increased in the presence of *S. equi* subsp. *zooepidemicus*. Unlike the dogs infected with CIV alone, the majority of dogs infected with CIV plus *S. equi* subsp. *zooepidemicus* exhibited clinical fever that lasted longer and had more-severe clinical signs, particularly a cough, that lasted longer. *S. equi* subsp. *zooepidemicus* was isolated with increased frequency in both nasal secretions and lung wash samples of dogs infected with CIV and *S. equi* subsp. *zooepidemicus*. Furthermore, lung lesion scores were more severe in dogs infected with CIV and *S. equi* subsp. *zooepidemicus*. These data strongly suggest that *S. equi* subsp. *zooepidemicus* is an opportunistic pathogen that contributes to the

severity of CIRD. Clinical disease and lung lesions were more severe in dual infection compared with infection with either CIV alone or *S. equi* subsp. *zooepidemicus* alone.

In natural *S. equi* subsp. *zooepidemicus* outbreaks in shelter and kenneled dogs, lung lesions were characterized by acute hemorrhagic pneumonia (12, 14). The lung lesions observed in the current study in dual-challenged dogs were much milder than the lesions in dogs during natural outbreaks. This could be attributed to multiple factors contributing to disease and pathology in the shelter environment compared with the current study, where the dogs were housed in a clean, controlled environment.

The canine influenza virus H3N8 vaccine used in this study has previously been shown to reduce the severity of clinical disease, virus shedding, and lung lesions following experimental CIV challenge (7). In the current study, the CIV (H3N8) vaccine significantly reduced the severity of clinical disease, viral shedding, bacterial shedding, and lung pathology in dogs infected with CIV and *S. equi* subsp. *zooepidemicus.* Hence, CIV vaccine could be used to aid in protecting dogs against CIV as well as against opportunistic bacterial pathogens, such as *S. equi* subsp. *zooepidemicus*. These findings are important because most, if not all, dogs will be infected with a variety of pathogens commonly found in the canine respiratory tract (e.g., *S. equi* subsp. *zooepidemicus*), and concomitant infection with CIV could further complicate respiratory disease and may result in mortality. Therefore, vaccination of dogs at risk for infection, such as those housed in shelters or kennels or those that are frequently boarded or go to dog day care and dog parks, is the best way to protect against CIV as well as against other secondary pathogens.

To achieve protection, it is important that dogs are vaccinated at least 3 weeks before exposure to CIV. This killed vaccine should be given in 2 doses no less than 2 weeks apart. Immunity can be expected approximately 7 days after the second dose. To ensure protection, dogs at risk for CIV, such as those that routinely spend time in multidog facilities (i.e., dog day care, boarding facilities, dog shows, other training facilities), should be given 2 doses of CIV (H3N8) vaccine 2 weeks apart and then held for 7 days before being placed in contact with other dogs.

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