Cross Protection of Vanguard 5L4-CV Vaccine against Virulent Canine Parvovirus-2c Circulating in the USA

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KEY WORDS: Canine Parvovirus, Vaccine, Crossprotection, CPV-2c strain

ABSTRACT

The objective of this study was to determine the ability of a modified live (ML) combination of Canine Distemper-Adenovirus Type 2-Parainfluenza-Parvovirus, inactivated Coronavirus vaccine and Leptospira Canicola-Grippotyphosa-Icterohaemorrhagiae-Pomona bacterin (Vanguard Plus 5 L4-CV; Pfizer Animal Health) to actively immunize 6- to 8- week old puppies against a virulent challenge with canine parvovirus (CPV) type 2c. Thirty CPV susceptible beagle pups were vaccinated twice, three weeks apart with either a 1.0 mL dose of the test vaccine or a negative control vaccine containing all the components previously cited except CPV. Five weeks after the second vaccination the dogs were challenged with 3.0 mL of CPV-2c (CPV-Arkansas) intranasally (0.5 mL per nostril), and orally (2mL) on Study Day 56. Dogs were observed daily for clinical signs, white blood cell count fluctuations, serological responses, and CPV-2c viral shedding. Dogs vaccinated with the vaccine containing CPV showed no clinical signs of infection, did not have lymphopenia, or shed challenge virus in their feces in contrast to negative control dogs, which displayed all the signs typical of infection with pathogenic CPV, including shedding of challenge virus in their feces. Dogs vaccinated with the material containing CPV also developed a high and protective serologic

response after the first dose of vaccine.

INTRODUCTION

Canine parvovirus (CPV) remains the most significant viral cause of enteritis in puppies over 2 months of age. The virus infects the intestinal epithelium, causing crypt dilation, necrosis, and villous atrophy. As a consequence, the absorption of food from the intestine is impaired. Canine parvovirus type 2 (CPV-2), one of the feline parvovirus (FPV) subspecies, first emerged in the late 1970s (Appel et al; 1979). In the early 1980s, the first CPV (antigenic type 2: CPV-2) strain was replaced by a new antigenic variant CPV-2a, with a third variant (CPV-2b) appearing shortly afterwards (Truyen 1999). During the late 1980s and into the early 1990s, these prototype CPV-2a and 2b viruses were replaced by other "new CPV-2a and 2b" variants (Ohshima 2009, Battilani et al 2001).

A new antigenic type (CPV-2c) was reported for the first time in Italy in 2001 (Buonavoglia et al 2001) and has since been detected in Western Europe (Decaro et al 2007), including Spain (Decaro et al 2005), and Germany; Asia (Vietnam and Japan); and South America (Pérez et al 2007). CPV-2c has also been isolated from samples collected in India (S. Nandi et al. 2010) and in the United States (Hong, C et al. 2007). The emergence and spread of CPV-2 variants with different epidemiological and antigenic properties has both evolutionary and clinical relevance worldwide. The strains of CPV in currently available live attenuated vaccines are derived from either CPV-2b isolates or the original type CPV-2 virus. The type 2 virus has been entirely replaced in the field by 2a, 2b and now 2c, and concerns have been voiced as to the level of protection afforded by these attenuated vaccines. The purpose of this study was to determine the ability of Vanguard® Plus 5L4-CV vaccine to cross-protect against a new CPV-2c strain isolated in the USA. The study was executed to 9CFR requirements for label approval registration purposes in the US.

MATERIALS AND METHODS

Vaccine and Challenge Viruses

The Vanguard® Plus 5L4-CV® vaccine containing live attenuated CPV-2 strain NL-35-D at minimum commercial titer and an experimental version of the same vaccine without live attenuated CPV-2 (negative control) supplied by Pfizer Animal Health Ltd., Lincoln NE, United States, were used. Both vaccines were prepared by adding a liquid preparation of inactivated canine coronavirus to the freeze-dried preparation. Both vaccines tested satisfactory for purity (sterility) and potency prior to study initiation. A dose of 1 mL per dog was administered by subcutaneous (SC) injection between the shoulder-blades at both vaccination times.

The challenge virus originated from a fecal sample isolated from a dog infected with CPV-2c, kindly provided by Prof. Ronald D. Schultz from the Department of Pathobiological Sciences, College of Veterinary Medicine, University of Wisconsin, Madison Wisconsin.

To increase virulence, the fecal sample was diluted in DMEM media, passed in dogs and purified in a prior study (data not shown). In this study, a single nasal-oral challenge dose of 3 mL induced all the characteristic signs of CPV disease with 100 % morbidity, and 75 % mortality. Four of five dogs demonstrated all four clinical signs characteristic of CPV infection starting 24 hours after challenge. All dogs shed CPV as determined by viral isolation from the fecal samples collected.

The resulting virus preparation was identified as CPV strain 2c via both a restriction fragment length polymorphism assay as well as nucleotide sequencing of a PCR fragment spanning codon 426 of the capsid gene. The challenge virus preparation was stored frozen (-70 ºC ± 10 ºC). Upon thawing, these preparations provided single oral-nasal challenge dose of 3.0 mL of the challenge material preparation, which was administered via the oral-nasal route to each dog. One half of a milliliter was administered in each nostril and 2 mL were administered orally.

Table 1. *Experimental Design*

Treatment Group	Group Description	Number of Dogs (N)	Route of Administration	Study Day						
				Vaccination	General Health Observations	Challenge	Rectal Temperature	Blood sample collections for WBC	Fecal Sample collections	Clinical Observations
T01	Controls	10	SC*	0 and 21	-7 to 55	56	54 to 70	54 to 69	54 to 70	56 to 70
T02	Vaccination	20								

** Subcutaneous*

Animals

Thirty healthy, CPV-susceptible, purpose-bred Beagle dogs 6 to 8 weeks of age at first vaccination and ≥ 1 kg of body weight participated in this study.

Experimental Design

This study was conducted in accordance with the 9CFR requirements for modified live vaccines against canine parvovirosis for label approval registration purposes in the US. Thirty clinically healthy specific pathogen free (SPF) Beagle dogs were randomly allocated to rooms, pens, and two treatments (negative control and vaccinated dogs, T01 and T02 respectively) as shown in Table 1 using a generalized block design. Blocking was based on date of birth and litter. Dogs were vaccinated with either an experimental Vanguard Plus 5L4-CV Control vaccine without CPV (negative controls or T01; n = 10 dogs) or with Vanguard Plus 5L4-CV® with CPV-2 (vaccinates or T02; n= 20 dogs).

The first dose of vaccine was administered when the dogs were 7-8 weeks old (Day 0). The second dose was administered when the dogs were 9-10 weeks old (Day 21). Each treatment group of dogs was housed in a completely separate controlled airspace with hygienic measures employed to prevent any cross-contamination within the testing facility. General health observations were collected from each dog from Day -7 to Day 53 of the study.

Thirty five days after the second vaccination (i.e., on Day 56), all dogs were challenged with CPV-2c and housed in individual cages. Clinical observations including rectal temperature, skin turgor to detect dehydration, presence or absence of anorexia, emesis, diarrhoea and lethargy were recorded twice daily starting 2 days before challenge (Day 54) until the end of the study (Day 70). Animals deemed unfit to continue the study were determined by a veterinarian based on refined humane endpoints for animals in pain (Stokes, 2002) and were euthanized using Beuthanasia® (Intervet) solution. Experimental clinical procedures were conducted in compliance with the animal welfare act and with the approval of the site IACUC.

Blood Sample Collection

Blood samples were collected from each animal for serology on Days 0 and 21, prior to the first and second vaccination respectively, pre-challenge on the day of challenge (Day 56), and the day before study completion (Day 69). Samples were collected from the jugular and/or cephalic vein into plain (without anticoagulant) tubes (Vacutainer® Becton Dickinson, Franklin Lakes, NJ), centrifuged, and serum was stored frozen

***Table 2**. Individual Animal Listing (Criteria for Infection)*

Treatment	Animal ID	Fever	Clinical Signs							Lymphopenia	Leukopenia *	Post challenge Viral Isolation**	Infected
			Diarrhea	Mucus in feces	Blood in feces	Vomit	Lethargy	Dehydration	Anorexia				
Controls (T01)	1030703	y	y	y	n	n	n	n	y	y		y	y
	1030707	y	y	y	y	y	y	y	y	y	y	y	y
	1030802	n	y	y	y	y	y	n	y	y	y	y	y
	1030905	y	y	y	y	y	n	y	n	y	y	y	y
	1031002	n	y	n	n	y	n	n	y	y		y	y
	1031004	n	y	y	y	y	n	n	y	y		y	y
	1031101	y	y	y	y	y	n	n	y	y	y	y	y
	1031104	y	y	y	y	y	n	n	y	y	y	y	y
	1060903	n	y	y	y	y	n	n	n	y		y	y
	1060904	y	y	y	y	y	n	n	n	y		y	y
Vaccinated (T02)	1030701	n	n	n	n	y	n	n	n	n	n	n	n
	1030702	n	n	n	n	n	n	n	n	n	n	n	n
	1030704	n	y	n	n	n	n	n	n	n	n	n	n
	1030705	n	n	n	n	n	n	n	n	n	n	n	n
	1030706	n	n	n	n	n	n	n	n	n	n	n	n
	1030801	n	n	n	n	n	n	n	n	n	n	n	n
	1030901	n	y	y	n	n	n	n	n	n	n	n	n
	1030902	n	n	n	n	n	n	n	n	n	n	n	n
	1030904	n	n	n	n	n	n	n	n	n	n	n	n
	1030906	n	y	y	n	y	n	n	n	n	n	n	n
	1031001	n	n	n	n	n	n	n	n	n	n	n	n
	1031005	n	n	n	n	n	n	n	n	n	n	n	n
	1031006	n	n	n	n	n	n	n	n	n	n	n	n
	1031102	n	n	n	n	n	n	n	n	n	n	n	n
	1031103	n	y	n	n	n	n	n	n	y	y	n	n
	1031105	n	n	n	n	n	n	n	n	n	n	n	n
	1060901	n	n	n	n	n	n	n	n	n	n	n	n
	1060902	n	n	n	n	n	n	n	n	n	n	n	n
	1060905	n	n	n	n	n	n	n	n	n	n	n	n

[1]Had at least 3 out of the 4 criteria for infection (i.e. lymphopenia, clinical signs, measurable viral shed ≥ $10^{3.3}$ ($TCID_{50}$ / gram).

[2] Had not more than one out of four criteria of infection.

** 5 of 10 were euthanized on Day 5.*

***Viral shedding.*

(-25 ºC ±5 ºC) until further testing.

Blood samples for white blood cell (WBC) counts were collected from each animal on study Day 54, 55, 56 (pre-challenge), and 57 to 69 inclusive. Blood was collected from the jugular and / or cephalic vein of each dog into EDTA tubes (Vacutainer® Becton Dickinson, Franklin Lakes, NJ), according to standard operating procedures at the site.

Clinical Signs

Clinical observations were initiated once daily on study Day 54 (2 days before challenge) to establish a baseline and twice daily on study Day 56 to 70. Dogs were examined for clinical signs indicative of disease associated with CPV including diarrhea, emesis, dehydration, mucus in stool, bloody stool, anorexia, lethargy, and dehydration (Table 2).

Serological Assays

The serological response to vaccination was assessed by quantification of CPV neutralizing antibodies. The neutralization titer was determined in a micro-titer serum neutralization (SN) test for the detection of neutralizing antibodies to CPV-2 developed at the test facility laboratory. Briefly, sera were tested in a SN test using dog kidney (DK) cells and 50 to 300 $TCID_{50}$ of the CPV-2 as the reference virus. Two-fold dilutions of the sera were analyzed in quadruplicates and incubated with the reference virus for 1 hour at room temperature. Each test plate included intra-assay positive and negative control sera to determine plate validity. Afterwards, 50 µL of virus-serum mixture was transferred to susceptible DK cells and further incubated at 37°C for 3-4 days in a humidified 5% CO2 atmosphere.

Cells then were fixed with ice-cold 80% acetone for 10 min and CPV-infected cells were identified by an imunoflorescence assay using a parvovirus fluorescent antibody (FA) conjugate. SN titers were calculated using the Spearman-Karber formula. The diluted virus used in each test was titrated to confirm that an acceptable amount of virus was used in the test. Tests were considered acceptable if the positive antiserum control exhibited a titer within the established four-fold range, and the negative antiserum control displayed characteristic viral FA in all wells.

The back-titration of the CPV-2 used as the reference virus in this test was conducted according to the 9CFR Section §113.317 requirements. Briefly, the virus back-titra-

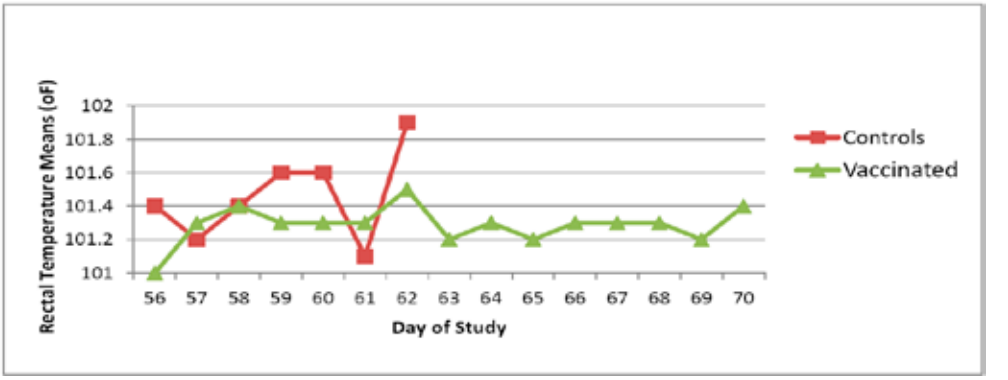
Table 3. *Antibody titer geometric means by treatment group and day of study*

Treatment	Day of study	Animals	Geometric Mean	Minimum	Maximum
Controls	0	10	1	1	1
	21	10	1	1	1
	56*	9	1	1	1
	61	5†	1	1	1
	62	5†	1	1	1
Vaccinates	0	20	1	1	1
	21	20	3990.8	2048	5793
	56*	19	2243.7	431	4871
	69	20	3723.5	1448	5793

**D56 had a duplicate sample number; these two results were flagged out of the analysis [1(T01) / 1 (T02)], Challenge day.*

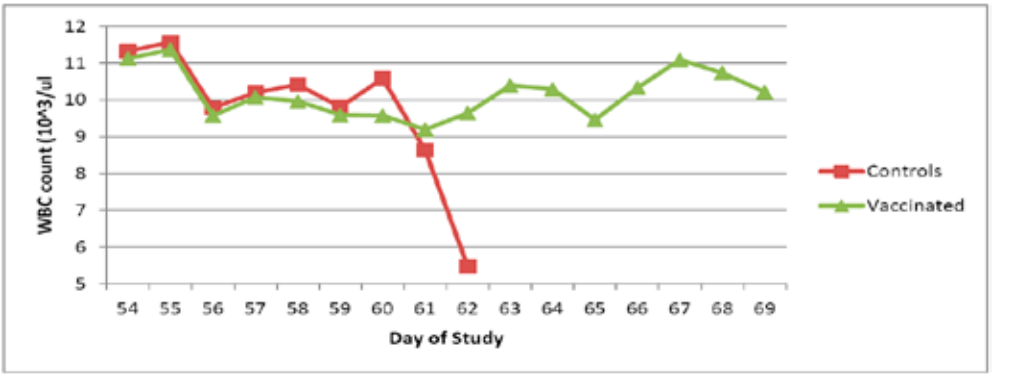
† Five dogs were euthanized for humane reasons five days after challenge, and the other five 6 days after challenge.

***Figure 1**. Rectal temperature (°F) arithmetic means by study day for each treatment group**



** Control dogs were euthanized 5 days after challenge on Day 61 (n=5) and 6 days after challenge on day 62 (n=5).*

***Figure 2**. WBC arithmetic mean values for each treatment group by study day**



** Control dogs were euthanized 5 days after challenge on Day 61 (n=5) and 6 days after challenge on day 62 (n=5).*

tion test was performed in microtiter plates. Tenfold serial dilutions of the challenge virus preparation were performed in order to obtain 10^{-1}, 10^{-2}, and 10^{-3} dilutions. From each dilution, eight wells were plated for a total of 16 wells per dilution. Negative cell controls were included on the back titration plate utilizing the diluent media. The back-titration plate was incubated at the same time the SN plates were.

White Blood Cell Counts

White blood cell (WBC) counts were performed three times pre-challenge, (2 and 1 days before challenge, and on the day of challenge), and 13 days post-challenge (1 - 13 days post challenge). Blood samples were collected from the jugular vein into EDTA-containing tubes (EDTA-Vacutaner® Becton Dickinson, Franklin Lakes, NJ). The total number of leukocytes [x 10^9/L] in each blood sample was determined with an Advia 120 Hematology System (WS-ADVIA120®Siemmens).

Fecal Virus Shedding

Individual fecal samples were collected from each dog daily from the day of challenge to the end of the study either from the pen or directly from the rectum of the animal. Each fecal sample (> 1 gram) was weighed and 4 times as much phosphate buffer saline (PBS) was added to make a 1:5 suspension. After homogenization, centrifugation at 1700 x G, and 0.2 um filtration each fecal supernatant was further diluted 1:64 in tissue culture media in accordance with 9 CFR §113.317. The resulting 1:320 preparation (or 3125 ug of feces / mL) was then subjected to 10-fold serial dilutions. Four replicate wells of susceptible DK cells were each inoculated with 0.1 mL of each dilution and incubated for 4

days. After immuno-staining, viable virus titers were calculated using the Spearman-Karber formula and reported either as negative (< $10^{3.0}$ $TCID_{50}$ / gram) or as positive (≥ $10^{3.3}$ $TCID_{50}$ / gram).

Data Analysis

Data were analyzed and summarized using SAS Release 9.1.3 (SAS Institute, Cary, NC) based on the 9 CFR, CHI § 113.317 requirements for modified live vaccines against canine parvovirosis. A control animal was considered infected if it met at least three of the following four criteria of infection during the observation period: 1. fever defined as rectal temperature ≥103.4°F; 2. lymphopenia defined as lymphocyte reduction ≥50 percent of pre-challenge normal (average of the three pre-challenge values); 3. Clinical signs such as diarrhea, mucus in feces, or blood in feces, and 4. Virus isolation ≥ 3.0 (≥$10^{3.0}$ $TCID_{50}$/gm). A vaccinated animal was considered infected if more than one of the above criteria of infection was observed.

As per 9CFR §113.317, the vaccine was considered efficacious if at least nineteen (19) of twenty (20) vaccinates survived the post challenge observation period showing no more than one of the criteria of infection described in the paragraph above.

Data were analyzed using a Cochran-Armitage test adjusting for block. All hypothesis tests were conducted at the 0.05 level of significance (two-sided).

RESULTS

All control animals were seronegative to parvovirus and free of CPV virus isolation until time of challenge. Rectal swabs, fecal and blood samples, as well as clinical observations were collected according to the study design from both treatment groups (Table 1). All ten control animals demonstrated infection [lymphopenia, fever (≥103.4) or clinical signs and measurable viral shed (≥ $10^{3.3}$ $TCID_{50}$ / gram)], while no animal in the vaccinated group demonstrated infection [19/20 animals survived the observation period without showing fever (≥ 103.4), and measurable viral shed ≥ $10^{3.3}$ ($TCID_{50}$ / gram).

One of the vaccinated animals showed either lymphopenia or a clinical sign during the post challenge period of observation. The individual listing of clinical signs characterizing infection (following 9CFR CH1, §113.317) is summarized in Table 2. Control dogs were euthanized 5 days after challenge on Day 61 (n=5) and 6 days after challenge on day 62 (n=5) for humane reasons based on refined endpoints (Stokes WS, 2002) after collecting the required data to show vaccine protection.

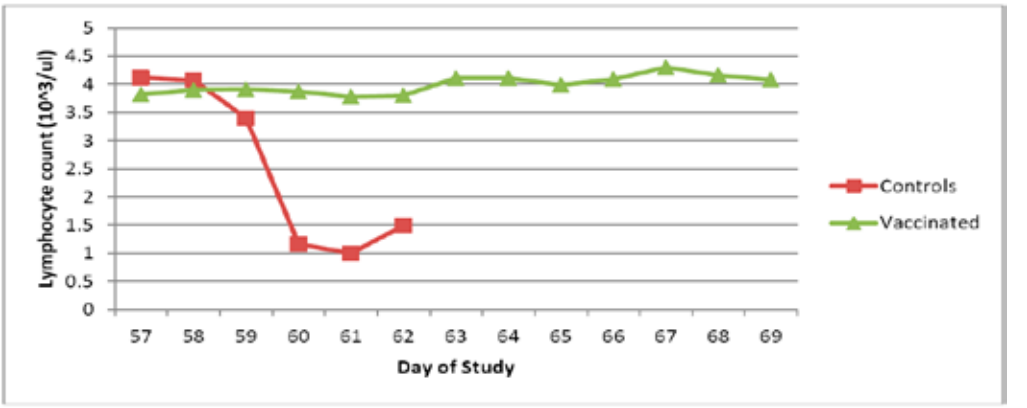
Clinical Signs

There was a significant difference (P≤0.05) in all of the individual clinical signs listed previously ever observed post-challenge between vaccinated and control dogs. The clinical symptoms subsequently observed from study Day 56 to 70 are summarized in Table 2. All ten control dogs exhibited diarrhea, nine exhibited vomiting and mucus in stool, seven had bloody stools, eight were anorexic, three animals exhibited lethargy, and two animals were dehydrated. One of the control dogs had an observation of other clinical signs, i.e, ocular discharge (Day 54 and 55) and one animal in the non vaccinated group exhibited diarrhea (D55) prior to challenge. Four of the vaccinated animals exhibited diarrhea (post challenge), two exhibited vomiting, and two had mucus in stools. There were no other clinical signs reported.

Body Temperature

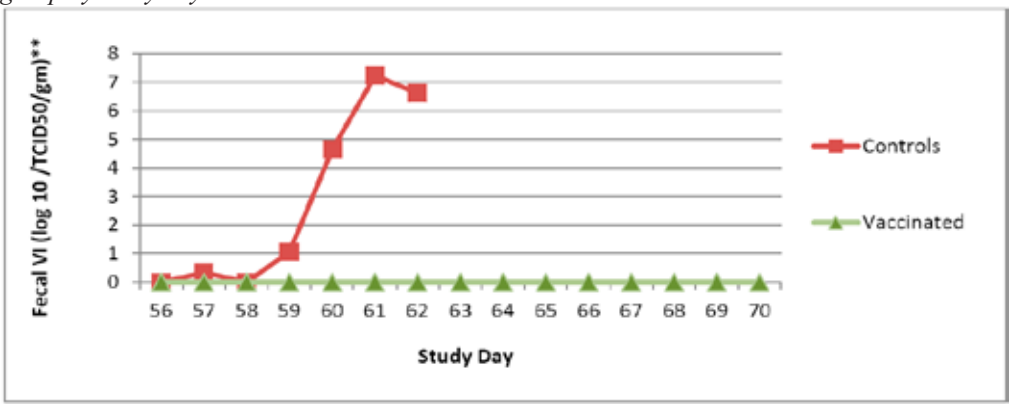
Rectal temperatures were measured twice daily beginning on study Day 56 through Day 70. In accordance with 9CFR , a rectal temperature of ≥103.4ºF was classified as febrile. Overall, before challenge arithmetic means of rectal temperatures were within the normal range for both treatment groups on all days. However, there was a significant difference between treatments in an animal ever being febrile post-challenge (P≤0.05). Six control animals were recorded as febrile on study day 60 (4 days after challenge). No animal in any treatment group was febrile at any other time point (Table 2; Figure

***Figure 3**. Lymphocytes arithmetic mean values for each treatment group by study day**



** Control dogs were euthanized 5 days after challenge on Day 61 (n=5) and 6 days after challenge on day 62 (n=5).*

***Figure 4.** Fecal live virus content (log 10 $TCID_{50}$ / gram) geometric means for each treatment group by study day**



** Control dogs were euthanized 5 days after challenge on Day 61 (n=5) and 6 days after challenge on day 62 (n=5).*
*** Undetectable levels (≤3) are represented as 0.*

1).

Lymphocyte Count

By Day 60 (Day 4 post-challenge) all control animals had lymphopenia (defined as a reduction in lymphocytes ≥ 50% of pre-challenge normal calculated as average of the three pre-challenge values). Arithmetic means for lymphocytes are shown in Figure 2.

White Blood Cell (WBC) Count

There was a significant difference ($P \leq 0.05$) in an animal ever being lymphopenic post-challenge between vaccinated and control animal treatments. Arithmetic means of WBC counts are shown in Figure 3.

Fecal Viral Levels

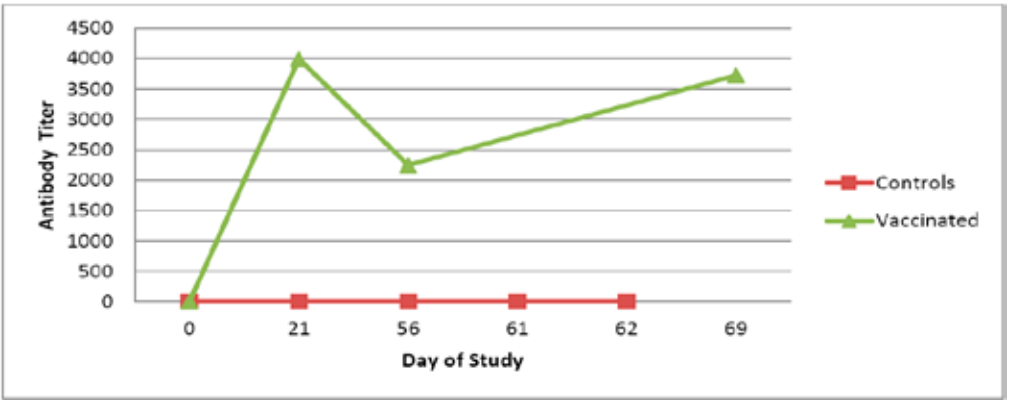
There was a significant difference ($P \leq 0.05$) between vaccinated and control animals in ever being positive for CPV post-challenge on study Day 60 and 61 (Figure 4).

Serology

Serum samples were assessed for CPV neutralizing antibodies. Serological titers to CPV on study Day 0 indicated that none of the dogs had been previously exposed to CPV. All of the vaccinated animals developed rising antibody titers Control animals did not develop detectable antibody titers. A summary of geometric mean antibody titers by treatment group and day of study is shown in Figure 5.

DISCUSSION

***Figure 5**. Serum antibody titer geometric means by treatment and study day*



** Control dogs were euthanized 5 days after challenge on Day 61 (n=5) and 6 days after challenge on day 62 (n=5).*

The CPV-2 strain in Vanguard Plus 5 L4 CV®vaccine has previously been shown to protect vaccinated dogs challenged with CPV, CPV-2a, and CPV-2b. Further investigation was needed to evaluate the effectiveness of this vaccine strain against the latest circulating variant, CPV-2c. A study conducted in Germany (Siedek et al 2011) in accordance with the EU Pharmacopeia using Vanguard® 7(Pfizer Animal Health) tested a modified live Canine Parvovirus antigenic type 2 strain, and demonstrated cross-protection against a CPV-2c strain circulating in Europe. In that study, control animals (n=2) began showing classical clinical signs associated with canine parvovirus 4 days post-challenge. Both control animals, as expected demonstrated typical signs of canine parvovirus infection, while the vaccinated animals (n=5) did not exhibit any abnormal clinical signs post-challenge.

In the current study, control animals challenged with CPV-2c were observed to have two distinct periods of increased rectal temperature (Fig. 1). The first wave was observed approximately 48 hours after the challenge, coinciding with lymphopenia, which was observed in all the control dogs (Fig. 3). Following a slight improvement in clinical signs lasting approximately 12-24 hours, leucopenia (Fig. 2) and a second distinct period of increased rectal temperature (≥103.4ºF) was observed in 60 % of the control animals.

All the control animals showed clinical signs (Table 2). Infection and destruction of lymphoid tissue is an important characteristic of both canine parvovirus enteritis (Meunier et al 1985b; and Decaro and Buonavoglia 2012) and feline panleukopenia (Carlson et al 1978). Plasma viraemia is associated with virus-mediated lympholysis. The results of this study indicate that viraemia preceded intestinal epithelial infection, and suggest that initial viral replication occurred in extra-intestinal lymphoid tissue, as reported previously with the other CPV variants (Meunier et al 1985b; and Decaro and Buonavoglia 2012).

All control dogs showed viraemia before the virus was detected in the feces (data not shown). Viraemia is the most critical event in the immunopathology of CPV enteritis because the virus reaches the intestinal epithelium from the bloodstream, not from the alimentary canal (Meunier et al 1985a,b; Patel and Heldens 2009; and Decaro and Buonavoglia, 2012). The magnitude and duration of the CPV viraemia correlates directly with the severity of disease and with fecal shedding (Williams et al, 2001; and Meunier et al, 1985b). The termination of the viraemia coincides with the onset of a circulating antibody response; therefore the humoral antibody level is the controlling factor in achieving protection and is a reliable indicator of CPV immunity (Williams et al 2001; and Meunier et al, 1985b).

In this study, vaccinated dogs were not only protected from clinical disease but also did not shed challenge virus. This finding corresponds with that reported by Greenwood et al (1995) in a similar study with another CPV vaccine. Viral shedding started 72 hours after challenge in 3 of the control animals, with control animals shedding large amounts of virus ($\geq 10^4$ $TCID_{50}$/gm of feces) by day 4 post challenge. The length of viral shedding was not determined in this study; five of the control dogs were euthanized on day 5 and the rest on day 6 after challenge for humane reasons based on refined endpoints (Stokes, WS; 2002). Spibey et al (2008) reported that the duration of virus shedding in control animals subjected to CPV-2c challenge was similar to that observed with the other CPV strains.

Dogs in the control group had the highest frequency of positive clinical signs observed vs. vaccinated animals, this being statistically significantly different ($p= 0.0004$). All of the control animals developed lymphopenia compared to one of the 20 vaccinated animals ($p<0.0001$). Six of ten control animals experienced pyrexia ($\geq$103.4 ºF), while none of the vaccinated animals experienced pyrexia ($p = 0.0005$). The control group had CPV isolated at the highest frequency, with 100% of dogs in this group positive for CPV isolation beginning on study Day 59 (3 days post challenge) and no detectable viral shedding in the vaccinated animals through study Day 70 ($p< 0.0001$).

The first dose of Vanguard Plus 5 L4 CV® vaccine generated a very high and protective antibody response, which was detected 21 days post vaccination (Figure 5). In support of this data, no viraemia was detected in these dogs. There was no anamnestic response following challenge in the vaccinated dogs, indicating that they had sterilizing immunity to CPV-2c (Figure 5). The humoral antibody level is the controlling factor in achieving protection, and is a reliable indicator of CPV immunity. This data, together with that of Spibey et al (2008), support the view that despite the minor differences between the original type 2 virus and the 2a, 2b and now 2c variants, dogs vaccinated with Vanguard Plus 5 L4 CV® will mount a robust immune response to CPV and are fully protected against challenge from any of the current CPV types.

Conclusion

Canine Distemper-Adenovirus Type 2 Coronavirus-Parainfluenza-Parvovirus Vaccine, Modified Live Virus and Killed Virus, Leptospira Canicola-Grippotyphosa- Icterohaemorrhagiae-Pomona Bacterin (Vanguard Plus 5 L4 CV®), actively immunized 6 to 8-week old, MDA free puppies against a virulent challenge of canine parvovirus type 2c (CPV-2c) and dogs vaccinated with this vaccine did not shed virus after challenge.

ACKNOWLEDGEMENTS:

The authors wish to thank Prof. Dr. Ronald D. Schultz from the Department of Pathobiological Sciences, College of Veterinary Medicine, University of Wisconsin, Madison Wisconsin who kindly provided us with a fecal sample isolated from a dog infected with CPV-2c, Dr. Albert Ramudo from Pfizer Animal Health Clinical Department, Kalamazoo, MI who wrote the study protocol for the challenge dose determination and Dr. Cass Tucker from Pfizer Animal Health Clinical Department, Kalamazoo, MI who conducted the challenge determination study and provided us with the information of the challenge dose used in this study.

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