

# Clinical Safety and Efficacy after In Vivo Challenge of a Killed, Monovalent Canine Influenza Virus (H3N8, Iowa05 Strain) Vaccine

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## ABSTRACT

### Objectives

To evaluate the safety of a killed canine influenza virus (CIV) vaccine, Iowa05 strain (H3N8), and its in vivo efficacy against a heterologous challenge strain (CO-08).

### Animals

The clinical safety study involved 691 privately owned, healthy dogs, 8 weeks to 19 years old, seen at 24 veterinary practices and 1 commercial dog breeder in 10 states within 3 geographic regions of the United States. Forty-eight purpose-bred beagles approximately 8 weeks old were used in the vaccination-challenge efficacy study.

### Procedure

Dogs in the safety study were vaccinated using 1 of 3 vaccine serials with a 1 mL dose administered subcutaneously (SC). Another 1 mL dose of the same serial was

administered approximately 3 weeks later. Veterinarians observed the vaccinated dogs for injection pain response, and owners were asked to report all adverse events. Dogs in the vaccination-challenge study were vaccinated with two 1 mL doses administered approximately 3 weeks apart via the SC route. Two weeks later, all dogs were challenged by aerosolization with heterologous CIV CO-08 and observed daily for clinical signs of respiratory disease. Dogs were euthanized and necropsied 4 or 11 days later. Lungs and tracheal bronchial lymph nodes were examined, and lung, lung lavage, and tracheal tissues collected for virus isolation, bacteriology, histopathological examination, and immunohistochemistry.

### Results

In the clinical safety study, 1359 vaccinations were administered and immediate animal discomfort was noted in 58 instances (4.3%). Veterinarians reported 51 adverse events (51/1359, 3.8%) in 47 dogs, and 17 of the 51 events (17/1359, 1.3%) were attributed to the vaccine. These affected 14

dogs from 9 sites. The events were generally transient, mild, and typical of those expected following vaccination. In the efficacy study, the vaccine significantly reduced the incidence and severity of lung lesions, and the incidence of clinical coughing and the incidence and duration of viral shedding compared with placebo control treatment. Mean diffuse (71 vs. 39.18) and total lung consolidation (74.98 vs. 40.19) scores, and total days of shedding (0.5 vs. 5.2 days) were significantly lower in the treated group than in the placebo group.

## Conclusions

The killed, monovalent CIV vaccine was safe when administered to dogs 8 weeks to 19 years old according to label directions. A very low incidence of mild and transient events was noted. The CIV vaccine demonstrated *in vivo* efficacy against a heterologous CIV challenge, reducing the incidence and severity of lung lesions, and the incidence of clinical coughing and viral shedding.

## INTRODUCTION

Canine influenza virus (CIV) causes a highly contagious respiratory infection in dogs. The first recognized outbreak occurred in racing greyhounds in Florida in 2004.<sup>1</sup> The incubation period for CIV usually lasts from 2 to 4 days, and viral shedding can continue for up to 10 days in some dogs.<sup>2</sup> Almost all exposed dogs will become infected, but 20% to 25% remain asymptomatic. The rest usually experience a mild form of canine influenza, characterized by a cough that persists for 10 to 21 days. Some dogs will have nasal discharge and a low-grade fever, and dogs weakened by the viral infection can develop severe, sometimes fatal, pneumonia. The mortality rate is estimated to be between 1% and 5%, but most dogs recover without treatment within 2 to 3 weeks. Susceptibility to CIV remains high because of its recent emergence and few dogs have developed natural immunity. Treatment consists of supportive care.

Spread of CIV occurs through aerosol-

ized respiratory secretions, contaminated objects, and people who move between infected and uninfected dogs.<sup>1</sup> Viable virus can be found on surfaces for up to 48 hours, on clothing for 24 hours, and on hands for 12 hours. Transmission generally occurs in areas where dogs congregate, such as veterinary, boarding and shelter facilities. The virus is easily killed with quaternary ammonium compounds and bleach solutions. Isolating exposed dogs for about 2 weeks can break the chain of transmission. In 2006, the American Veterinary Medical Association called for the development of a vaccine against CIV.<sup>3</sup> The two studies reported herein were conducted in support of licensing for a CIV vaccine.

The first study was designed to evaluate the clinical safety of a killed, adjuvanted monovalent CIV vaccine in client-owned dogs treated at veterinary hospitals throughout the United States. A second study was undertaken to determine the vaccine's *in vivo* efficacy in the face of heterologous CIV challenge. A number of criteria must be met for a valid test of CIV vaccine efficacy. First, placebo-vaccinated dogs must remain seronegative to the vaccine strain until challenge. Additionally, at least 80% of these dogs must develop signs of CIV infection following challenge, demonstrated by clinical signs, lung lesion scores, or isolation of CIV from nasal swabs or tissue samples. Criteria for satisfactory demonstration of efficacy include seroconversion (>4-fold increase in titers) to the vaccine strain in at least 90% of dogs vaccinated with the CIV test vaccine. Additionally after challenge, vaccinated dogs must also demonstrate significant reduction in 1 or more clinical signs, or lung consolidation scores, or significant reduction in shedding of CIV H3N8 compared to dogs in the placebo group.

## MATERIALS AND METHODS

### Clinical Safety Study

#### Study Design

Twenty-four small animal veterinary practices and 1 commercial dog breeder in

10 states representing 3 geographic regions of the United States were recruited to enroll privately owned, healthy dogs in the study. Each site administered 1 serial of the test vaccine to enrolled animals, so no masking was necessary. Practices enrolled eligible dogs as young as 8 weeks old and obtained written consent from owners. Dogs were considered to have completed the study if owners did not contact the practice or return animals for examination during the 10 days following the second vaccination.

## **Animals**

Dogs of any breed or cross-breed, male or female, intact, neutered or spayed, and approximately 8 weeks or older were enrolled. Only healthy dogs were considered eligible for enrollment, and owners had to be willing to provide written informed consent. Animals were excluded if they were younger than 8 weeks old, known or suspected pregnant, known or suspected of having a history of anaphylactoid vaccine reactions, or not expected to survive for at least 3 weeks after the first vaccination. All dogs remained with their owners during the study.

**Vaccine Dosage and Administration:** The sterility and potency of the experimental CIV vaccine was determined before it was released for administration. Each dog received two 1 mL doses approximately 3 weeks apart by subcutaneous injection. The injections were administered by staff at the veterinary practices during routine visits.

## **Observations**

Immediately after vaccination, the veterinarian observed the dog for injection pain response or other immediate responses to vaccination. If pain was observed, it was described as vocalization, scratching or biting at injection site, aggression, or escape attempt. Vocalization and scratching or biting were categorized as minor, moderate, or severe. Aggression and escape attempts were recorded as present or not present. Animals were also observed for abnormal attitude, which was considered an adverse event if it occurred. If other vaccines, medications or

treatments were administered at the same time, these were recorded. Veterinarians documented all reports of unexpected or abnormal clinical signs made by owners and, based on professional judgment, determined if the dog should be brought in for examination. An animal history and brief analysis of the complaint, including diagnosis and any laboratory tests or treatment, were recorded by the veterinarian. For every adverse event reported, the veterinarian decided if the incident was related to the vaccination.

## **Statistical Analysis**

Frequency distributions were calculated overall for animals enrolled and completing the study. They were also calculated for each vaccine administration (first vs. second), acute responses, and adverse events by investigator, geographic region, serial, age (<8 to 10 weeks of age or >11 weeks of age), and sex.

## **In Vivo Efficacy Challenge Study**

### **Study Design**

This study used a complete random block design with 2 replicates of 24 purpose-bred dogs each. All dogs were vaccinated with either the investigational CIV or placebo vaccine on study days 0 and 21, and whole venous blood samples for serum were collected on days 0, 21, 35 (before challenge), 39 and 46. Serology and gene sequencing results are reported in a companion article (in press). Nasal swabs were collected from all dogs on day 1 and day 35 before challenge and daily until the end of the study. Both nostrils were swabbed and the swab suspended in sterile viral medium for transport on ice to the laboratory. Necropsy occurred on day 39 (4 days post-challenge) for 4 animals in each group, and for the remaining dogs on day 46 (11 days post-challenge). All study personnel responsible for vaccine administration and daily clinical observations, sample collection, and gross evaluation of tissues at necropsy, as well as all laboratories, were masked to treatment groups.

## Animals

Forty-eight male or female purpose-bred beagles approximately 8 weeks of age were acclimated to a research facility for 10 days, dewormed, and received canine parvovirus vaccine and *Bordetella bronchiseptica* intranasal vaccine before the study began. Animals were examined and certified as healthy by a veterinarian before being randomly assigned to the treatment or placebo group. Animals were housed in 12 pens per room, with 1 dog from each group in each pen. The facilities met USDA Animal Welfare Regulations (Code of Federal Regulations 9, Chapter 1, Subchapter A – Animal Welfare) and Institutional Animal Care and Use Committee (IACUC) guidelines. The dogs were fed Hill's Science Diet Puppy dry food (Hill's Pet Nutrition, Topeka, KS) and had access to fresh water *ad libitum*. All dogs were offered canned food at the time of daily observations. Following CIV challenge on day 35, they were scored as anorexic if they refused canned food.

## Dosage and Administration

All dogs received two 1.0 mL doses of CIV or placebo vaccine, administered subcutaneously 3 weeks apart on days 0 and 21. The vaccine contained CIV strain A/Ca/Iowa/13528/05. Before administration, both the CIV and placebo vaccines were tested for sterility, and the CIV vaccine was also tested for potency.

## CIV Challenge

All animals were challenged with a heterologous strain of CIV 2 weeks after the second vaccination, on day 35. This CIV strain was isolated from a dog in Colorado in 2008 by inoculation of embryonated eggs at Cornell University, New York. Challenge material was prepared by the Pfizer Biologics Development laboratories. The strain was characterized by sequencing and identity testing and satisfactorily passed purity testing. The challenge virus was administered to all dogs via aerosolization with a nebulizer in Plexiglas® chambers for 30 minutes. The live viral titer of the challenge material was

determined before and after challenge.

## Observations

General health observations were recorded daily for each dog. Immediate reactions to vaccination were also recorded. Injection sites were observed for vaccine-associated reactions on the day of vaccination, just before second vaccination, for 3 days after each vaccination, and then weekly. A record was made if injection sites were painful, hot, hard to the touch, or swollen.

After challenge, on days 36 to 46, each dog was evaluated daily for clinical signs of respiratory disease associated with CIV, including nasal discharge, ocular discharge, coughing, sneezing, retching, and depression. Tympanic body temperature was also recorded. Positive clinical signs were categorized as mild, moderate or severe. Presence or absence of anorexia was noted.

## Necropsy

In accordance with random allotment, 4 animals in each group were euthanized with an overdose of sodium barbiturate 4 days after challenge (day 39), and the remaining animals were euthanized in the same manner 11 days after challenge (day 46). A veterinary pathologist conducted a gross evaluation of the lung lobes for lesions characteristic of CIV. To determine the amount of total lung consolidation, each lobe was examined for the presence of diffuse lung consolidation (an indicator of viral pneumonia), discrete lung consolidation (an indicator of non-viral pneumonia), and total lung consolidation. Percentages of diffuse, discrete and total lung consolidation were calculated according to the following equation:

$$\text{Percent consolidation} = 0.53((0.35 \times \text{right cranial lobe}) + (0.15 \times \text{right middle lobe}) + (0.40 \times \text{right caudal lobe}) + (0.10 \times \text{accessory lobe})) + 0.47((0.30 \times \text{left cranial lobe}) + (0.25 \times \text{left middle lobe}) + (0.45 \times \text{left caudal lobe})).$$

All tonsils and tracheobronchial lymph nodes were also examined for gross lesions by a board-certified veterinary pathologist.

Abnormal gross lesions were characterized as present, enlarged, or discolored. Lung lavage, lung, and trachea tissues on day 39 were aseptically collected, and lung and trachea tissues on day 46. Right caudal lung lobes were lavaged for CIV isolation on necropsy day 39. Portions of the right cranial lung lobe tissue samples were aseptically collected for CIV isolation (day 39 only), bacteriology and *Mycoplasma* culture, and PCR for viruses other than CIV. Right middle lung lobes were insufflated for preparations of histopathology and immunohistochemistry slides. Trachea samples were also collected on day 39 and 46 for culture of CIV (day 39 only), bacteria and *Mycoplasma* culture, histopathology, immunohistochemistry, and PCR for viruses other than CIV. Samples for histopathology and immunohistochemistry were fixed in 10% buffered formalin and transported to Laboratory for processing. Stained slides for histopathology and immunohistochemistry were forwarded to a board-certified veterinary pathologist at Pfizer Animal Health for examination and archiving. Lung and trachea tissue samples were placed in centrifuge tubes and forwarded to the Michigan State University, Diagnostic Center for Population and Animal Health for PCR testing of canine adenovirus (CAV-2), canine distemper virus (CDV), and canine parainfluenza virus 2 (CPiV-2).

### **Virus Isolation**

Swabs, lung lavage fluid, trachea and lung tissue samples were assayed for the presence of CIV. In addition, swab and lavage samples were quantitatively cultured in the Pfizer Animal Health laboratories and results reported as  $\text{Log}_{10} \text{TCID}_{50}/\text{mL}$ . All CIV isolations were performed on >95% confluent monolayer of MDCK cells. Immediately before virus inoculation, cell monolayers were washed twice with Dulbecco's modified Eagle medium (DMEM) supplemented with 2 mM L-glutamine and 20 mg/L gentamicin to remove residual serum. Dilutions for virus titration were prepared in virus titration medium or VTM

(DMEM supplemented with 2 mM L-glutamine, 0.125  $\mu\text{g}/\text{L}$  LAH, 0.25% trypsin, 20 mg/L gentamicin, 5 mg/L amphotericin B, 100 U/mL penicillin, and 100  $\mu\text{g}/\text{mL}$  streptomycin). Tubes containing swabs immersed in virus transport media were thawed in a room temperature water bath. Samples were vortexed for 10 to 15 seconds. Two hundred  $\mu\text{L}$  of serially diluted samples were plated onto quadruplicate plates of washed MDCK cells in a 96-well tissue culture plate. All plates were incubated for 3 days at 37°C in 5% CO<sub>2</sub>. Following incubation, plates were fixed with 80% acetone for 15 minutes at room temperature and stained with 0.1  $\mu\text{g}/\text{well}$  of primary antibody MAB722P (Maine Biotechnology Services, Portland, ME), followed by detection with fluorescein isothiocyanate goat anti-mouse IgG (secondary antibody). Wells were scored positive for CIV if they demonstrated fluorescence using fluorescent microscopy, and  $\text{Log}_{10} \text{TCID}_{50}/\text{mL}$  were calculated using the method of Spearman-Kärber.

Quantitative virus isolation from lung lavage samples collected on day 39 was performed at the Pfizer Laboratory Sciences, Assay Development Laboratory. Virus isolation and titration were performed in VTM as described previously. Lavage fluids were adjusted to a minimum of 5 mL using VTM, samples were vortexed and centrifuged at 1500Xg for 20 minutes and the supernatant was sterile filtered using a 0.2  $\mu\text{m}/0.8 \mu\text{m}$  filter. Samples were diluted and quantitative CIV testing was performed as described previously.

Qualitative testing on bronchoalveolar lavage (BAL), lung and tracheal samples was also conducted. Filtered BAL fluids (200  $\mu\text{L}$ ) were directly inoculated into 25 cm tissue culture flasks of confluent MDCK cells. Tissue samples collected on day 39 (4 days after challenge) were transported on wet ice to the same laboratory, processed using sterile tissue grinders, and centrifuged at 1500Xg for 20 minutes. For qualitative virus isolation, supernatant was trypsin-treated by dilution with 10% trypsin/VTM

and inoculated into 25 cm tissue culture flasks of confluent MDCK cells. All cultures were incubated for 5 days. Flasks were frozen. For subculturing of the virus, flasks were thawed and 60 µL of supernatant from each flask was transferred into 8 replicate wells of a washed 96-well plate of confluent monolayers of MDCK cells in 200 µL of VTM, and incubated for 5 days at 36°C in a CO<sub>2</sub> humidified chamber. Following incubation, cells in the plates were fixed with 80% acetone and stained as described previously. Qualitative assays were reported as positive for virus staining or negative for no virus detected.

### **Bacteriology and Culture**

Lung and trachea bifurcation samples were collected aseptically. Lung samples were seared with a heated metal spatula, incised with a sterile scalpel and swabbed using a sterile, cotton-tipped applicator. The inner surface of the trachea samples were swabbed using a sterile swab. Lung and trachea swabs were used to inoculate a 5% sheep blood agar (BAP), chocolate blood agar (choc) and a brain-heart infusion (BHI) broth. Additionally, a MacConkey (MAC) agar plate was inoculated for trachea swabs only. The BAP and choc plates were incubated overnight at 37°C in 5% CO<sub>2</sub>. The BHI and MAC plates were also incubated at 37°C but without additional CO<sub>2</sub>. Primary bacterial pathogens of interest included *Streptococcus canis*, *Bordetella bronchiseptica*, and *Pasteurella spp.* All plates were read following overnight incubation, any growth recorded, and bacteria identification started. Standard microbiological procedures were used to confirm identification of canine respiratory pathogens.

For detection of *Mycoplasma spp.*, swabs were used to inoculate *Mycoplasma* agar and a 3 mL *Mycoplasma* enrichment broth. These were incubated at 37°C in 5% CO<sub>2</sub>. After 7 days of incubation, *Mycoplasma* plates were examined for the presence of “fried-egg” like colonies. After 48 hours of incubation, the enrichment broths were subcultured onto *Mycoplasma* agar and

these were examined after another 7 days of incubation.

### **Histopathology**

Samples were placed into 10% neutral buffered formalin and stored in labeled containers, which were kept at ambient room temperature for approximately 48 hours. Samples were subsequently transferred into 70% ethanol for a maximum of 7 days before processing. Tissues were processed into paraffin blocks, slides were prepared and stained with hematoxylin and eosin. The lung and trachea samples were evaluated by standard immunohistochemical methods for the presence of influenza A-H3 antigen.<sup>4</sup> Slides were prepared and shipped to the Pfizer Animal Health study pathologist for routine evaluation.

### **Extraneous Virus Testing**

Lung and trachea samples for extraneous virus PCR testing were cooled and shipped at refrigeration temperature to the virology laboratory at the Diagnostic Center for Population and Animal Health, Michigan State University (East Lansing, MI) for detection of viruses other than CIV.

### **Statistical Analysis**

Data were entered into the centralized data management system (SAS Version 9.1.3, SAS Institute, Cary, NC) by Pfizer Animal Health Biometrics personnel. Lung consolidation was analyzed with a general linear mixed model. The fixed effects were necropsy day, treatment, and treatment-by-necropsy day interaction. The random effects were room, block within room and necropsy day, and residual. If the treatment-by-necropsy day interaction was significant, treatments were compared within necropsy days, otherwise they were compared across necropsy days. Least squares means (LSMs), standard errors (SEs) and 95% confidence intervals (CIs) were calculated for treatments at the same level the treatment comparisons were made, as well as the minimums and maximums. The stratified mitigated fraction was calculated for nec-

ropsy day 46 data only, along with its 95% confidence limits. Room was the stratification variable.

Frequency distributions of the presence or absence of CIV isolation from the swabs, trachea, lung lavage and lung tissue were calculated for each treatment group and time. The number of days that CIV was detected in the nasal swabs after challenge was calculated and analyzed using a general linear mixed model. The fixed effect was treatment; the random effects were room, block within room, and residual. Least squares means, SEs, 95% CIs, minimums and maximums were calculated for each treatment. The geometric means, 95% CIs and SEs for the swab data were calculated by back-transforming the LSMs and SEs from a general linear mixed model for repeated measures. The fixed effects were treatment, time points, and treatment-by-time interaction. The random effects were room, block within room, treatment-by-block within room interaction, and residual.

Frequency distributions of injection-site reactions and clinical observations were calculated for each group and time. Percentages of clinical signs were determined and prevented fractions calculated for coughing, nasal discharge and ocular discharge at necropsy on day 46. The United States Dept. of Agriculture method and the PROC GLIMMIX method were used to calculate prevented fractions. Descriptive statistics, including mean, median, SE, minimum and maximum of the tympanic membrane temperature were calculated for each group and time. Means and SEs were calculated using a general linear mixed model for repeated measures. The fixed effects were treatment, time, and treatment-by-time interaction; the random effects were room, block within room, treatment-by-block within room interaction, and residual. Animals were also classified as febrile ( $>39.5^{\circ}\text{C}$ ) or not febrile ( $<39.5^{\circ}\text{C}$ ) for each time. Frequency distributions were calculated for each group and time.

Frequency distributions for canine respiratory, bacterial and *Mycoplasma* pathogens

were calculated for each group and necropsy day. Frequency distributions of each necropsy observation were calculated for each tissue type scored by group and necropsy day.

## RESULTS

### Clinical Safety Study

A total of 691 dogs were enrolled at 26 sites with 186 dogs at the minimum age of 8 to 10 weeks. Of the 691 dogs, 668 (96.7%) completed the study and 23 (2.3%) did not. Reasons for not completing the study ranged from owner noncompliance to animals having been sold, and 1 animal euthanized due to a severe parvovirus infection. In the southeast, 304 dogs were enrolled; 206 were enrolled in the northeast, and 181 in the west. Use of the 3 serials was evenly distributed, with 226, 245, and 220 dogs receiving serial 1, 2, and 3, respectively. For dogs  $\geq 11$  weeks of age, the mean age was 4.3 years, with a standard deviation of 3.4 years and median of 3 years. Overall, 357 females and 334 males were enrolled. Dogs representing more than 90 breeds or breed crosses were enrolled. The time between vaccinations ranged from 16 to 84 days, with 94.5% (631) of the dogs receiving both injections within 16 to 27 days.

A total of 845 medications, vaccinations, or treatments were administered in addition to the CIV vaccine. More than 600 concomitant vaccinations were administered at the same time, although not in the same location. Other vaccines included monovalent rabies, canine distemper, *Bordetella bronchiseptica*, and core antigen combination vaccines, such as those for canine distemper, infectious canine hepatitis, canine adenovirus type 2, canine parainfluenza, and canine parvovirus. Approximately 167 medications were administered concomitantly, including antimicrobials, heartworm preventives, antiparasitics, pain and sedation medicines. Simultaneous medical procedures included blood draws, fecal examinations, dental and aural cleaning, anal gland expression, nail trimming, and microchip insertion.

**Table 1.** Most Frequent Clinical Signs Associated with Adverse Events in Clinical Safety Study\*

	Total Number	Number Attributed to CIV Vaccine
Vomiting	21	7
Diarrhea	11	2
Depression	9	4
Anorexia/inappetence	8	4
Injection site pain or swelling	6	4
Total	55	21

\*Note: Individual animals with adverse events may have exhibited more than 1 clinical sign per adverse event.

Animal discomfort at the time of CIV vaccine administration was noted in 58 of the 1359 (3.4%) vaccinations. Of these 58 animals, 39 were less than 12 weeks of age. No discomfort was reported in the remaining 1301 vaccinations. During 4 of the 1359 vaccinations, animal discomfort resulted in scratching or biting at the site of vaccine administration; in 3 cases the reaction was classified as minor, in 1 case as moderate. Fifteen aggressive or escape attempts were noted. Vocalization was the most common sign of immediate discomfort, noted during 49 of 1359 (3.6%) vaccinations. Vocalization was reported in 41 dogs (22 breeds or cross breeds) from 16 sites. Forty instances (2.9%) were classified as minor vocalization, 8 (0.6%) as moderate, and 1 (0.1%) as severe. None of the dogs exhibited abnormal attitude immediately following vaccination.

Fifty-one adverse events were reported in 47 of the 691 dogs that received 1359 injections (3.76%, Table 1). Of these, 17 adverse events in 14 dogs at 9 sites were attributed to CIV vaccine administration (17/1359, 1.25%). Vomiting was the most prevalent clinical sign, followed by diarrhea and depression with some dogs exhibiting more than 1 clinical sign. This was true for dogs 8 to 10 weeks old, as well as for older dogs. Vomiting was more likely for 1 serial (12 instances) than for the other 2 (4 and 5 instances each). Eleven adverse events were noted in male dogs and 6 in females.

Six dogs that experienced 7 adverse events received concomitant medications or treatments. The reported adverse events were typically mild and transient.

### In Vivo Efficacy Challenge Study

The only immediate reaction to injection was on day 0 when 1 dog in the placebo group vocalized during dose administration. Swelling at the site of vaccine administration was noted in 2 dogs in the vaccine group on days 22 and 23, and 1 dog in the same group on day 24. The swellings were small, transient, and resolved quickly with no need for treatment. None of the injection-site swellings was painful, hot, or hard to touch.

Serological response: On study day 0, the HAI titers for all dogs were <8 HAI units/25  $\mu$ L against the vaccine strain of CIV, indicating that none of the dogs had been exposed to CIV prior to vaccination. Additionally, all dogs in the placebo control group were serologically negative at pre-challenge on day 35. For animals receiving the CIV vaccine, detectable titers were measured for all dogs on day 35 with back-transformed least square mean values against the vaccine strain of 46.6 HAI units/25  $\mu$ L. This value represents a significant rise in serological titer associated with vaccination.

After challenge, coughing and ocular discharge were the most prevalent clinical observations in all dogs, with nasal dis-

**Table 2.** Number (%) of Dogs in the Challenge Study Having Cough, Ocular Discharge or Nasal Discharge After Challenge, and Prevented Fraction Calculated by Two Methods

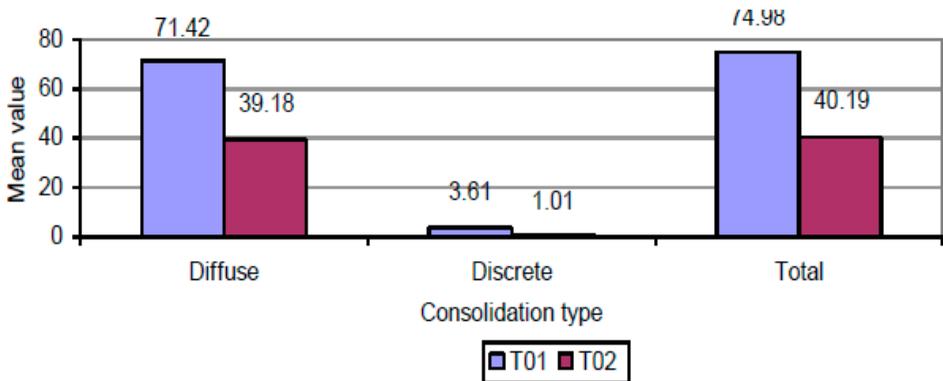
	Placebo Group	Vaccinated Group	Prevented Fraction USDA Method	Prevented Fraction PROC GLIMMIX
Coughing	13 (65)	2 (10)	61.1 (95% lower bound exact: 30.9) (95% upper bound exact: 82.8)	86.3 Unprotected fraction Vaccine group: 0.09 Placebo group: 0.66
Ocular Discharge	12 (60)	5 (25)	46.7 (95% lower bound exact: 4.9) (95% upper bound exact: 75.2)	59.8 Unprotected fraction Vaccine group: 0.24 Placebo group: 0.6
Nasal Discharge	1 (5)	0 (0)	5 (95% lower bound exact: -14.2) (95% upper bound exact: 24.9)	Not determined

charge less frequent (Table 2). Retching was observed in 2 dogs in the placebo group on days 38, 40 and 42. Percentages of clinical signs were determined and prevented fractions calculated for coughing, nasal discharge, and ocular discharge at necropsy on day 46 (figure ). Using either method of calculation, the test vaccine prevented cough and ocular discharge compared with placebo. These data demonstrate that vaccination with CIV vaccine prevents the development of cough and ocular discharge, clinically relevant signs of respiratory disease. Mean tympanic membrane temperatures were within the normal range for both groups on all days. However, 1 dog in the

vaccinated group was febrile the day before challenge, and 2 dogs in the placebo group were febrile, 1 each on days 37 and 46.

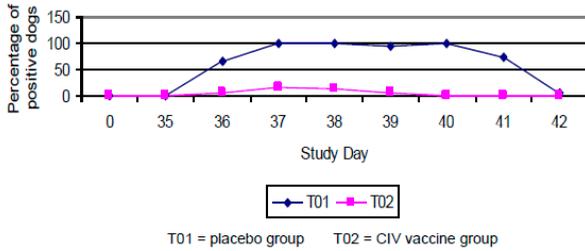
Mean lung consolidation scores are graphed in Figure 1. Lung consolidation was observed in all but 3 dogs in at least 1 lung lobe at necropsy. Dogs in the test vaccine group had significantly lower mean diffuse (39.18,  $P=0.0317$ ) and total lung (40.19,  $P<0.0238$ ) consolidation scores than dogs in the placebo group (71.42 diffuse and 74.98 total). The mitigated fractions were 11 (4-18 95% CI) and 20 (6-34 95% CI) for diffuse and total lung consolidation, respectively. These data support the efficacy of the CIV test vaccine.

**Figure 1.** Lung Consolidation Scores



Dogs in the test vaccine group had significantly lower diffuse ( $P=0.0317$ ) and total lung ( $P<0.0238$ ) consolidation scores than dogs in the placebo group.

**Figure 2.** Percentage of Animals with Positive Canine Influenza Virus Isolation from Nasal Swabs by Study Day



Enlarged tonsils were noted in 2 dogs in the placebo group and 1 dog in the test vaccine group, while discoloration of the tonsils was seen in 10 animals in the placebo group and 6 in the vaccine group. In all cases, enlargement and discoloration were categorized as mild. Gross examination revealed tracheobronchial lymph node pathology in 44 of the 48 of the dogs. Twenty-three dogs in the placebo group and 11 dogs in the vaccine group had enlarged lymph nodes. Twenty dogs in the placebo group and 18 in the vaccine group had discoloration. The overall frequency of enlargement and discoloration was similar across groups.

No CIV was isolated from any of the nasal swabs collected on days 1 or 35, indicating that dogs were free of CIV before challenge (Figure 2). CIV was isolated from all dogs in the placebo group on days 37, 38, and 40. Eight dogs from the test vaccine group were positive for CIV, 1 animal for 2 days and the remaining dogs for a single time. One day after challenge (day 36), 16/24 (66.7%) of the dogs in the placebo group and 1 (4.2%) dog in the vaccine group were CIV positive. The number of dogs in the placebo group with CIV isolation peaked on days 37 and 40. By contrast, the number of dogs testing positive in the vaccine group peaked on day 37 (4/24) and was declining by day 38. Geometric mean CIV titers from nasal swabs were significantly higher in the placebo group than the vaccine group on days 37 to 40 (data not shown). The mean number of days of viral shedding (i.e., days of CIV isolation from nasal swabs)

was significantly higher in the placebo group (5.2 days) than in the vaccine group (0.4 days,  $P < 0001$ ). CIV was isolated from all the tissue samples from dogs in the placebo group, and from none of the samples from dogs in the vaccine group.

No bacteria were isolated from any of the lungs of dogs in the vaccine group. In the placebo group *B. bronchiseptica* was isolated from 1 dog, *S. canis* from 1 dog, and *Mycoplasma* species from 10 dogs. Bacteria and *Mycoplasma* were much more likely to be found in tracheal samples, which are not considered sterile. On day 39, *Mycoplasma* was cultured from 2 dogs in the placebo group and *B. bronchiseptica* from 2 dogs in the vaccine group. On day 46 in the placebo group, cultures from 7 dogs had *B. bronchiseptica*, 16 had *Mycoplasma*, 1 had *Staphylococcus pseudintermedius*, and 1 had *S. canis* in the trachea. On the same day in the vaccine group, cultures from 13 dogs had *B. bronchiseptica* and 1 had *S. pseudintermedius*. All samples were negative for CDV and CPiV-2. Two animals in each group were positive for CAV-2 in the lungs on first extraction and negative on second extraction. The PCR values were very low and not duplicated on subsequent extractions. These results reflected low-level, focal infections of CAV.

Examples of the marked microscopic differences observed on histopathological examination of tissues from the trachea, bronchi, and alveoli of dogs in the vaccine and placebo groups can be seen in Figure 3. Dogs in the placebo group (right side of Figure 3) had marked loss of ciliated respiratory epithelial cells, loss of goblet in the trachea and bronchi, as well as epithelial inflammation and individual cell necrosis, and peribronchial inflammation. The alveolar spaces in the lungs of unvaccinated dogs were filled with neutrophils and alveoli had cells and proliferation of type II pneumocytes (type II hyperplasia). These hallmark lesions associ-

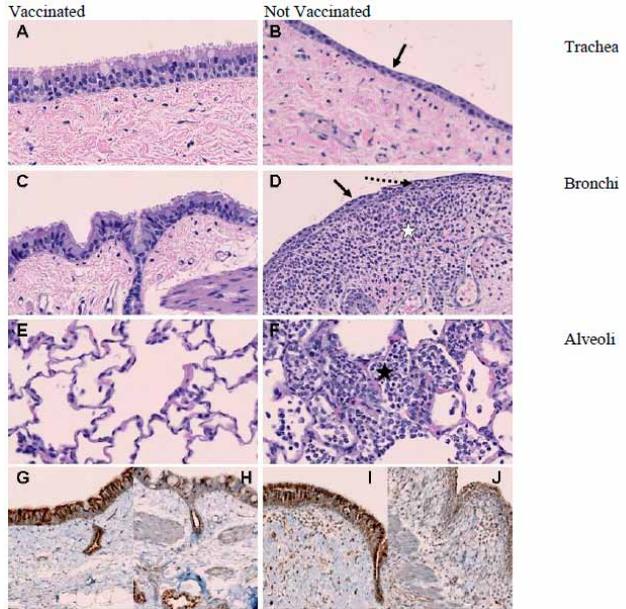
ated with an influenza Type A infection were observed in 24 of 24 control dogs and only 1 of 24 vaccinated dogs. Examination of slides from the lung and trachea for immunohistochemistry demonstrated that both groups had similarly intense staining for identification of influenza A H3 antigen in the lungs and trachea. This is indicative of the circulation of the influenza virus in all dogs post-challenge.

## DISCUSSION

The first study was designed to evaluate the clinical safety of a killed, monovalent CIV vaccine in typical veterinary practices. Consequently, the vaccine was administered during routinely scheduled veterinary visits along with more than 800 other vaccines, medications, or treatments. Despite this, the overall incidence of adverse events attributable to the CIV vaccine was low. The events were typically mild and transient, with the vaccine being well tolerated by dogs of numerous breeds and a wide range of ages. In this study, the CIV vaccine achieved clinically acceptable levels of safety when used according to label instructions.

Vaccine efficacy is measured by seroconversion, significant reduction in clinical and pathological signs of infection, and significant reduction in viral shedding among vaccinated animals compared to unvaccinated animals. The serological results for dogs in the efficacy study are discussed in a companion article (in press), but vaccinated dogs had HAI titers to all CIV strains, with 6- to 20-fold increases in titers. Vaccinated dogs had significantly fewer clinical signs of respiratory disease than dogs that received placebo. Preventive fractions demonstrated that vaccination with CIV Iowa05 H3N8

**Figure 3. Microscopic Appearance of Tissues from Dogs in the Vaccinated and Placebo Groups 4 Days after Challenge with Canine Influenza Virus**



Marked loss of cilia (arrows) is observed in trachea and bronchi, epithelial inflammation and individual cell necrosis (dashed arrows), and peribronchial inflammation (white star) in animals receiving placebo vaccine. H3 antigen is present in trachea (G & I) and bronchi (H & J) of vaccine- and placebo-treated dogs.

vaccine successfully reduced the incidence and severity of lung lesions, and the incidence of clinical coughing and viral shedding. Vaccinates shed virus for less than half a day, and no CIV was isolated from the tissues of any of them 4 days after challenge. Thus, the vaccine met all of the required conditions of efficacy.

Like many types of influenza, CIV has a relatively low mortality rate and most infected dogs eventually recover without treatment. However, the disease spreads easily and rapidly where dogs are in close contact. In this study, vaccination successfully reduced the incidence of clinical coughing and viral shedding; outcomes that can be expected to curb transmission of the virus. It is also important to note that damage to the respiratory cilia, epithelium and alveoli occurred within 4 days of exposure to CIV in unvaccinated dogs. Such damage facilitates the development of opportunistic bacterial infections. Indeed, *B. bronchiseptica*, *S.*

*canis*, and *Mycoplasma spp.* were isolated from the lungs of untreated dogs, whereas bacteria were not isolated from the lungs of vaccinated dogs. *B. bronchiseptica* tends to be mildly pathogenic, and *Mycoplasma spp.* rarely colonize airways effectively in the absence of epithelial damage. However, damage from CIV or other viral infections often creates ideal conditions for the development of more virulent bacterial infections. Impairment of lung defenses, including mucociliary function, is recognized as a predisposing factor for bronchopneumonia caused by bacteria.<sup>4</sup> Agents that impair mucociliary clearance include as viral infections, exposure to cold and toxic gases, and ciliary dyskinesia.<sup>4</sup> Thus, vaccination may not only help control CIV, but may also ameliorate the bacterial pneumonias that can take hold in virus-damaged respiratory tissues.

## CONCLUSIONS

The killed, monovalent CIV (H3N8, Iowa05) vaccine was safe when administered to dogs 8 weeks to 19 years old according to label directions. A very low incidence of mild and transient events was noted. The vaccine also demonstrated in vivo efficacy against a contemporary heterologous CIV challenge. The vaccine reduced the incidence and severity of lung lesions, and the incidence of coughing and viral shedding. This evidence supports the use of

this killed, monovalent CIV vaccine as an aid in the control of disease associated with CIV infection.

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