

Evidence for immunisation failure in vaccinated adult dogs infected with canine parvovirus type 2c

Nicola Decaro¹, Costantina Desario¹, Gabriella Elia¹, Vito Martella¹, Viviana Mari¹, Antonio Lavazza², Manuela Nardi³, Canio Buonavoglia¹

¹Department of Animal Health and Well-being, Faculty of Veterinary Medicine of Bari, Valenzano, Bari, Italy;

²Istituto Zooprofilattico Sperimentale di Lombardia ed Emilia Romagna, Sezione di Brescia, Brescia, Italy;

³Istituto Zooprofilattico Sperimentale di Lombardia ed Emilia Romagna, Sezione di Mantova, Mantova, Italy

SUMMARY

An outbreak of canine parvovirus type 2c (CPV-2c) infection in vaccinated adult dogs is reported. The disease occurred in a breeding kennel in Italy and affected 11 dogs aged between 6 months and 2.5 years, that had been repeatedly administered vaccines containing a type 2 (old type) CPV strain. CPV infection was demonstrated in all diseased dogs by an immunochromatographic test. A CPV strain was isolated from the intestinal content of a 20-month-old pregnant Bernese mountain bitch that underwent a fatal outcome. The strain was characterised as CPV-2c by means of real-time PCR assays using minor groove binder probes. The present report provides further concerns about the real efficacy of type 2-based vaccines against the antigenic variants of CPV and stresses the need for developing new vaccines prepared with the variants currently circulating in the dog population.

KEY WORDS: Canine parvovirus, Adult dogs, Immunisation failure

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INTRODUCTION

Canine parvovirus (CPV) is responsible for acute gastroenteritis in pups, with a high rate of mortality (Carmichael and Binn, 1981). The original type, which emerged in the late 1970s, was rapidly replaced by two antigenic variants, types 2a and 2b (Parrish *et al.*, 1985, 1991), and in 2000 a third type CPV-2c was detected (Buonavoglia *et al.*, 2001), which is progressively replacing the old variants in Italy (Martella *et al.*, 2004, 2005b; Desario *et al.*, 2005; Decaro *et al.*, 2005c, d, 2006a) and has also been reported in other countries (Nakamura *et al.*, 2004; Decaro *et al.*, 2006b, 2007; Perez *et al.*, 2007; Hong *et al.*, 2007).

Commonly, CPV causes disease in unvaccinated 1-6 month-old pups. Vaccinated pups are usual-

ly protected from the disease and from infection, unless immunisation fails due to the presence of high titres of maternally derived antibodies (Decaro *et al.*, 2004a, 2005a; Elia *et al.*, 2005). Similarly, adult dogs (≥ 1 year) are not usually susceptible to CPV infection due to vaccination or previous infections with a field virus (Truyen, 1999, 2006). However, preliminary observations have shown that CPV-2c is also isolated from sick or dead dogs older than 6 months and up to 2 years (C. Buonavoglia, personal observation). In the present report, we describe an outbreak of severe gastroenteritis induced by CPV-2c in adult dogs repeatedly vaccinated using a classical, type 2-based vaccine.

MATERIALS AND METHODS

Clinical case

In January 2007, a severe outbreak of acute gastroenteritis occurred in a breeding kennel located in Mantua, Italy. During the outbreak, the kennel housed 60 Bernese mountain dogs, 35 dachs-

Corresponding author

Nicola Decaro

Department of Animal Health and Well-being

Faculty of Veterinary Medicine of Bari

S.P. Casamassima Km. 3 - 70010 Valenzano, Bari, Italy

E-mail: n.decaro@veterinaria.uniba.it

hunds and 3 collies. The dogs were allocated in three separate facilities about 25 metres apart:

- 1) facility no. 1 housed dogs aged between 6 months and 4 years, including 12 Bernese mountain dogs and 12 dachshunds;
- 2) facility no. 2 housed 3 adult collies, 4 Bernese mountain bitches with their offspring (27 pups in total) and 1 dachshund bitch with 6 pups;
- 3) facility no. 3 housed 33 pups, including 17 Bernese mountain dogs and 16 dachshunds.

All pups were vaccinated regularly according to the following schedule: one dose of a monovalent CPV-2 high-titre vaccine at 42 days of age and two doses of a tetravalent vaccine against canine distemper, parvovirus, adenovirus and leptospirosis at 57 and 90 days of age, respectively. The adult dogs received a yearly booster vaccination using the same tetravalent vaccine. Both vaccines contained the same type 2 (old type) CPV strain. Clinical signs of gastroenteritis were only observed in facility no. 1 which consisted of 16 separate pens (Table 1). The first case occurred on 12/13/2006 in a pregnant dachshund bitch that showed bloody faeces and cough but recovered rapidly. The disease spread slowly to other closed pens, causing initially mild and subsequently more severe clinical signs between 12/31/2006 and 01/02/2007. The last case was observed on 01/05/2007 in an 18-month-old dog. In all, 11 dogs aged between 6 months and 2.5 years became ill, with the most severe clinical signs observed in one 8-month-old and three 18-20-month-old Bernese mountain dogs. Despite the severity of the disease observed in some dogs, only one 20-month-old Bernese mountain pregnant bitch (40/07) succumbed to the disease. On the basis of clinical signs, a presumptive diagnosis of CPV infection was tentatively made, which was confirmed by an immunochromatographic test (SNAP[®] Canine Parvovirus Antigen Test, IDEXX Laboratories, Inc., Maine, USA) carried out on the faecal samples of all dogs during the acute phase of the disease.

Electron microscopy

The intestinal content of the dead pregnant bitch (40/07) was subjected to negative staining electron microscopy (EM) examination. The sample was suspended in distilled water (10% w/v) and clarified by centrifugations at 4,000 \times g for 20 min and 9,300 \times g for 10 min. Then, 85 μ l of the supernatant were ultracentrifuged in Airfuge

Beckman for 15 min at 21 psi (82,000 \times g) and pelleted on a formvarcoated grid. After negative staining with 2% sodium phosphotungstate (pH 6.8), the grid was observed under a TEM Philips CN10 operating at 80 kV at 19,000-39,000 magnification.

Real-time PCR assays for detection and characterisation of CPV

Sample 40/07 was tested by a TaqMan-based real-time PCR assay for detection of CPV (Decaro *et al.*, 2005d) and by two minor groove binder (MGB) probe assays for characterisation of the parvovirus variants (Decaro *et al.*, 2006a). The template was prepared by homogenising (10% w/v) the intestinal content in Dulbecco's minimal essential medium (D-MEM) and subsequent boiling for 10 min and chilling on ice, as previously described (Decaro *et al.*, 2005d). The TaqMan and MGB probe assays were carried out in a 25- μ l reaction containing 10 μ l of template or standard DNA (both in duplicates), 12.5 μ l of IQTM Supermix (Bio-Rad Laboratories Srl, Milan, Italy), 600 (TaqMan assay) or 900 (MGB probe assays) nM of primers and 200 nM of probes. The thermal protocol was as follows: activation of iTaq DNA polymerase at 95°C for 10 min, 45 cycles of denaturation at 95°C for 30 s and primer annealing-extension at 60°C for 1 min. All reactions were conducted in an i-Cycler iQTM Real-Time Detection System (Bio-Rad Laboratories Srl) and the data were analysed with the appropriate software (version 3.0).

Virus isolation

The intestinal content homogenate was clarified by centrifuging at 1500 \times g for 15 min and the supernatant was treated with antibiotics (penicillin 5000 IU/mL, streptomycin 2500 μ g/mL, amphotericin B 10 μ g/mL) at 37°C for 30 min and inoculated onto freshly trypsinised A-72 canine cells grown in D-MEM containing 5% foetal calf serum. The inoculated cells were incubated at 37°C in the presence of 5% CO₂ and observed daily at the light microscope for the occurrence of cytopathic effect (cpe). After three days of incubation, an immunofluorescence (IF) assay was carried out using a dog anti-serum for CPV and a rabbit anti-dog IgG conjugated with fluorescein isothiocyanate (Sigma Aldrich srl, Milan, Italy).

TABLE 1 - Dogs housed in facility no. 1 and clinical evolution of the CPV outbreak.

Pen no.	No. of dogs	Breed	Age	Pregnancy	Clinical signs	Date of onset of clinical signs	Outcome
1	4	Bernese mountain dog	4 y	NP	None	/	/
2	2	Dachshund	4 y	NP	None	/	/
3	3	Dachshund	3 y	NP	None	/	/
4	1	Dachshund	2.5 y	27 d	Bloody faeces, cough	12/13/2006	Recovery in 1 d Full-term pregnancy
5	1	Bernese mountain dog	2 y	NP	Bloody faeces, cough	12/24/2006	Recovery in 2 d
6	1	Bernese mountain dog	18 mo	NP	Haemorrhagic diarrhoea	01/05/2007	Recovery in 5 d
7	1	Dachshund	18 mo	NP	Bloody faeces, anorexia	12/20/2006	Recovery in 2 d
8	1	Dachshund	18 mo	NP	None	/	
9	1	Bernese mountain	20 mo	20 d	Severe haemorrhagic diarrhoea	12/31/2006	Death in 3 d
10	1	Bernese mountain	18 mo	NP	Mucous diarrhoea	01/03/2007	Recovery in 3 d
11	2	Bernese mountain dog	11 mo	NP	Bloody faeces, cough	12/15/2006	Recovery in 2 d
12	1	Bernese mountain dog	8 mo	NP	Haemorrhagic diarrhoea	01/02/2007	Recovery in 8 d
13	1	Dachshund	8 mo	NP	None	/	/
14	1	Bernese mountain dog	6 mo	NP	Vomiting, cough	01/02/2007	Recovery in 3 d
15	1	Dachshund	6 mo	NP	Vomiting, cough	12/31/2006	Recovery in 4 d
16	2	Dachshund	6 mo	NP	None	/	/

NP, not pregnant; y = years; mo = months; d = day/days.

Screening for other canine pathogens

Sample 40/07 was also tested by (RT-)PCR or real-time (RT-)PCR for detection of other common canine viral enteric pathogens, including reoviruses (Leary *et al.*, 2002; Decaro *et al.*, 2005b),

rotaviruses (Gouvea *et al.*, 1994), caliciviruses (Jiang *et al.*, 1999; Marsilio *et al.*, 2005), canine adenoviruses (CAV) (Hu *et al.*, 2001), canine distemper virus (CDV) (Elia *et al.*, 2006), canid herpesvirus type 1 (Schulze and Baumgartner,

1998), and canine coronavirus (CCoV) (Decaro *et al.*, 2004b). *Leptospira* spp. was searched for using PCR (Gravekamp *et al.*, 1993). Standard bacteriological investigations were also carried out for detection of aerobic and anaerobic pathogens by plating the faecal samples onto MacConkey's agar (Oxoid S.p.A., Garbagnate Milanese, Italy). Detection of the most common enteric parasites was achieved using zinc sulphate flotation. The Ziehl Nielsen staining was performed for detection of *Cryptosporidium* spp.

Serology

In order to evaluate the immunity status of the vaccinated dogs against the viral antigens contained in the vaccines used, one month after the onset of the outbreak, sera were collected from 7 dogs, aged between 6 months and 4 years, and tested for antibodies to CAAdVs, CDV and CPV. Four dogs had remained healthy during the outbreak and the other 3 dogs had displayed gastroenteritis and resulted CPV-positive by the immunochromatographic test.

Antibodies to CAAdVs and CDV were detected by virus neutralisation (VN) assays using Madin Darby canine kidney (MDCK) and African green monkey kidney (VERO) cells, respectively, and 100 TCID₅₀ per each virus. Detection of CPV antibodies was carried out using a haemagglutination inhibition (HI) test, as described previously (Desario *et al.*, 2005).

RESULTS

EM examination indicated the presence of parvovirus-like particles in the intestinal content of the dead pregnant bitch (40/07). By the TaqMan assay, a CPV strain was detected in the specimen at a titre of 6.78×10^7 DNA copies/mg of intestinal content and this strain was characterised as type 2c by the MGB probe assays. By virus isolation, the intestinal content induced a mild cpe at the first passage on A-72 cells that was more evident in the second and third passages. The nuclear fluorescence observed at the UV microscope by the IF assay using a CPV specific serum confirmed the successful isolation of the CPV-2c strain. Other canine viral and bacterial pathogens of the dog were not detected in the tested sample. The dogs bled for serology exhibited VN antibody titres

ranging from 1:4 to 1:128 against CDV and from 1:16 to 1:256 against CAAdVs and HI antibody titres from 1:640 to 1:10,240 against CPV. The highest HI antibody titres (1:10,240) were found in dogs which recovered from gastroenteritis.

DISCUSSION

CPV-2c, first isolated in Italy in 2000, is now spreading in the canine population worldwide (Nakamura *et al.*, 2004; Decaro *et al.*, 2006b, 2007; Perez *et al.*, 2007; Hong *et al.*, 2007). Although CPV causes gastroenteritis mainly in 1-6 month-old pups, the CPV-2c outbreak described in this paper involved young as well as adult dogs, with one case of mortality in a 20-month-old Bernese mountain dog. Sporadic cases of parvovirus in adult dogs associated with CPV-2c infection have been reported (Buonavoglia *et al.*, 2001; Cavalli *et al.*, 2001). Nevertheless, in the described outbreak most adult dogs housed in the same facility were affected although the vaccination against CPV had been carried out systematically using classical type 2-based vaccines. This CPV outbreak shows two main atypical aspects:

- 1) the age of the affected dogs and
- 2) the infection of dogs regularly vaccinated.

It could be speculated that the vaccines used were of poor quality or, alternatively, they were stored or administered incorrectly. However, although the batches of the vaccines employed were not examined, the presence of antibodies specific for CAAdVs and CDV in the sera of both symptomatic and asymptomatic dogs should rule out this hypothesis. The interference of an exceptionally long-lasting maternal derived immunity with the active immunisation of dogs has been also considered. However, even this hypothesis can be rejected since the vaccination at 90 days of age and, for some dogs, that at one year of age should have induced a protective immunity. It is rather likely that a highly pathogenic CPV-2c strain was able to infect adult dogs, causing disease and in one case even the death, as the immunity induced by the CPV-2 vaccine strain was not able to ensure adequate protection against a field strain.

Protection elicited by CPV-2 based vaccines against the field variants still represents a "vexata quaestio" as current opinions are highly divergent. Many authors have suggested that the old

type-2 based vaccines are still protective against the variants currently circulating in the field (Larson and Schultz, 1997). On the other hand, other researchers believe that the immunity induced by CPV-2 vaccines is effective against the homologous (vaccine) virus but significantly lower against the variants, thus allowing an aggressive strain to cause infection and even mortality in dogs "regularly" vaccinated (Martella *et al.*, 2005a). It has been shown that there is a one-way cross-reactivity between the antigenic variants and the original CPV-2 (Pratelli *et al.*, 2001). Sera raised against CPV-2 displayed low VN titres against the heterologous virus (CPV-2b) in comparison to those obtained when sera raised against CPV-2b were run against the original CPV-2. Similar experiments were carried out for evaluation of serological cross-reactivity between CPV-2 and CPV-2a, CPV-2b and CPV-2c in sera of dogs and rabbits (Cavalli *et al.*, 2007). Animals administered CPV-2 showed a significantly lower VN antibody titres against the heterologous viruses with respect to the homologous virus. Recently, the efficacy of the classical CPV-2 vaccine against a field type 2c strain has been proved (Spibey *et al.*, 2006). However, in that experiment the challenge was carried out in controlled conditions at 28 days post-vaccination, when antibodies commonly reach the highest levels (Decaro *et al.*, 2004a).

Nothing is known about the protection induced by the original type against CPV-2c after a long period interval between vaccination and challenge, when the type-2 antibody titres could be not enough efficient to prevent infection and disease caused by a field CPV-2c. There is concern that the antigenic differences between the original type 2 and its variants may decrease the effectiveness of the CPV-2 based vaccines (Greenwood *et al.*, 1995; Yule *et al.*, 1997; Pratelli *et al.*, 2001). Therefore, it would be useful to prepare vaccines using the CPV variants circulating in the field, taking into account that CPV-2c has been frequently associated with disease in adult dogs.

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