Canine Parvovirus (CPV) Type 2b Vaccine Protects puppies with Maternal Antibodies to CPV when Challenged with Virulent CPV-2c Virus

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ABBREVIATIONS

CPV=canine parvovirus

$FAID_{50}$=50% fluorescent antibody infectious dose

FPV=feline panleukopenia virus

MDA=maternal derived antibody

MDCK=Madin-Darby canine kidney

SN=serum neutralization

$TCID_{50}$=50% tissue culture infective dose

HI=hemagglutination inhibition

ABSTRACT

This study was conducted to evaluate canine parvovirus disease prevention efficacy of the minimum immunizing dose of the CPV-2b fraction of a multivalent vaccine when administered at approximately 6 weeks of age to pups with maternal CPV-2b antibodies. A second dose was administered 4 weeks later. Pups were challenged with a virulent strain of CPV-2c virus 2 months after the second vaccination. Efficacy was evaluated by monitoring the pups for various clinical observations and laboratory testing of parvovirus infection, including mucous stool, bloody stool, diarrhea, fever, death, leukopenia, lymphopenia, CPV-2b serum neutralization titer, and detection of CPV in the feces. Upon a severe challenge with a virulent CPV-2c virus, four of five (80%) control pups had at least three of four clinical signs of CPV infection while 19 of 20 (95%) vaccinated pups had not more than one sign of CPV infection. The response of the control pups confirmed the virulence of the challenge and validity of the study. The response of the vaccinated pups demonstrated the efficacy of the CPV-2b vaccine, even in puppies with maternal antibody, which was one of the main objectives of this study. The outcome of this study was consistent with the 9CFR requirements necessary to support an additional label claim that the vaccine

aids in the prevention of disease caused by CPV-2c when administered to puppies as young as 6 weeks of age with maternal CPV antibodies.

INTRODUCTION

Canine Parvovirus (CPV) caused a severe pandemic when it was first identified in 1978 as an emerging disease that caused hemorrhagic enteritis associated with leukopenia and high mortality. Named CPV type 2 (CPV-2) to distinguish it from the antigenically unrelated CPV type 1, also known as minute virus of canines,[1] CPV-2 spread rapidly worldwide. CPV-2 is closely related to feline panleukopenia virus (FPV), and most likely evolved from FPV or another closely related virus.[2,3] While closely related, phylogenetic analysis revealed CPV-2 differs from FPV by 16 nucleotide amino acid substitutions.[3]

In 1979, CPV evolution continued with the emergence CPV-2a, which varies from CPV-2 by seven amino acid substitutions and one epitope.[2-4] CPV-2a essentially replaced CPV-2 worldwide by 1982.[5] Another variant, CPV-2b, emerged in 1984 and became the predominant CPV variant worldwide by 1988.[4] CPV-2b differs from CPV-2a only in the changed residue 426-Asn to Asp.[4,5] In 2000, the latest variant, CPV-2c, was first detected in Italy[6] and subsequently detected in Vietnam,[7] Spain,[8] Uruguay,[9] North America,[10,11] Portugal,[12] Japan,[13] and Greece.[14] This latest variant, CPV-2c, differs from both CPV-2b and CPV-2a in the same codon for residue 426, which changed to Glu.[2] Thus, there is not a great deal of antigenic difference among CPV-2a, CPV-2b, and CPV-2c compared to the difference between CPV-2 and the three variants.

The predominant CPV-2 subtype varied geographically and changed over time. In Italy CPV-2a still predominated in 2001 and 2002, but CPV-2b and CPV-2c were also co-circulating.[15] Similar results were noted in samples collected in 2008 and 2009 in Greece.[14] In contrast, samples collected in the United States in 2006 and 2007 were predominately CPV-2b in one study[11] and nearly equally split between CPV-2b and CPV-2c in another study[10] with relatively few occurrences of CPV-2. Approximately equal distribution between CPV-2b and CPV-2c was also documented in Portugal in 2006 and 2007.[12] CPV-2b predominated in a recent India study[16] and from 1997 to 2006 CPV-2b predominated in Japan.[13] A Brazilian study of CPV variants in 37 samples collected from 1995 to 2009 and a United Kingdom study of 255 samples collected from 2006 to 2008 revealed both CPV-2a and CPV-2b, but not CPV-2c.[17,18] In contrast, in Uruguay, CPV-2c predominated in 2006.[9]

Before a canine origin CPV vaccine was developed, veterinary practitioners used FPV vaccine to inoculate dogs, which provided some protection.[19-21] Inactivated CPV-2 vaccines followed,[22] but protective immunity was short-lived compared to the subsequently developed modified live CPV-2 vaccines, which were still blocked by maternal derived antibody (MDA).[23] Since the appearance of CPV variants, concerns were raised with modified-live CPV vaccine based on the original type CPV-2 virus regarding efficacy against variants,[24] especially CPV-2b[16] and CPV-2c,[8] and it was suggested CPV vaccines should be updated to include one of the more recent variants. Field experience certainly indicates some dogs fully vaccinated with original type CPV-2 products succumb to illness due to CPV-2 variants.[9,10,14] In January 2007, an outbreak of CPV-2c was reported in Italy in adult dogs that had been repeatedly vaccinated with original type CPV-2 vaccines.[25]

A study comparing unvaccinated control puppies with puppies vaccinated with CPV-2 or CPV-2b vaccine demonstrated both vaccines performed equally well in preventing disease in puppies challenged with CPV-2b and CPV-2c, but all of the puppies in that study were free of CPV MDA at the time of vaccination.[26] Similarly, studies of specific pathogen-free Beagles without MDA showed original type CPV-2 vaccination provided some indication of cross protection against CPV-2c challenge[27] or CPV-2b and

CPV-2c challenge.[28]

While those studies attempted to address some of the cross protection concerns regarding original type CPV-2 versus CPV-2b vaccination protection against CPV-2b and CPV-2c infection, they did not address concerns about MDA interference, which has been the primary efficacy problem for CPV vaccination of puppies.[29,30] However, based on results of a seroconversion after CPV-2b vaccination study of puppies with demonstrable CPV MDA, Pratelli et al concluded that vaccination with the CPV-2b variant was more effective at overcoming MDA than modified-live, 10^7 $TCID_{50}$/dose, CPV-2 vaccines.[31] Although the current CPV-2b vaccine strain in the study vaccine was previously approved for efficacy against a CPV 2b challenge[42] in pathogen free puppies, the main focus of the current study is to investigate its ability to protect against CPV2c challenge in pups with MDA at the time of initial vaccination.

MATERIALS AND METHODS

Puppies were selected at about 4 weeks of age, 2 weeks before the first vaccination, by screening for CPV-2b serum neutralization (SN) antibody titers (titers are expressed in reciprocal dilutions). Previously, a large number of dogs sourced from Class A suppliers were screened over a period of 1 year and the SN titers were determined. Dogs with typical MDA titers were selected based on the titers of two thirds of the range from the lowest to the highest titer. Thus, dogs were selected with titers of 64 to 400, which would result in SN antibody titers between 2 and 128 on the day of the first vaccination (Day 0) based on the estimated half-life of MDA for CPV of 9.7 days.[29] The goal was to document that the pups had serum MDA present when the first vaccination was administered at about 6 weeks of age.

The second vaccination was administered four weeks after the initial vaccination (Day 28). Serum samples were taken for CPV-2b SN testing on Study Days 0, 7, 28, 41, 56, 70, and 84. The pups were challenged with virulent CPV-2c two months after the second vaccination (Day 84). The four clinical and laboratory criteria of CPV disease: 1) clinical observations, 2) body temperature (fever), 3) lymphocyte count (lymphopenia), and 4) fecal CPV isolation were monitored daily starting 2 days before challenge to establish baseline and for 10 days after challenge to detect susceptibility to or immunocompetence against CPV-2c disease.

Vaccine and Challenge Virus

A prototype Duramune Lyme®+Max 5-CvK/4L vaccine was produced and tested according to the Outline of Production with the exception of CPV, which was formulated at the minimum immunizing dose. The experimental freeze-dried modified live virus fractions containing canine distemper virus, canine adenovirus Type 2, canine parainfluenza virus, and CPV were reconstituted with 1.0 mL liquid inactivated canine coronavirus vaccine, Borrelia burgdorferi bacterin, and four serovars of Leptospira (canicola, grippotyphosa, icterohaemorrhagiae, and pomona) bacterial extracts. The challenge virus was originally isolated in California in 2007 from a CPV-diseased dog and later confirmed to be a CPV 2c virus by Dr. Sanjay Kapil of the Oklahoma State University Diagnostic Lab. It was determined that the challenge virus had the single substitution in the VP2 gene at amino acid residue 426.[10] The challenge virus was frozen at -80˚C until use, standardized and 1.5 ml was administered by intranasal and oral routes.

Test Animals and Vaccination

Immediately prior to the study, a physical examination was conducted, and only puppies deemed healthy were included in the study. Twenty-five puppies, 6 weeks of age with CPV maternal antibody, were obtained from a Class A commercial source and was randomized and allocated into two treatment groups. Puppies in good health before initiation of the study were selected to have had CPV SN antibody titers between 2 and 128 on Day 0 based on SN antibody screening at 4 weeks of age. Twenty of the puppies were allocated to a vaccinated group (Group A)

and were housed in an isolation facility for the duration of the vaccination phase of the test period. The remaining five puppies were assigned to a placebo control group (Group B) and were housed in a separate isolation facility. Puppies in both groups were observed daily for 3 days following each vaccination for adverse reactions. Puppies from Group A were vaccinated subcutaneously with 1.0 ml of the test vaccine on Day 0 and again 28 days later. The control puppies in Group B were administered a placebo of phosphate buffer saline.

Serology Testing

Blood samples were collected for serological evaluation of SN antibody titers from each dog before each vaccination on Day 0 and 28 and further on Days 7, 41, 56, 70, and 84 to monitor the antibody response. Collected serum samples were stored at below -60° C prior to SN testing and determination of SN antibody titers. Briefly, serum samples were heat-inactivated at 56 ± 2°C for 30 minutes. Assays were performed by combining 0.05 ml of serum in serial two-fold dilutions with an equal volume of a virus suspension containing approximately 100 tissue culture infective dose 50 ($TCID_{50}$) of the test virus. Serum-virus mixtures were incubated for 60 minutes at 36 ±1°C, after which cell suspensions of canine kidney cells were added to the virus-sera mixture.. The plates were incubated in a humidified carbon dioxide chamber at 36 ±1°C for 4 to 6 days, fixed in cold acetone, stained with a fluorescein-conjugated specific antiserum, and observed under an immunofluorescence microscope. Serum neutralization titers were calculated by the method of Reed and Muench.[32]

Clinical Assessments

Approximately 8 weeks after the second vaccination, all 25 puppies were challenged orally and intranasally with a virulent type 2c strain of CPV. During the challenge phase of the study, all dogs were randomized and housed in individual cages in an isolation facility.

Study personnel were blinded to the treatment groups and the pups were observed daily from 2 days before to 14 days after challenge for the presence or absence of diarrhea, mucous or blood in the stool, or death, clinical signs that are associated with CPV. Rectal temperatures for all animals were recorded 2 days before challenge to establish the base line; on the day of challenge; and daily for 10 days after challenge. Daily blood and fecals were collected 2 days before challenge, on the day of challenge, and then daily for 10 days.

Lymphocyte Counts

Approximately 2 mL of whole blood were collected into an EDTA tube from each puppy daily from 2 days before challenge and on the day of challenge in order to establish the base line counts. Samples were further taken and counted daily and for 10 days after challenge. Testing was conducted using an Abbott Cell-Dyne® blood cell counter for lymphocyte counts. A puppy was considered to have lymphopenia when there was a single occurrence of 50% reduction or greater of counts compared to pre-challenge baseline following challenge.

Fecal Virus Isolation

Isolation of CPV from fecal samples was performed by inoculating fecal filtrates onto Madin-Darby canine kidney (MDCK) cells. Fecal samples were collected daily either directly from the puppies, or from the cages of challenged dogs for 2 days before, on the day of, and 10 days after challenge.

A 10% (w/v) fecal suspension was prepared by mixing the thawed fecal material with growth medium. The fecal suspension was clarified by low-speed centrifugation, and the supernatant was collected. An equal volume of chloroform was added to the fecal supernatant. The chloroform/fecal supernatant was mixed well, allowed to separate into phases, and the aqueous phase was collected and titrated for CPV using a tissue culture assay. Briefly, a suspension culture of MDCK cells were planted in 96-well microtiter plates in 0.1 mL volumes. Plates were inoculated with 0.1 mL of ten-fold serial dilutions of the fecal supernatant, and then incubated for 4 to 6 days at 36 ± 1°C.

Plates were read by direct fluorescent antibody staining with antibody specific to CPV conjugated with fluorescein isothiocyanate. Results of the isolation were calculated according to the method of Reed and Muench, and expressed as $FAID_{50}$ per/mL.[32]

Criteria for a Valid Study

For the study to be considered valid, the design and results had to meet certain criteria defined in 9CFR §113.317 (c), (3), (i). At least 80% of placebo control canines had to demonstrate at least three of the following four CPV clinical parameters: 1) clinical signs such as diarrhea, mucus, or blood in feces; 2) rectal temperature greater than 103.4 °F and at least 1 degree of above pre-challenge baseline; 3) lymphopenia of greater than 50% of pre-challenge baseline; , and 4) isolation of CPV in feces during the post-challenge observation period. The criteria to achieve satisfactory efficacy against disease caused by CPV strain 2c in the presence of MDAs is that ≥ 95% of vaccinates must survive to the conclusion of the study without exhibiting more than one of the CPV clinical parameters.

RESULTS

Clinical Observations and Challenge Results

For the challenge phase of the study, four of the five control pups (80%) were positive after challenge for at least three of the four clinical disease criteria for CPV.

One of the placebo control pups died 8 days post-challenge. Samples of duodenum, jejunum, ileum, lung, liver, and spleen were collected and submitted to Iowa State University Veterinary Diagnostic Laboratory for bacteriology, histopathology, and fluorescent antibody testing for CPV. Necropsy results confirmed CPV as the cause of death. The control group results confirmed the virulence of the challenge and demonstrated the validity of the challenge dose.

None of the 20 vaccinated pups died. One puppy was positive for 2 criteria; 9 puppies were positive for a single criterion; and 10 were not positive for any of the criteria. Thus, 19 of the 20 vaccinated pups (95%) were positive for not more than one of the four criteria, which demonstrated the efficacy of the vaccine.

In the placebo control group the following observations were made in 4/5 of the puppies: bloody diarrhea (2-day duration); bloody diarrhea noted 1 day followed by mucoid, bloody diarrhea the next; mucoid, bloody diarrhea (3-day duration) followed by bloody diarrhea the next day; and one puppy had mucoid, bloody diarrhea (3-day duration) followed by death the next day. In the vaccinated group 5/20 of the pups had an abnormal stool; 1-day duration for 4/5 and 2-day duration for one of the pups.

For the fever criterion, a single occurrence of pyrexia, defined as rectal temperature of ≥ 103.4º F and at least 1.0º F above pre-challenge baseline, was considered as positive. A single occurrence of fever was noted in three of five (60%) of the control pups and 1 of 20 (5%) of the vaccinated pups.

For the lymphopenia criterion, a single occurrence of lymphopenia, defined as ≤ 50% of the pre-challenge baseline was considered positive. All five (100%) of the control pups and 3 of 20 (15%) of the vaccinated pups were positive for lymphopenia. Of the lymphopenic pups, one control pup had lymphopenia for 4 consecutive days; two control pups (one of which died) had lymphopenia for 3 days; and two control pups and all three vaccinated pups were lymphopenic for only a single day.

For the CPV viral shedding criterion, a single occurrence of viral isolation at > 1.8 $\log_{10}$ $FAID_{50}$/mL (50% fluorescent antibody infectious dose/mL) during the observation period was considered positive, which occurred in all five (100%) of the control pups and 1 of 20 (5%) of the vaccinated pups. Of those positive for this criterion, the control pups were positive for 4-8 days, whereas the vaccinated pup was positive on only a single occasion.

In summary, four of the five control pups (80%) were positive for at least three of the

four clinical disease criteria for CPV after challenge, none of the 20 vaccinated pups died and 19 of 20 vaccinates were positive for not more than one criterion. These results were consistent with 9CFR section 113.317, (c), (3), (i) criteria for immunogenicity of parvovirus vaccine.

CPV Serology

The results of CPV-2b SN titer testing performed prior to challenge revealed that SN titers ranged from 4 to 64 on Day 0 when the first vaccination was administered, which met study inclusion criteria (≥ 2 and ≤ 128) for these puppies. Divergence of SN titer data between control and vaccinate groups is evident from after Day 41. The GMT of the vaccinates reached titers greater than 6,000 on Day 56, and a value of greater than 15,000 on Day 84.

DISCUSSION

The objective of this study was to evaluate the CPV-2c efficacy of the CPV-2b fraction of a multivalent vaccine when initially administered to 6-week-old pups seropositive for maternal CPV antibodies and administered again 4 weeks later. Maternal antibodies, transferred to offspring via breast milk immunoglobulins IgG, IgM, and IgA and absorbed by the intestinal lining, provide neonatal immunity from a wide variety of diseases including CPV. Colostrum is particularly rich in immunoglobulins IgG and IgM. Secretory IgA is present throughout the lactation period.

Since the 1990s, several strategies have been used to create potentiated CPV vaccines to overcome the MDA-induced susceptibility period, including use of more current variants, more immunogenic strains, raising the viral antigenic mass or titer per dose, and lowering the serial passage.[33,34] The use of a high-titer vaccine of 10^7 $TCID_{50}$/mL (50% tissue culture infective dose) has been advocated by some.[34] But one study revealed vaccination with a modified–live CPV-2b vaccine with a titer of $10^{4.5}$ $TCID_{50}$/ per dose was quite effective at overcoming MDA, which calls into question why the comparatively lower titer CPV-2b vaccine would be more efficacious than the high titer CPV-2 vaccine regarding overcoming MDA.[31] Obviously, the titer or concentration of viral antigenic mass is but one of the factors involved. However, the titer of a vaccine may not be relevant if the vaccine immunogenicity differs greatly from the field variant or the vaccine virus is too attenuated to the point of being poorly immunogenic. There is a balance reached when creating a modified live vaccine that is immunogenic enough to be effective across variants and in the face of MDA, but sufficiently attenuated that it does not cause clinical disease. The result of balancing between these extremes determines the minimum immunizing dose of a vaccine, which is of course influenced by virulence of the viral strain used, viral attenuation (number of passages from the virulent infective virus), and the antigen load of the vaccine.

When evaluating the efficacy of CPV vaccination of puppies with MDA, a low level of MDA, which may not be detected by hemagglutination inhibition (HI), may prevent an immune response to CPV in puppies and may prevent an immune response to CPV in puppies and be insufficient to prevent clinical disease [29,35] or fecal shedding and viral transmission.[36,37] While the level of CPV MDA that interferes with immunization is less than can be detected by HI, SN assays such as viral neutralization and plaque reduction titers are more sensitive and can reveal the presence of CPV MDA in amounts that will interfere with the immunological response to CPV vaccine in puppies.[29,38] In addition, SN provides more accurate results regarding specificity than HI testing for antibody titer against virus that is either homologous to or heterologous with the variants.[39,40, 41]

A study that compared the CPV-2 and CPV-2b HI and SN titers of puppies inoculated with CPV-2 or CPV-2b vaccines demonstrated that CPV-2b titers resulting from original type CPV-2 vaccine were lower than those resulting from CPV-2b vaccine.[39] In another study antigenic analysis by cross HI

testing of field strains (CPV-2a and CPV-2b) with serum samples obtained 3 weeks after vaccination revealed CPV-2 vaccinated pups had consistently lower titers than CPV-2b vaccinated puppies.[13] The later report involved the same CPV-2b vaccine fraction studied herein, and the results prompted the authors to conclude that serum from CPV-2b vaccinated puppies reacted more efficiently when exposed to field strains CPV-2a and CPV-2b, than that of CPV-2 vaccinated pups.

A severe outbreak of CPV-2c in a litter of seven unvaccinated 40-day-old puppies in a breeding kennel facility with bitches that were routinely vaccinated with multivalent vaccines containing the original type CPV-2 calls into question the CPV-2c efficacy of colostral MDA resulting from CPV-2 vaccine.[8] According to Decaro et al, the morbidity and mortality facts associated with their case report, that the entire litter was involved with signs of hemorrhagic diarrhea, vomiting, and, death within 3-5 days of onset are best explained by poor CPV-2 MDA protection against the CPV-2c variant. The HI titers of pups in that report are unknown.

The efficacy of CPV vaccines developed for protection against maternal antibody depends upon vaccine strain selection, attenuation levels, and product formulations that may affect the efficacy elicited by the specific product. This study demonstrated the CPV-2c disease prevention efficacy of the CPV-2b fraction of a multivalent vaccine when initially administered to 6-week-old pups seropositive for maternal CPV-2b antibodies and repeated in 4 weeks.

CONCLUSION

The present study clearly confirms the dogs vaccinated at 6 weeks of age with Duramune Lyme®+Max 5-CvK/4L with the CPV-2b fraction at minimum immunizing dose when administered subcutaneously were protected from virulent CPV-2c challenge in presence of MDAs to CPV. As such, the results support an additional label claim that the vaccine aids in the prevention of disease caused by the CPV-2c strain, in the presence of MDAs to canine parvovirus.

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REFERENCES

1. Carmichael LE, Schlafer DH, Hashimoto A. Minute virus of canines (MVC, canine parvovirus type-1): pathogenicity for pups and seroprevalence estimate. *J Vet Diagn Invest* 1994;6:165-174.
2. Hoelzer K, Parrish CR. The emergence of parvoviruses of carnivores. *Vet Res* 2010;41:39.
3. Hoelzer K, Shackelton LA, Parrish CR, et al. Phylogenetic analysis reveals the emergence, evolution and dispersal of carnivore parvoviruses. *J Gen Virol* 2008;89:2280-2289.
4. Parrish CR, Aquadro CF, Strassheim ML, et al. Rapid antigenic-type replacement and DNA sequence evolution of canine parvovirus. *J Virol* 1991;65:6544-6552.
5. Parrish CR, Have P, Foreyt WJ, et al. The global spread and replacement of canine parvovirus strains. *J Gen Virol* 1988;69 (Pt 5):1111-1116.
6. Buonavoglia C, Martella V, Pratelli A, et al. Evidence for evolution of canine parvovirus type 2 in Italy. *J Gen Virol* 2001;82:3021-3025.
7. Nakamura M, Tohya Y, Miyazawa T, et al. A novel antigenic variant of Canine parvovirus from a Vietnamese dog. *Arch Virol* 2004;149:2261-2269.
8. Decaro N, Martella V, Desario C, et al. First detection of canine parvovirus type 2c in pups with haemorrhagic enteritis in Spain. *J Vet Med B Infect Dis Vet Public Health* 2006;53:468-472.
9. Perez R, Francia L, Romero V, et al. First detection of canine parvovirus type 2c in South America. *Vet Microbiol* 2007;124:147-152.
10. Kapil S, Cooper E, Lamm C, et al. Canine parvovirus types 2c and 2b circulating in North American dogs in 2006 and 2007. *J Clin Microbiol* 2007;45:4044-4047.
11. Hong C, Decaro N, Desario C, et al. Occurrence of canine parvovirus type 2c in the United States. *J Vet Diagn Invest* 2007;19:535-539.
12. Joao Vieira M, Silva E, Oliveira J, et al. Canine parvovirus 2c infection in central Portugal. *J Vet Diagn Invest* 2008;20:488-491.
13. Ohshima T, Hisaka M, Kawakami K, et al. Chronological analysis of canine parvovirus type 2 isolates in Japan. *J Vet Med Sci* 2008;70:769-775.
14. Ntafis V, Xylouri E, Kalli I, et al. Characterization of Canine parvovirus 2 variants circulating in Greece. *J Vet Diagn Invest* 2010;22:737-740.
15. Martella V, Cavalli A, Pratelli A, et al. A canine parvovirus mutant is spreading in Italy. *J Clin Microbiol* 2004;42:1333-1336.
16. Nandi S, Anbazhagan R, Kumar M. Molecular

characterisation and nucleotide sequence analysis of canine parvovirus strains in vaccines in India. *Vet Ital* 2010;46:69-81.

17. Castro TX, Costa EM, Leite JP, et al. Monitoring of canine parvovirus (CPV) strains detected in vaccinated puppies in Brazil. *Res Vet Sci* 2011;90:336-340.
18. Clegg SR, Coyne KP, Parker J, et al. Molecular epidemiology and phylogeny reveal complex spatial dynamics in areas where canine parvovirus is endemic. *J Virol* 2011;85:7892-7899.
19. Appel MJ, Scott FW, Carmichael LE. Isolation and immunisation studies of a canine parco-like virus from dogs with haemorrhagic enteritis. *Vet Rec* 1979;105:156-159.
20. Wierup M, Olson P, Hedhammar A, et al. Evaluation of a killed feline panleukopenia virus vaccine against canine parvoviral enteritis in dogs. *Am J Vet Res* 1982;43:2183-2187.
21. Pollock RV, Carmichael LE. Use of modified live feline panleukopenia virus vaccine to immunize dogs against canine parvovirus. *Am J Vet Res* 1983;44:169-175.
22. Povey C. Development of a vaccine incorporating killed virus of canine origin for the prevention of canine parvovirus infection. *Can Vet J* 1982;23:15-21.
23. Carmichael LE, Joubert JC, Pollock RV. A modified live canine parvovirus vaccine. II. Immune response. *Cornell Vet* 1983;73:13-29.
24. Truyen U. Evolution of canine parvovirus--a need for new vaccines? *Vet Microbiol* 2006;117:9-13.
25. Decaro N, Desario C, Elia G, et al. Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c. *New Microbiol* 2008;31:125-130.
26. Larson LJ, Schultz RD. Do two current canine parvovirus type 2 and 2b vaccines provide protection against the new type 2c variant? *Vet Ther* 2008;9:94-101.
27. Spibey N, Greenwood NM, Sutton D, et al. Canine parvovirus type 2 vaccine protects against virulent challenge with type 2c virus. *Vet Microbiol* 2008;128:48-55.
28. Siedek EM, Schmidt H, Sture GH, et al. Vaccination with canine parvovirus type 2 (CPV-2) protects against challenge with virulent CPV-2b and CPV-2c. *Berl Munch Tierarztl Wochenschr* 2011;124:58-64.
29. Pollock RV, Carmichael LE. Maternally derived immunity to canine parvovirus infection: transfer, decline, and interference with vaccination. *J Am Vet Med Assoc* 1982;180:37-42.
30. Buonavoglia C, Tollis M, Buonavoglia D, et al. Response of pups with maternal derived antibody to modified-live canine parvovirus vaccine. *Comp Immunol Microbiol Infect Dis* 1992;15:281-283.
31. Pratelli A, Cavalli A, Normanno G, et al. Immunization of pups with maternally derived antibodies to canine parvovirus (CPV) using a modified-live variant (CPV-2b). *J Vet Med B Infect Dis Vet Public Health* 2000;47:273-276.
32. Reed L, Muench H. A simple method of estimating fifty percent endpoints. *Am J Hyg* 1938;27:493-497.
33. Greene CE, Schultz RD. Immnoprophylaxis In: Greene CE, ed. Infectious diseases of the dog and cat. St. Louis: Saunders Elsevier, 2006;1069-1119.
34. Burtonboy S, Charlier P, Hertoghs J, et al. Performance of high titre attenuated canine parvovirus vaccine in pups with maternally derived antibody. *Vet Rec* 1991;128:377-381.
35. Fiscus SA, Mildbrand MM, Gordon JC, et al. Rapid enzyme-linked immunosorbent assay for detecting antibodies to canine parvovirus. *Am J Vet Res* 1985;46:859-863.
36. Decaro N, Campolo M, Desario C, et al. Maternally-derived antibodies in pups and protection from canine parvovirus infection. *Biologicals* 2005;33:261-267.
37. Elia G, Cavalli A, Cirone F, et al. Antibody levels and protection to canine parvovirus type 2. *J Vet Med B Infect Dis Vet Public Health* 2005;52:320-322.
38. Carmichael LE, Joubert JC, Pollock RV. Hemagglutination by canine parvovirus: serologic studies and diagnostic applications. *Am J Vet Res* 1980;41:784-791.
39. Pratelli A, Cavalli A, Martella V, et al. Canine parvovirus (CPV) vaccination: comparison of neutralizing antibody responses in pups after inoculation with CPV2 or CPV2b modified live virus vaccine. *Clin Diagn Lab Immunol* 2001;8:612-615.
40. Cavalli A, Martella V, Desario C, et al. Evaluation of the antigenic relationships among canine parvovirus type 2 variants. *Clin Vaccine Immunol* 2008;15:534-539.
41. Luff PR, Wood GW, Hebert CN, et al. Canine parvovirus serology: a collaborative assay. *Vet Rec* 1987;120:270-273.
42. Study on file.