

## Clinical and virological findings in pups naturally infected by canine parvovirus type 2 Glu-426 mutant

Nicola Decaro,<sup>1</sup> Costantina Desario, Marco Campolo, Gabriella Elia, Vito Martella, Dominga Ricci, Eleonora Lorusso, Canio Buonavoglia

**Abstract.** An outbreak of canine parvovirus type 2 infection caused by the Glu-426 mutant in 2 litters of pups is reported. The infected pups ( $n = 6$ ) were monitored daily for evidence of clinical signs and hematological changes and for the evaluation of viral shedding in the feces. The disease induced by the Glu-426 mutant was mild in all the infected pups. Vomiting and hemorrhagic diarrhea were not observed; however, the pups developed mucoid diarrhea (3.5 median days), depression (1.5 median days), and relative leukopenia and lymphopenia (2.5 median days). Fever and loss of appetite were observed only in 2 pups. Virus was detected in the feces for 4.5, 6.5, and 46 median days by hemagglutination, virus isolation on cell cultures, and real-time polymerase chain reaction (PCR), respectively. By real-time PCR, the highest viral DNA titers were detected in the feces of both litters at day 10, reaching median values of more than  $10^{10}$  DNA copies/mg of feces.

**Key words:** Dogs; infection; parvovirus Glu-426 mutant.

### Introduction

Canine parvovirus (CPV) disease emerged in 1978 as an epizootic gastroenteritis of dogs characterized by depression, loss of appetite, vomiting, diarrhea (from mucoid to hemorrhagic), and leukopenia.<sup>1,9,20,21</sup> Subsequently, the virus was isolated in cell cultures and referred to as CPV-2 to distinguish it from the unrelated minute virus of canines (MVC or CPV-1), which is responsible for neonatal death in pups.<sup>6,13</sup> Canine parvovirus-2 belongs to the feline parvovirus subgroup of the genus *Parvovirus*, together with feline panleukopenia virus (FPLV), mink enteritis virus, raccoon parvovirus, raccoon dog parvovirus, and blue fox parvovirus.<sup>5</sup> The origin of CPV-2 is still unknown, although its derivation from FPLV in cats or from FPLV-like viruses in wild carnivores has been hypothesized.<sup>39–41</sup> Canine parvovirus-2 is able to replicate in canine and feline cells in vitro and in dogs, whereas replication of FPLV can be observed only in cats and feline or mink cell cultures.<sup>42</sup>

After the initial appearance of CPV-2, 2 antigenic variants were identified in the 1980s, CPV type 2a and 2b, which have completely replaced the original type 2<sup>11,28,29</sup> and are variously distributed worldwide.<sup>8,15,17,25,31,34,37,43,44</sup> These new types differ from the original type 2 by amino acid changes affecting the capsid protein and by their extended host range, which includes canine and feline cells in vitro and dogs and

cats. Subsequently, the emergence of further mutations in the CPV capsid protein has been reported in several countries.<sup>4,19,36,43,44</sup> In Italy, a mutant of CPV-2b has been identified, which presents the substitution Asp426Glu occurring in a strategic epitope of the capsid that differentiates this mutant from the classical type 2b CPVs.<sup>7</sup> The Glu-426 mutant is widely distributed in Italy and is currently cocirculating with types 2a and 2b.<sup>23</sup> Recently, antigenic and genetic analyses of the CPV-2 strains isolated in the past 3 years have revealed that the Glu-426 mutant is currently replacing CPV type 2b in the Italian dog population.<sup>16</sup> This type of CPV-2 mutant also has been detected in other countries.<sup>27</sup>

Whether the Asp426Glu change represents an advantage for viral spread has not yet been determined. However, a less-severe clinical course and a lower mortality rate have been observed in Glu-426 mutant-infected dogs than in reported outbreaks caused by type 2a and type 2b CPVs (C. Buonavoglia, unpublished data). This study describes the clinical behavior and the pattern of viral shedding by real-time polymerase chain reaction (PCR) in dogs naturally infected with the CPV Glu-426 mutant.

### Materials and methods

**Animals.** Two litters of German shepherds (A and B), each comprising 3 pups, were monitored throughout the study. The 2 litters, 10 and 9 wk of age, respectively, were housed in a breeding kennel in Apulia (Italy), together with 12 pedigreed dogs (7 males and 5 females). The dogs were not kept strictly separated but were managed by the same attendant. Both litters had not been vaccinated yet, whereas all adult dogs were systematically vaccinated with modified

From the Department of Animal Health and Well-being, Faculty of Veterinary Medicine of Bari, Valenzano, Bari, Italy.

<sup>1</sup>Corresponding Author: Nicola Decaro, Department of Animal Health and Well-being, Faculty of Veterinary Medicine of Bari, S.p. per Casamassima km 3, 70010 Valenzano, Bari, Italy.

**Table 1.** Sequence and position of oligonucleotides used in the study.\*

Primer/probe	Sequence 5' to 3'	Sense	Position†	Amplicon size (bp)
555-for‡	CAGGAAGATATCCAGAAGGA	+	4003–4022	583
555-rev‡	GGTGCTAGTTGATATGTAATAACA	–	4561–4585	
CPV-For§	AAACAGGAATTAACTATACTAATATATTTA	+	4104–4135	93
CPV-Rev§	AAATTTGACCATTTGGATAAACT	–	4176–4198	
CPV-Pb§	FAM-TGGTCCTTTAACTGCATTAATAATGTACC-TAMRA	+	4143–4172	

\* FAM = 6-carboxyfluorescein; TAMRA = 6-carboxytetramethylrhodamine.

† Oligonucleotide position is referred to the sequence of strain CPV-b (accession M38245).

‡ Conventional PCR.<sup>7</sup>

§ Real-time PCR.<sup>16</sup>

live canine distemper virus, canine adenovirus type 2, and CPV-2 and killed *Leptospira icterohemorrhagiae* and *canicola* vaccines. In March 2004, 1 pup (litter A) with mild diarrhea was presented to our institute and found to be infected with CPV-2 by a hemagglutination (HA) assay carried out on fecal samples. The CPV strain was characterized as type 2b by a hemagglutination inhibition (HI) assay using a panel of 4 monoclonal antibodies ([Mabs] A4E3, B4A2, C1D1, and B4E1)<sup>a</sup>, as described previously.<sup>34</sup> Molecular analysis with restriction fragment length polymorphism (RFLP) using *Mbo*II enzyme<sup>b</sup> characterized this CPV-2b strain as Glu-426 mutant, which was confirmed by sequence analysis of the PCR product generated with primer pair 555for–555rev<sup>7</sup> (Table 1).

After the onset of diarrhea in the first pup, the other 3 pups of litter A and the 3 pups of litter B, still negative for CPV-2, were monitored for the occurrence of Glu-426 mutant infection during an observation period of 60 days.

**Clinical studies and sampling.** Pups were examined daily for the evidence of clinical signs. Rectal temperatures were recorded daily for 20 days, and blood samples were taken for total white blood cell (WBC) counts as well as differential neutrophil and lymphocyte determinations. Serum samples were collected weekly for the evaluation of the antibody response to CPV-2 using a HI test. Fecal samples were collected daily for 60 days for detection of CPV-2 by HA, virus isolation in cell cultures, and real-time PCR analysis.

**Virus isolation.** Fecal samples were homogenized (10% wt/vol) in Dulbecco minimal essential medium and clarified by centrifuging at 10,000 × *g* for 5 min. The supernatants

were treated with antibiotics and inoculated onto freshly trypsinized A-72 canine cells. After an incubation period of 3 days, the cell cultures were tested by an immunofluorescence (IF) test using a Mab to CPV-2 (A4E3).<sup>a</sup> The cell cultures that tested negative by the IF assay were then passaged, and this procedure was repeated for a total of 3 passages. Samples were considered negative if the IF test remained negative after the third blind passage.

**Evaluation of CPV Glu-426 mutant shedding in the feces by real-time PCR.** Deoxyribonucleic acid preparations from fecal samples were analyzed by homogenization (10% wt/vol) in phosphate-buffered saline, followed by boiling for 10 min before chilling on ice. Serial 10-fold dilutions of the fecal extracts in distilled water were used in a real-time PCR assay for detection and quantitation of CPV-2 DNA, as described previously.<sup>16</sup> In brief, real-time PCR was carried out in a 25- $\mu$ l reaction containing 12.5  $\mu$ l of master mix<sup>c</sup>, 600 nM of primers CPV-For and CPV-Rev, 200 nM of probe CPV-Pb (Table 1), and 10  $\mu$ l of DNA. Serial 10-fold dilutions (representing from 10<sup>9</sup> to 10<sup>2</sup> DNA copies/10  $\mu$ l of standard DNA) of a plasmid<sup>a</sup> containing the nearly full-length genome of CPV-2 were used to generate a standard curve. The thermal cycle protocol used was done as follows: activation of *Taq* DNA polymerase at 95 C for 10 min and 40 cycles consisting of denaturation at 95 C for 15 sec, primer annealing at 52 C for 30 sec, and extension at 60 C for 1 min.

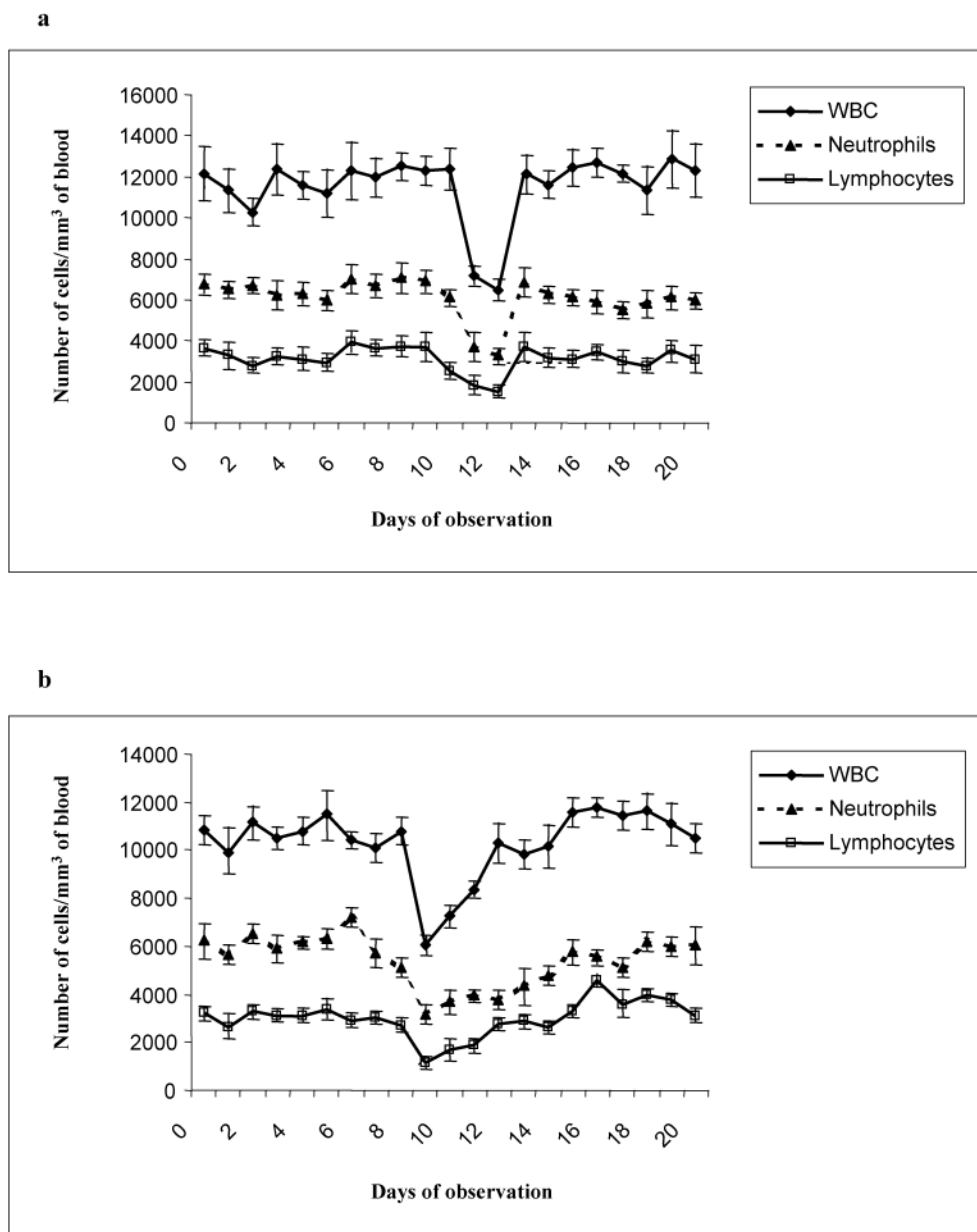
## Results

**Clinical findings and hematology.** All the 6 pups monitored throughout the study developed clinical signs of CPV-2 infection (Table 2), although none showed hemorrhagic diarrhea or vomiting and all recovered in a few days. The onset of clinical signs was registered at days 5 and 7 in pups of litters A and B, respectively. Mucoid diarrhea was observed for 3.5 median days, from days 8 to 10 in litter A and from days 8 to 11 in litter B. Two pups of litter A (A1 and A3) had a fever at days 8 and 9, with a peak at day 8 (39.8 C and 39.5 C, respectively), and loss of appetite for 3 days, from days 8 to 10 (pup A1) and from days 9 to 11 (pup A3). Fever and anorexia were not observed in pup A2 and in pups of litter B. Depression

**Table 2.** Clinical signs observed in pups infected with CPV-2 Glu-426 mutant.\*

Clinical signs	Pups					
	A1	A2	A3	B1	B2	B3
Vomiting	–	–	–	–	–	–
Diarrhea	+	+	+	+	+	+
Fever	+	–	+	–	–	–
Loss of appetite	+	–	+	–	–	–
Depression	+	+	+	+	+	+
Leucopenia	+	+	+	+	+	+

\* + = positive; – = negative.



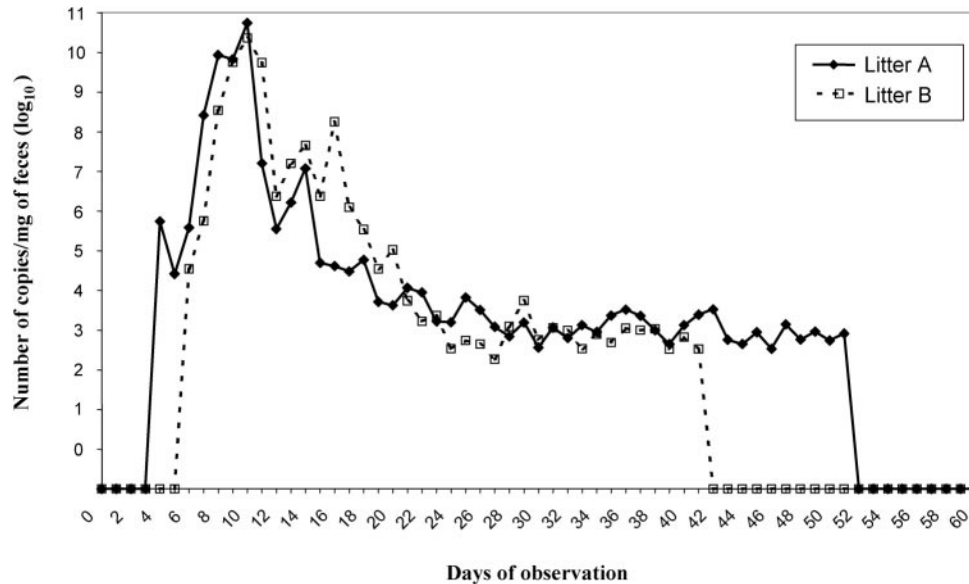
**Figure 1.** The  $\bar{x} \pm \text{SE}$  of leukocyte counts of litters A **a**, and B **b**, infected with CPV Glu-426 mutant.

was observed for 1.5 median days, but it was more severe in pup A1, lasting for 3 days, from days 8 to 10, versus 2 days (days 8–9) in pups A2 and A3 and for 1 day in pups of litter B (day 9 in pup B1 and day 10 in pups B2 and B3). All pups experienced a mild leukopenia for 2.5 median days, from days 9 to 11 in pups of litter A, with a peak ( $\bar{x} \pm \text{SE} = 6,027 \pm 399$  WBC/mm<sup>3</sup>) at day 9, and from days 11 to 12 in pups of litter B, with a peak ( $\bar{x} \pm \text{SE} = 6,427 \pm 353$  WBC/mm<sup>3</sup>) at day 13 (Fig. 1a). Similarly, a transient lymphopenia was observed for 2.5 median days, from days 9 to 11 in litter A, being maximum ( $\bar{x} \pm \text{SE} = 1,140 \pm 140$  lymphocytes/mm<sup>3</sup>) at day 9, and from days 11 to 13 in litter B, peaking at day 13 ( $\bar{x} \pm \text{SE} = 1,480$

$\pm 188$  lymphocytes/mm<sup>3</sup>) (Fig. 1b). Neutrophil counts showed similar patterns in both the litters (Fig. 1a, 1b).

**Fecal shedding of CPV Glu-426 mutant.** Canine parvovirus-2 infection was confirmed in all the pups by HA and real-time PCR. The occurrence of the same Glu-426 mutant, which had infected the first pup, was demonstrated in fecal samples collected from each pup by RFLP with *Mbo*II<sup>b</sup> and confirmed by sequence analysis of the PCR products generated with primer pair 555for–555rev<sup>7</sup> (data not shown).

Canine parvovirus-2 was detected by HA in all the fecal samples for 4.5 median days, from days 7 to 11 (litter A) or 8 to 11 (litter B). Canine parvovirus-2 was detected by virus isolation in cell cultures for 6.5



**Figure 2.** Number of copies (median  $\log_{10}$  titers) of CPV-2 DNA detected in the fecal samples of the infected pups by real-time PCR.

median days, from days 6 to 13 and 8 to 13 in litters A and B, respectively. In contrast, by real-time PCR, the DNA of CPV Glu-426 mutant was detectable for 46 median days, from days 4 to 54 in litter A and from days 6 to 46 in litter B, reaching a peak at day 10 in both litters, with median titers of  $5.49 \times 10^{10}$  and  $2.30 \times 10^{10}$  DNA copies/mg of feces, respectively (Fig. 2).

**Serology.** Hemagglutination inhibition antibody titers to CPV-2 were detected in all pups, reaching a peak at day 14, and remaining high throughout the observation period (Table 3).

### Discussion

Several reports have described the clinical features, changes in hematological parameters, and viral shedding in dogs naturally<sup>3,18,30</sup> or experimentally<sup>2,10,22,24,32,33</sup> infected with CPV-2 (original type). Despite the existence of several studies on the antigenic characterization and geographic distribution of type 2a and 2b

CPVs, limited data are available on the pathobiology of CPV-2 variants in dogs as compared with the original type 2. However, it seems that type 2a and 2b CPVs commonly cause a more severe disease with respect to the original type 2 (L. E. Carmichael, personal communication). In contrast, the pathogenicity of the CPV variants for cats has been investigated, although the results remain controversial.<sup>14,26,35</sup> This report describes an outbreak of CPV Glu-426 mutant infection, characterized by a mild clinical course and rapid recovery of the infected pups. Vomiting and hemorrhagic diarrhea did not occur in any infected pup, whereas fever and loss of appetite were observed only in 2 pups of litter A. All infected pups experienced a relative leukopenia and lymphopenia, with WBC and lymphocyte counts dropping below the baseline values at days 9–11 (litter A) and 11–13 (litter B).

Using real-time PCR, prolonged fecal shedding of the Glu-426 mutant DNA was observed. In previous studies, viral shedding in the feces had been detected up to 12 days by virus isolation in cell cultures<sup>32</sup> and for up to 7 days by HA.<sup>12</sup> This study confirms previous viral isolation data because virus isolation and HA tests were able to detect CPV-2 in the feces of the infected pups for 6.5 and 4.5 median days, respectively. However, the higher sensitivity of real-time PCR allowed the detection of viral nucleic acid for a much longer period, although titers dropped to about  $10^4$  DNA copies/mg of feces toward the end. Because prolonged transmission of CPV-2 to susceptible dogs may occur, these findings are of particular significance considering the high stability of CPV-2 in the environment. Previously, it has been reported that recovery is

**Table 3.** HI antibody titers in pups infected with CPV-2 Glu-426 mutant.\*

Pups	Days of observation				
	0	7	14	21	28
A1	<10	160	5,120	5,120	5,120
A2	<10	80	1,280	1,280	1,280
A3	<10	80	1,280	1,280	1,280
B1	<10	<10	640	1,280	1,280
B2	<10	<10	640	640	640
B3	<10	<10	1,280	1,280	1,280

\* Values are expressed as the reciprocal of the highest dilution of serum that inhibited agglutination by 8 hemagglutinating units of CPV-2.

associated with cessation of viral shedding<sup>33</sup> and that convalescent dogs were not contagious for contact dogs after 18<sup>12</sup> or 25<sup>32</sup> days after infection. Conversely, persistent or periodic shedding of CPV-2 in feces has been observed only rarely.<sup>38</sup> Because real-time PCR detects viral nucleic acid but not infectious virus, whether CPV-2 DNA in the feces of recovering pups is associated with the shedding of infectious virus can only be hypothesized. In fact, in the late stage of the CPV-2 infection (starting from days 8–10 after infection) specific antibodies in the intestinal lumen frequently sequester most of the CPV-2 virions, thus preventing parvoviral binding to cellular receptors and subsequent growth in cell cultures. Consequently, attempts to isolate CPV-2 from the feces in this stage of infection are frequently unsuccessful (C. Buonavoglia, unpublished observation). The results of this study seem to confirm previous data because the CPV Glu-426 mutant was successfully isolated in cell cultures for few days (6.5 median days) and only up to day 13, although high viral DNA amounts were detectable by real-time PCR for a much longer period. These findings underscore the need to investigate the epidemiological role of the CPV-2–recovering pups by *in vivo* transmission trials, such as housing susceptible pups in close contact with pups that are HA- and virus-isolation negative but real-time PCR positive.

### Sources and manufacturers

- a. C. R. Parrish, Cornell University, Ithaca, NY.
- b. *MboII*, MBI Fermentas GMBH, St. Leon-Rot, Germany.
- c. IQ<sup>®</sup> Supermix, Bio-Rad Laboratories Srl, Segrate (MI), Italy.

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