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The administration of a single dose of a multivalent (DHPPiL4R) vaccine prevents clinical signs and mortality following virulent challenge with canine distemper virus, canine adenovirus or canine parvovirus

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ABSTRACT

Four challenge studies following vaccination of dogs with a multivalent vaccine containing canine parvovirus (CPV-2b), adenovirus (CAV-1/-2) and distemper (CDV) are described. Six week old puppies received a single vaccination while non-vaccinated control dogs received water. In each respective trial, groups of dogs were challenged 21 days after vaccination with heterologous viral isolates. Clinical observations, rectal temperature measurements, and blood and swab samples for analysis were collected throughout the study.

Dogs in all studies had normal temperatures and general health up to challenge. Clinical signs of infection and temperatures outside the normal range were observed in non-vaccinated dogs challenged with CDV, CPV, CAV-1 and CAV-2; vaccinated dogs remained clinically normal after challenge. All dogs were sero-negative prior to vaccination, non-vaccinated dogs remaining negative until challenge. Vaccinated dogs all sero-converted by 21 days after vaccination, with further increases seen after challenge. Non-vaccinated dogs sero-converted following challenge with CPV or CAV-2; no final blood samples were taken in the CDV and CAV-1 studies. Rectal swab analysis showed prevention of CPV shedding in vaccinated compared to non-vaccinated dogs, and nasal swab analysis following CAV-2 challenge showed longer duration and higher amount of viral shedding for non-vaccinated dogs.

In conclusion, we demonstrated that a single administration of a minimum titre, multivalent vaccine to dogs of six weeks of age is efficacious and prevents clinical signs and mortality caused by CAV-1 and CDV; prevents clinical signs and significantly reduces virus shedding caused by CAV-2; and prevents clinical signs, leucopoenia and viral excretion caused by CPV.

Introduction

Domestic dogs are at higher risk of contracting infectious diseases, and in particular viral infections, if they are not immune to the specific agent [1]. The widespread use of vaccines has led to reduced incidence of common infections; however, continuing evolution of vaccine technology results in products of varying efficacy [2] or range of protection. Vaccines have been grouped into core, non-core or non-recommended categories; with canine distemper, canine parvovirus and canine adenovirus considered core vaccine components recommended to be administered every three years [3]. These recommendations have been expanded and form the basis of the World Small Animal Veterinary Association (WSAVA) Guidelines for the Vaccination of Dogs and Cats [4].

Canine parvovirus (CPV) infection of dogs results in a contagious enteric disease leading to high rates of mortality or severe morbidity [5]. All naïve dogs are susceptible to infection with those under one year of age having the highest risk of developing severe disease. Since CPV2 was first identified [6], there have been multiple variations of virus strains which have increased in prevalence. Canine parvovirus type 2a [7], CPV-2b [8] and CPV-2c have been identified [9], and are becoming more frequently isolated [10,11]. Canine adenovirus type 2 (CAV-2) infection ranges from essentially non-apparent to a mild form of respiratory disease; however, the virus is considered to be one of the main causes of infectious

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tracheobronchitis in dogs [1]. The canine adenovirus type 1 (CAV-1) results in a more general infection, with predominant clinical signs being nausea, vomiting and diarrhoea [12]. Although CAV-2 was initially thought to be derived from CAV-1, later analysis [13] revealed they are genetically distinct. Despite this, dogs vaccinated with vaccines containing CAV-1 or CAV-2 were found to be cross protected [14,15]. As vaccines containing CAV-1 do have safety issues [12], current vaccines as recommended by the WSAVA [4] contain CAV-2. Canine distemper virus (CDV) is another highly contagious virus which results in acute to sub-acute systemic disease and a high mortality rate in dogs [16], although the host range [17] includes a variety of species. The introduction of efficacious vaccines has reduced the incidence of canine distemper disease in dogs although the possibility still exists that infection outbreaks can occur, even in vaccinated animals [16].

In this paper we describe the efficacy of a new multivalent canine vaccine containing the core viral components – CDV, CPV and CAV – in addition to canine para-influenza, rabies and four *Leptospira interrogans* and *kirschneri* serovar antigens. In four separate trials, dogs free of antibodies to the vaccine antigens received a single vaccination and then were challenged with different strains of each core viral antigen (distemper, adenovirus 1 and 2, and parvovirus). The impact of vaccination on clinical variables, serology and for some components (CPV and CAV-1) re-isolation of challenge virus was examined by comparing vaccinated dogs to non-vaccinated dogs in each study.

Materials and methods

This study reports the results of four separate trials with all studies designed to be compliant with the respective European Pharmacopeia monographs 01/2008:0448 (distemper), 01/2008: 1951 (adenovirus) and 01/2008:0964 (parvovirus). The studies were carried out in accordance with the Act on Animal Health and Animal Welfare of The Czech Republic, and had been approved by Bioveta a.s. and Zoetis ethical review committees.

Animals

In the studies determining efficacy to CDV, CAV-1 and CPV, seven dogs aged 6 weeks old, were enrolled into each study with five dogs vaccinated and two dogs receiving water for injection acting as controls. In the CAV-2 study, 20 dogs were enrolled with 10 dogs vaccinated and 10 dogs receiving water for injection as controls. All dogs were confirmed to be free of antibodies (methods described in laboratory analysis) against the respective challenge virus.

Vaccine

An experimental vaccine batch was produced which contained live CDV, CPiV, CAV-2, CPV-2b (DHPPi); inactivated *L. interrogans* sv Canicola, Icterohaemorrhagiae and Bratislava, and *L. kirschneri* sv Grippotyphosa, and rabies virus (L4R). The DHPPi component was freeze-dried while the L4R component was a liquid containing adjuvant (aluminium hydroxide). The control product was sterile water. Administration (1 ml) was by the subcutaneous route behind the left shoulder blade on day 0 using standard aseptic technique. Where appropriate vaccine antigens were formulated at minimum titre and maximum passage.

Challenge

The challenge isolates were different strains to the vaccine antigens and had been used previously in validation studies. CDV isolate Snyder Hill was obtained from the American Type Culture Collection; CAV-1 isolate Mirandola and the CAV-2 isolate Manhattan were obtained from the Animal and Plant Health Inspection Services at the Centre for Veterinary Biologics; and CPV 2b isolate 212/98 was obtained from the University of Bari, Italy. For the CDV (10^{-1} dilution of virus provided which was of unknown titre) and CAV-1 ($10^{5.8}$ $TCID_{50}$/mL) studies 1 mL of challenge material was administered by the intravenous route; for CPV ($10^{6.8}$ $TCID_{50}$/mL) a 2 mL dose was administered with 1 mL orally and 1 mL intranasally (0.5 mL per nostril); for CAV-2 ($10^{5.3}$ $TCID_{50}$/mL), 1 mL of challenge material was administered intranasally.

Observations and samples

Rectal temperatures (°C) of all animals were recorded on days-2, -1, 0 (prior to and 4 h after vaccination) and then daily thereafter for a period of seven days. Further measurements were recorded on day 21 (prior to and 4 h after challenge administration), then daily until the end of the study.

Clinical observations were performed once daily from day-2 until the end of the study. Clinical observations on days 0 and 21 were performed prior to vaccine and challenge strain administration respectively, with additional observations approximately 4 h after challenge strain administration. Observations performed following challenge administration assessed developing clinical disease. However, for animal welfare reasons specific clinical endpoints were defined for each challenge whereby animals would be euthanased prior to reaching end-stage clinical disease.

Blood samples were collected into plain blood tubes from each animal prior to test material (vaccine or control) administration on day 0, prior to challenge administration on day 21, and at the end of the study on day 35 for CAV-2 and CPV or day 42 for CDV and CAV-1. For the CPV study further blood samples (0.5–1 ml) for white blood cell (WBC) counts were collected from each animal 4, 2 and 0 days before and then 3, 5, 7, 10, 12 and 14 days post-challenge administration.

To determine virus shedding in the CPV study, faecal swabs were collected into sterile tubes on day 21 before CPV-2b challenge administration (before challenge) and then 3, 5, 7, 10, 12 and 14 days after CPV-2b challenge. In the CAV-2 study, two nasal swab samples (one from each nostril) were collected into sterile tubes from each animal prior to challenge administration on day 21 and then daily from day 23 (2nd day after challenge) until day 31 (10th day after challenge).

Laboratory analysis

Serum samples from the respective studies were examined for the presence of antibodies to CDV, CPV-2, CPV-2b, CAV-1 and CAV-2 by serum-neutralisation test. Briefly, duplicate two-fold dilutions of test and control sera in MEM cultivation medium were prepared in micro titre plates; approximately 100 $TCID_{50}$ of the respective virus was added followed by incubation at 37 °C for 1 h in 5% CO_2. Susceptible cells (VERO – CDV; MDCK – CAV; CRFK – CPV) were added as appropriate; with a further incubation at 37 °C for 3–7 days in 5% CO_2. The end point was assessed as the serum dilution where more than 50% of the characteristic cytopathic effect was attenuated. For the CPV analysis the haemagglutination assay was used to definitively confirm virus presence and visualisation; the end point was assessed as the serum dilution where inhibition of haemagglutination was observed.

For the CPV study, whole blood samples were analysed for leukocyte counts by staining cells with Tűrk's solution and counting them in a Bűrkerusing standard counting chamber. Faecal samples were examined for virus presence and titre by re-isolation on a

susceptible cell line (A72). Briefly, samples were suspended in PBS and centrifuged, with supernatants diluted two-fold on microtitre plates. The A72 cells were added (400–600,000 cells/well) and plates incubated at 37 °C in 5% CO_2. After 5 to 7 days virus presence was confirmed by haemagglutination assay. Porcine erythrocytes were added to all wells and plates were incubated for 1 h at 2–8 °C. The titre is calculated and expressed in $TCID_{50}$/mL. For the CAV-2 study nasal swab samples were examined for virus presence and titre by virus re-isolation on MDCK cells.

Statistical analysis

Body temperatures were classified into hypothermic (<37.0 °C), normal (37.0 to 39.5 °C) and hyperthermic (>39.5 °C). Abnormal clinical data and the number of days with abnormal clinical signs were summarised for vaccinates and controls pre-challenge and post challenge. Descriptive statistics for antibody titres against relevant antigens including the geometric mean, minimum and maximum were calculated for vaccinates and controls at each time point.

In the CPV study, for each animal the arithmetic mean of WBC counts taken up to 4 days pre-challenge was calculated to obtain the baseline value; post-challenge counts were compared with the baseline value thereby calculating the percentage reduction. Leucopoenia was defined as a decrease in WBC greater than 50% of the baseline value. The geometric mean of the maximum titres excreted in faeces of control animals were calculated and compared to the maximum titre excreted in faeces of each vaccinated animal.

In the CAV-2 study clinical observations were scored and the total clinical scores, the number of days of virus shedding, and the AUC (area under the curve) of virus shedding in the challenge phase of the study were calculated for each animal. The rank transformed total clinical scores, number of days of virus shedding and the logarithm transformed AUC values were analysed with a general linear mixed model (fixed effect was treatment, random effects were pen, block within pen and residual). The mean and median clinical scores were calculated for each treatment as well as the minimum and maximum.

Results

Canine distemper virus

No abnormal clinical observations were observed in the period following administration of vaccine or control product. All dogs remained in good health and their rectal temperatures stayed within the physiological range (37.0 to 39.5 °C). Following challenge both control dogs showed abnormal clinical signs due to canine distemper infection, with fever, anorexia, apathy and gastrointestinal disturbances seen from three days after challenge. Both dogs were subsequently euthanased nine and ten days after challenge respectively due to progression of clinical signs in accordance with pre-defined welfare endpoints. No abnormal clinical signs indicative of distemper infection or rectal temperatures outside the physiological range were observed in vaccinated dogs.

Antibody titres to CDV were first detected in vaccinated dogs on day 21 and continued to rise until day 42 when sampling ceased. Controls remained sero-negative until after challenge on day 21, as controls were euthanased prior to day 35 (study completion) no final (day 35) blood sample was obtained (Table 1).

Canine parvovirus

Following vaccination, one vaccinated dog developed a swelling at the injection site. Furthermore, one control dog had a temperature below the lower limit (37.9 °C) two days before and six days after the administration of water for injection, which was not thought clinically relevant in the context of this study. No other abnormal clinical signs were observed in the pre-challenge period. Following challenge both control dogs showed typical clinical signs of canine parvovirosis such as apathy, anorexia and diarrhoea from six days until 10 days after challenge. A considerable decrease in temperature from 38.7 °C to 37.0 °C in 24 h was also observed in one control dog which was clearly related to the challenge (data not shown). The vaccinated dogs neither showed any abnormal clinical signs nor temperatures after the challenge (data not shown).

Antibody titres to CPV-2 and CPV-2b were first detected in vaccinated dogs on day 21 and continued to rise until day 42 when sampling ceased. Controls remained sero-negative until after challenge on day 21, sero-converting by day 42 (Table 2).

Following challenge none of the vaccinated dogs showed any decrease in WBC below 50% of the mean pre-challenge values. The WBC counts of both control dogs decreased more than 50% from pre-challenge values. 12 days post-challenge they had decreased more than 60% from baseline, and were below the lower limit of the normal physiological range (data not shown).

Results achieved for examination of faeces by virus re-isolation are summarised in Table 3. No virus excretion was detected in the vaccinated animals at any time point following challenge. Both control animals excreted CPV from three to fourteen days after challenge. When the samples were examined by haemagglutination assays (data not shown) small amounts of excreted CPV ranging from 4 to 16 HAU were detected in three of five vaccinated animals on one day between three to five days after challenge. No virus excretion was observed in the remaining two vaccinated animals and in any of the vaccinated animals from seven days after challenge. Both control animals excreted CPV from three to fourteen days after challenge.

Canine adenovirus type 1

Following vaccination no abnormal clinical signs were observed and the rectal temperatures of all dogs remained within the normal physiological range. After challenge, both control dogs showed abnormal clinical signs due to canine hepatitis infection from five

Table 1
Geometric mean antibody titres against canine distemper virus, by time period and treatment group.

Treatment group	Virus neutralising antibody titres		
	Day 0	Day 21	Day 42
Vaccinated ($n = 5$)	<2	9.2	48.5
Control ($n = 2$)	<2	<2	NA

NA – both control dogs were euthanased prior to sample collection day.

Table 2
Geometric mean antibody titres against canine parvovirus type 2 and type 2b, by time period and treatment group. Titres against CPV-2 and CPV-2b are expressed as the reciprocal dilution of sera.

Treatment group	CPV detected	Virus neutralising antibody titre		
		Day 0	Day 21	Day 42
		VN	VN	VN
Vaccinated ($n = 5$)	CPV-2	<2	485.0	2940.7
Control ($n = 2$)		<2	<2	1810.2
Vaccinated ($n = 5$)	CPV-2b	<2	3377.9	8914.4
Control ($n = 2$)		<2	<2	3620.4

VN = virus neutralisation.

Table 3
CPV re-isolation from faecal swab samples, by time period and treatment group. Titres are expressed as the $TCID_{50}$.

Animal	Treatment group	Examination of faeces samples – haemagglutination assay						
		Day 21	Day 24	Day 26	Day 28	Day 31	Day 33	Day 35
1	Vaccinated	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$
2		$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$
3		$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$
4		$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$
5		$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$
6	Control	$<10^{2.1}$	$<10^{2.1}$	$10^{2.8}$	$10^{4.8}$	$10^{3.8}$	$10^{3.8}$	$10^{2.8}$
7		$<10^{2.1}$	$<10^{2.1}$	$<10^{2.1}$	$10^{3.8}$	$10^{4.8}$	$10^{2.8}$	$10^{3.1}$

days after infection, with apathy, anorexia, recumbency and jaundice observed. Elevated temperatures were observed in both control dogs on day three to six following challenge (peak temperatures 40.0 °C and 39.8 °C, respectively). Both dogs were euthanased prior to study completion five and six days after challenge according to prescribed endpoints. No abnormal clinical observations were observed in vaccinated dogs after challenge, except for a slight temperature increase above the upper range limit (39.6 °C) in one vaccinated dog six days after challenge.

Antibody titres to CAV-1 and CAV-2 were first detected in vaccinated dogs on day 21 and continued to rise until day 42 when sampling ceased. Controls remained sero-negative until after challenge on day 21, no samples were obtained after challenge as both control dogs were euthanased prior to study completion (Table 4).

Canine adenovirus type 2

No abnormal clinical observations were observed in the period following administration of vaccine or control product, with all dogs remaining in good health. Rectal temperatures remained within the physiological range during the vaccination phase and also during the 14 day period following challenge administration. Following challenge no abnormal clinical symptoms were observed in vaccinated dogs, but all controls showed signs of infection with nasal and ocular discharge, depression and coughing seen most frequently. The mean total score of abnormal clinical observations was significantly higher in control (10.1; range 6–15) than vaccinated dogs (0.0; $P < 0.0001$).

Antibody titres to CAV-2 were first detected in vaccinated dogs on day 21 and continued to rise until day 42 when sampling ceased. Controls remained sero-negative until after challenge on day 21, sero-converting by day 35 (Table 5).

Analysis of nasal swabs showed that none of the vaccinated or control dogs had virus in their nasal cavities prior to challenge on day 21 (titres were less than $10^{2.1}$ $TCID_{50}$/mL which is considered negative). Following challenge virus was re-isolated from five vaccinated and seven control dogs; virus titres ranged from $10^{2.1}$ to $10^{3.8}$ $TCID_{50}$/mL in vaccinates and $10^{2.1}$ to $10^{6.1}$ $TCID_{50}$/mL in control dogs. The number of days of viral shedding was significantly higher in control (Least Squares Mean (LSM) = 4.7) than in vaccinated (LSM = 0.9) dogs ($P < 0.0001$). The area under the curve of CAV-2 virus titres was also significantly higher in non-vaccinated (back-transformed LSM = 23.9) than in vaccinated (back-transformed LSM = 11.7) dogs ($P < 0.0001$).

Table 4
Geometric mean antibody titres against CAV-1 and CAV-2, by time period and treatment group. Titres are expressed as the reciprocal dilution of sera.

Treatment group	Virus neutralising antibody titres					
	Day 0		Day 21		Day 42	
	CAV-1	CAV-2	CAV-1	CAV-2	CAV-1	CAV-2
Vaccinated ($n = 5$)	<2	<2	18.4	32.0	147.0	128.0
Controls ($n = 2$)	<2	<2	<2	<2	NA	NA

NA – both control dogs euthanased prior to sample collection day.

Table 5
Geometric mean antibody titres against CAV-2 by time period and treatment group. Titres are expressed as the reciprocal of dilutions.

Treatment group	Virus neutralising antibody titres		
	Day 0	Day 21	Day 35
Vaccinated ($n = 10$)	<2	21	181
Controls ($n = 10$)	<2	<2	119

Discussion

In this paper we have demonstrated a rapid three week onset of immunity following a single dose of a multivalent vaccine to sero-negative dogs at minimum age and subsequent protection of vaccinated animals following virulent challenge with heterologous isolates of canine distemper, canine adenovirus (types 1 and 2) and canine parvovirus.

As has been previously described [17,18], infection of non-vaccinated dogs with the respective CDV, CPV, CAV-1 or CAV-2 viruses resulted in specific clinical signs, mortality, leucopoenia or virus shedding. The non-vaccinated dogs in each study remained sero-negative at the pre-challenge sampling time point thus demonstrating that no concurrent infection occurred, and if completing the study they were found to sero-convert at the subsequent sampling time point after challenge. In contrast, in each of the four studies all vaccinated dogs were found to have (positive) antibody titres to the respective challenge virus by three weeks following the single vaccination; the antibody titres obtained contributed to protection against virulent challenge with CPV, CDV, CAV-1 and CAV-2.

The successful sero-conversion of dogs in each of the four studies to vaccination, with generation of protective antibodies, is in contrast to other studies with puppies that had maternally derived antibodies prior to vaccination [19]. Maternally derived immunity is considered the primary cause of vaccine failure in young dogs [20–22]. To overcome interference by maternally derived antibodies, puppies are recommended to be vaccinated repeatedly between 6 and 16 weeks of age. In the four studies reported here, dogs were sero-negative prior to vaccination and thus only one vaccination was required to result in full protection.

Protection against CPV was assessed by challenge with a heterologous CPV2b strain. There are currently two more antigenic types of CPV circulating in the field, CPV2a and CPV2c. They differ genetically from CPV2b through point mutations [23]. In vitro, cross serum-neutralisation and haemagglutination studies have shown that antibody titres against heterologous antigenic types can be significantly lower than those to homologous types. Despite

these differences however no definitive evidence for a lack of cross-protection against parvovirosis has been found in vivo to date [21].

Conclusion

A single administration of a minimum titre, multivalent vaccine to dogs of six weeks of age is efficacious and prevents both clinical signs and mortality caused by CAV-1 and CDV; prevents clinical signs and significantly reduces virus shedding caused by CAV-2; and prevents clinical signs, leucopoenia and viral excretion caused by CPV.

Conflict of interest

All authors are either employees (SMW, JI, AT, VK, CS, JS and GS) or technical consultants (ES) of Zoetis or employees of Bioveta (EP), and the vaccine described in this paper is marketed by Zoetis.

SMW, ES, CS, EP, AT and VK contributed to experimental design; VK and JI performed data review and analysis; JS and GS provided project mentorship and support; SMW prepared the manuscript; all authors have read and approved the content.

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