An Inactivated H3N2 Canine Influenza Virus (CIV) Vaccine Aids in the Prevention of Clinical Disease and Virus Shedding in Dogs Challenged with Virulent H3N2 CIV

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ABSTRACT

Canine influenza virus (CIV) is an etiologic agent of canine infectious respiratory disease (CIRD). In March of 2015, a strain of H3N2 subtype CIV, previously found only in Asia, emerged in Chicago, IL, USA. The virus triggered localized outbreaks of CIRD in Illinois, and rapidly spread to dogs in at least 30 different U.S. states. In response to this outbreak and the high probability that current H3N8 CIV vaccines would not prevent disease caused by the newly emergent virus, we developed a monovalent, inactivated H3N2 CIV vaccine. In this study, we evaluated the safety and efficacy of the vaccine and showed that vaccinated dogs were protected from clinical disease and shed less virus following challenge with a virulent H3N2 CIV strain compared to placebo vaccinated dogs. The vaccine was administered subcutaneously, twice, 3 weeks apart, and did not cause adverse systemic or administration site reactions. Collectively, our findings support the use of this vaccine to aid in the reduction of clinical disease caused by H3N2 CIV.

INTRODUCTION

Canine influenza viruses (CIV) cause a highly transmissible, acute respiratory disease in domestic dogs (Canis familiaris). [1, 2] Common clinical signs of infection include a low-grade fever, lethargy, anorexia, mucopurulent nasal and/or ocular discharge, and cough.[3] Infectious virus is shed from infected animals in respiratory secretions for up to 10 days post-exposure, and virus can spread rapidly among naïve animals housed

in close proximity and in areas where dogs tend to congregate.[4, 5, 6, 7] Treatment consists of supportive care combined with antimicrobial administration if a secondary bacterial infection is suspected. Previously healthy dogs often recover without complication 2-3 weeks after infection; However, animals can develop severe pneumonia, particularly when co-infected with other respiratory pathogens.[3]

Two subtypes of CIV (H3N8 and H3N2) infect and are readily transmitted between dogs. H3N8 subtype CIV was first recognized in 2004 following the transmission of an equine influenza virus (EIV) from horses to racing greyhounds in the U.S.[1] The virus subsequently spread to other dog breeds in at least 40 U.S. states and now circulates primarily in large kennels and boarding facilities in the northeastern states.[8] H3N2 subtype CIV is an avian-origin virus originally detected in South Korea in 2007.[2] Since that time, closely related strains of H3N2 CIV have been isolated from dogs in China and Thailand, as well as from dogs and cats in South Korea.[9, 10, 11] In March of 2015, a strain of H3N2 CIV emerged in Chicago, IL, where it was associated with outbreaks of canine respiratory illness.[12] Within 6 months of emergence, the virus infected over 1,600 dogs in 30 U.S. states, and it continues to circulate in large metropolitan areas.[13]

Monovalent, inactivated virus vaccines against the H3N8 CIV have been licensed for sale in the U.S. since 2009.[14] Whether these vaccines protect dogs from disease caused by the newly emergent H3N2 CIV strains is not known. However, sequence comparisons have shown that the HA and NA proteins of the two virus subtypes are approximately 85% and 39% identical, respectively, while the remainder of the viral proteins share 84% to 97% identity.[15] This high degree of divergence in the viral surface antigens suggests that the H3N8 CIV vaccines may not be fully efficacious against infection with H3N2 subtype CIV. Consequently, we developed an inactivated H3N2 CIV vaccine and evaluated its safety and efficacy in dogs. The vaccine was safe when administered twice at 3-week intervals to 6-week old puppies, and it prevented clinical disease and infectious virus shedding in dogs challenged with virulent H3N2 CIV. Thus, our data support the use of this monovalent vaccine to protect dogs against disease caused by the newly emergent H3N2 subtype of CIV.

MATERIALS AND METHODS

Viruses and Cells

Viruses were isolated from nasal swabs in specific pathogen free (SPF) embryonic chicken eggs and amplified either in eggs to generate the challenge virus strain or in Madin-Darby Canine Kidney (MDCK) cells to establish a vaccine master seed. Results of purity and sterility testing for both viruses were satisfactory.

Virus Titrations

Virus titers were measured by hemagglutination (HA) assay or 50% infectious dose assay as described.[16] Chicken red blood cells (cRBCs) were used in HA assays, and HA titers were recorded as the reciprocal of the highest dilution at which 50 µL of the virus solution completely agglutinated the cRBCs. Infectious dose assays were incubated for 3-4 days at 37°C, and eggs or MDCK cells were scored for the presence of infectious virus by HA assay or by the presence of virus-induced cytopathic effect, respectively. The Spearman-Karber method was used to estimate 50% infectious virus doses.[17]

Viral Genome Sequencing and Sequence Analyses

Viral genomic RNA was extracted from virus-laden fluid and amplified by RT-PCR using universal primer pairs.[18] Nucleotide sequences were determined by the Sanger method, and contiguous sequence alignments were assembled using Sequencher v5.1 software. Sequences from other H3N2 CIV isolates were downloaded from the NCBI Influenza Virus Resource Database. Bioinformatic analyses were performed in BioEdit Sequence Alignment Editor v7.0.4.1.

Table 1: Study Groups

Group	Vaccine	Vaccination Route	Vaccination Frequency	No. of dogs	Challenge
1	2X CIV	Subcutaneous	Twice, 21 days apart	10	H3N2 CIV
2	1X CIV	Subcutaneous	Twice, 21 days apart	10	H3N2 CIV
3	Placebo	Subcutaneous	Twice, 21 days apart	10	H3N2 CIV

Vaccine Formulation

Vaccines were formulated as monovalent, aqueous solutions containing a 1X or 2X dose of whole, inactivated H3N2 CIV and a proprietary adjuvant. The placebo vaccine lacked antigen, but it contained adjuvant at the same concentration as the test vaccines.

Animals

Institutional Animal Care and Use Committee approval was obtained prior to the initiation of the study. Thirty CIV seronegative 6-week old, commercial source beagles were randomized to three vaccination groups, containing 10 dogs each, using litter and gender as randomization factors. The dogs in each group were commingled and housed in isolation rooms throughout the study. Water was available ad libitum, and food was provided in an amount and manner that provided the nutrient and energy requirements for the dogs.

Vaccine Administration and Challenge

Dogs in Groups 1 and 2 received 2X and 1X doses of the H3N2 CIV vaccine, respectively (Table 1). Dogs in Group 3 received a placebo vaccine. Each dog was vaccinated twice, 21 days apart, with 1 mL of vaccine, subcutaneously over the scapula. The dogs were monitored for injection site reactions and temperature elevations for 3 days after each vaccination and on days 14 and 27 (day prior to challenge). Blood samples were collected on days 0 (prior to vaccination), 7, 14, 21 (prior to vaccination), 27, and 35.

Seven days after the second vaccination, the dogs were challenged with virulent H3N2 CIV via aerosolization in a closed chamber using a commercial nebulizer. During challenge, dogs were randomized to challenge chambers using treatment group and litter as blocking factors such that each treatment group, litter, and sex (if possible) was represented in each challenge chamber run. After challenge, dogs were randomized to post-challenge (PC) pens such that each pen contained dogs from all three treatment groups and each chamber run from the challenge phase.

The dogs were observed by personnel blinded to treatment group for cough, fever, mucopurulent nasal discharge, and other clinical signs for 7 days. Nasal swabs for virus isolation were collected on days 3, 4, and 5 PC. A dog was classified as having disease due to CIV if it developed cough in addition to either fever or mucopurulent nasal discharge. A dog was considered febrile when the rectal temperature was $\geq 39.7°C$ and 0.5°C above baseline (day 0 rectal temperature). The challenge was considered valid when at least 60% of the placebo vaccinated dogs developed disease due to CIV.

Hemagglutination Inhibition (HAI) Assay

Heat-inactivated serum samples were incubated with 2.5% v/v washed cRBCs for 30 min at room temperature, after which cRBCs were pelleted by centrifugation. The pellet supernatant was used to perform the HAI assay as described[16] using a final concentration of 0.25% v/v cRBCs. The HAI titer of each sample was recorded as the reciprocal of the highest serum dilution that completely inhibited cRBC agglutination by 8 HA units of H3N2 CIV. A titer of <4 was considered negative for CIV serum antibody.

Table 2. *Number of dogs expressing clinical signs of CIV disease post-challenge*

Vaccine Group	Cough	Fever	Mucopurulent Nasal Discharge
2X CIV (n=10)	0	1	0
1X CIV (n=10)	0	0	0
Placebo (n=10)	9	5	10

Table 3. *Total days with cough*

Vaccine Group	Number of days of cough			
	2	3	4	5
2X CIV (n=0)	-	-	-	-
1X CIV (n=0)	-	-	-	-
Placebo (n=9)	6	1	1	1

Table 4. *Total days with mucopurulent nasal discharge*

Vaccine Group	Number of days of mucopurulent nasal discharge				
	2	3	4	5	6
2X CIV (n=0)	-	-	-	-	-
1X CIV (n=0)	-	-	-	-	-
Placebo (n=10)	2	2	3	1	2

Table 5. *Total days with fever*

Vaccine Group	Number of days of fever	
	1	2
2X CIV (n=1)	1	-
1X CIV (n=0)	-	-
Placebo (n=5)	4	1

Detection of Virus in Nasal Secretions

Fluids expressed from nasal swabs were inoculated into the allantoic cavity of three embryonic chicken eggs. Eggs were incubated for 3 days at 37°C and tested for the presence of virus by HA assay. Specimens were considered positive for virus if at least one of three eggs contained detectable virus. Specimens were considered negative for virus if at least two eggs were viable at the time of testing, and no eggs contained detectable virus. Positive samples and samples for which two or three eggs were inviable at the time of testing were tested by $TCID_{50}$ assay.

Statistical Analyses

All statistical analyses were performed using SAS v9.4, StatXact 10, and/or PF package in R 3.1.1. All tests were two-sided, and statistical significance was declared at a P value of 0.05 or less. The incidences of positive CIV recovery from nasal secretions of each test vaccine group and the placebo vaccine group were compared using Fisher's exact tests by day. The proportion of animals that exhibited positive CIV recovery post-challenge in each test vaccine group was also compared against the placebo vaccine group using a Fisher's Exact test, and the prevented fraction (PF) was calculated.

A positive incidence of CIV recovery for each animal was defined as one or more occurrences of positive CIV recovery post-challenge in each animal. The proportions of animals that seroconverted in each test vaccine group post-vaccination were compared against the placebo vaccine group using Fisher's Exact tests for days 14, 21, 27, and 35. Incidence of disease was compared between each test vaccine group and the placebo vaccine group using Fisher's exact test. The PF of each test vaccine and its 95% confidence interval (CI) were also

Table 6. 2X dose and 1X dose H3N2 Vaccine Group Prevented Fractions for CIV disease

Vaccine Group	Number of dogs with disease	P-value (Fisher's Exact Test)	Prevented Fraction (95% CI)
2X CIV (n=10)	0	0.0001	1.00 (0.67, 1.00)
Placebo (n=10)	9		
1X CIV (n=10)	0	0.0001	1.00 (0.67, 1.00)

Table 7. Incidence of nasal CIV shedding

Vaccine Group	Number of dogs with positive nasal shedding		
	3 DPC (Day 31)	4 DPC (Day 32)	5 DPC (Day 33)
2X CIV (n=10)	0[§a]	2[§b]	0[§a]
1X CIV (n=10)	1[§c]	1[§c]	0[§a]
Placebo (n=10)	10	10	10

[§]significant difference between test vaccine and placebo
[a] p<0.0001 [b] p=0.0007 [c] p=0.0001

calculated.

RESULTS AND DISCUSSION

Virus Isolation and Genetic Characterization

Two strains of H3N2 CIV were isolated from pet dogs in the Chicago, IL area and separately amplified to generate virus for clinical challenge studies or vaccine production. The viral HA and NA genes encoded by the two virus strains were sequenced, and the deduced amino acid sequences of the gene products were aligned to analogous H3N2 CIV sequences in the public databases to determine the genetic relatedness of the viral surface antigens to those from previous H3N2 CIV isolates. The challenge and vaccine virus HA proteins differed at two amino acid positions and shared the highest degree of homology (99.2% or 99.6% identity) with the HA proteins encoded by another US H3N2 CIV isolate (A/Canine/IL/12191/2015), as well as two isolates from South Korea (99.2% identity to CY005, CY009).[19]

Similarly, the NA protein sequences from the three U.S. viruses were identical and highly homologous (97.0% - 97.2% identity) to those from the aforementioned South Korean viruses. Based on these data, we conclude that the viruses isolated in this study are representative strains of the H3N2 subtype CIV that emerged in the U.S. during 2015.

Injection Site Reactions and Clinical Signs

No systemic or injection site adverse reactions were observed following vaccination of dogs with the H3N2 CIV vaccine at 1X and 2X doses. Prior to challenge, no dogs in any group showed clinical signs of disease. After challenge, the first clinical signs of disease (cough, mucopurulent nasal discharge, and fever) were observed two days post-challenge (DPC), which is consistent with reports of dogs with natural and experimental CIV-induced disease (Table 2).[2, 4]

Cough and mucopurulent nasal discharge were the predominant clinical signs of disease, with most dogs in the placebo group coughing for 2 days and showing mucopurulent discharge for 4 days (Tables 3 and 4). Ninety percent (90%) of the dogs in the placebo group were observed with cough, and 100% of the placebo group developed mucopurulent nasal discharge. The

Table 8. *2X dose and 1X dose H3N2 Vaccine Group Prevented Fractions for nasal shedding*

Vaccine Group	Number of dogs with positive nasal shedding	P-value (Fisher's Exact Test)	Prevented Fraction (95% CI)
2X CIV (n=10)	2	0.0007	0.80 (0.46, 0.96)
Placebo (n=10)	10		
1X CIV (n=10)	2	0.0007	0.80 (0.46, 0.96)

Table 9. *Dogs seropositive (≥ 4) for CIV antibodies*

Vaccine Group	Number of dogs seroconverted					
	Day 0*	Day 7	Day 14	Day 21*	Day 27#	Day 35†
2X CIV (n=10)	0	0	1	7§a	10§b	10
1X CIV (n=10)	0	0	0	2	10§b	10
Placebo (n=10)	0	0	0	0	0	6

**Vaccination days—sample collected prior to vaccination*
#One day prior to challenge †Seven days post-challenge
§significant difference between test vaccine and placebo
[a] p=0.0031 [b] p<0.0001

days with highest frequency were 3 DPC for cough (8 dogs) and 4 DPC for mucopurulent nasal discharge (10 dogs). No animals in the 2X CIV or 1X CIV vaccine groups developed cough or mucopurulent discharge. The highest frequency of fever occurred in six dogs; five in the placebo group and one in the 2X CIV vaccine group 2 DPC. Most dogs had one instance of fever, while one dog in the placebo group had 2 days of fever (Table 5). Rectal temperatures for dogs with fever ranged from 39.7°C to 40.4°C.

Ninety percent (90%) of the dogs in the placebo group met the case definition for clinical disease, thereby validating the challenge. The incidence of disease in dogs administered the H3N2 CIV vaccines was significantly reduced compared to the placebo vaccinated dogs (p=0.0001) (Table 6). Clinical disease was prevented in 100% of the CIV vaccinates, underscoring the effectiveness of the vaccines at preventing clinical disease.

Nasal Shedding and Serology

Nasal swabs were collected to assess the capability of the test vaccines to prevent viral shedding. In this study, the collection days also corresponded with the days on which the highest frequency of cough and mucopurulent nasal discharge were observed. All dogs in the placebo group exhibited nasal shedding of CIV. However, shedding was significantly reduced in the 2X and 1X dose groups on each collection day (Table 7). The prevented fraction with respect to reduction in shedding was 80% for the 1X and 2X CIV vaccines (Table 8).

Lee et al. (2010) demonstrated that vaccination reduced viral shedding from 8 days to 4 days post-challenge. Similarly, the CIV vaccines administered in this study reduced shedding to undetectable levels in 100% of the CIV vaccinated dogs at 5 DPC. Our data are also consistent with the observation that dogs not showing clinical signs of disease may still shed virus.[14] We found that 90% of the placebo dogs exhibited clinical disease, while 100% of the placebo dogs shed infectious virus. This result emphasizes the importance of limiting virus shedding as part of strategies to control virus transmission.

All animals were seronegative prior to the initiation of the study, and all dogs in the placebo group were seronegative prior to challenge (Table 9). On day 21, the proportion of dogs that seroconverted in the 2X

CIV group was significantly higher than in the placebo group (p=0.0031), and on day 27, all dogs in the 2X and 1X CIV groups had seroconverted. Hence, seroconversion correlated with protection of the vaccinated dogs from clinical disease.

CONCLUSIONS

This study demonstrates that subcutaneous vaccination of dogs with an inactivated H3N2 CIV vaccine was well tolerated, produced seroconversion, and successfully protected 100% of vaccinates from clinical disease caused by challenge with virulent virus. Notably, the vaccines also reduced nasal shedding of infectious challenge virus, substantiating their use as safe and effective measures to alleviate signs of disease caused by CIV and potentially control virus transmission.

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