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First Detection of Canine Parvovirus Type 2c in Pups with Haemorrhagic Enteritis in Spain

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With 1 table

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Summary

Canine parvovirus type 2 (CPV-2), the aetiological agent of haemorrhagic enteritis in dogs, includes three antigenic variants, types 2a, 2b and 2c. CPV-2c has been detected initially in Italy and subsequently in Vietnam. We report the first identification of this novel antigenic variant in Spain, where it caused an outbreak of fatal enteritis in basset hound pups in association with canine coronavirus type I and type II. We suggest that this new antigenic variant of CPV-2 could spread throughout Europe and that there is a subsequent need to update current CPV vaccines.

Introduction

Canine parvovirus type 2 (CPV-2) is responsible for haemorrhagic enteritis in dogs (Carmichael and Binn, 1981). CPV-2 emerged in the late 1970s but in a few years it was replaced completely by two antigenic variants, named types 2a and 2b, that are characterized by amino acid substitutions occurring in the capsid protein (VP1/VP2) gene (Parrish et al., 1988). While the original type 2 is no longer circulating in the dog population and is present only in vaccine formulations (Parrish et al., 1991), its antigenic variants are distributed worldwide (Mochizuki et al., 1993; De Ybanez et al., 1995; Greenwood et al., 1996; Truyen et al., 1996, 2000; Sagazio et al., 1998; Steinel et al., 1998; Buonavoglia et al., 2000; Pereira et al., 2000; Buonavoglia et al., 2001; Martella et al., 2004, 2005b; Decaro et al., 2005a,c, 2006b; Desario et al., 2005). Real-time polymerase chain reaction (PCR) assays based on minor groove binder (MGB) probe technology have been developed for rapid characterization of the antigenic variants, taking advantage of single nucleotide polymorphisms occurring in the VP1/VP2 gene (Decaro et al., 2006b). An additional MGB probe assay has also been established for discrimination between vaccine and field strains of CPV (Decaro et al., 2006a).

In 2000, a mutant with the change Asp426Glu (Table 1) occurring in a strategic residue for the antigenicity of CPV-2 was detected in Italy (Buonavoglia et al., 2001). Such a mutant is currently spreading in dog population in Italy and progressively replacing types 2a and 2b (Martella et al., 2004, 2005b; Decaro et al., 2005a,c, 2006b; Desario et al., 2005). Recently, CPV-2c has been detected in Vietnam (Nakamura et al., 2004), whereas to date there are no reports in other European countries.

The pathogenicity of the new mutant has been investigated in dogs infected naturally (Decaro et al., 2005b) and a monoclonal antibody has been developed that is able to distinguish between type 2b and Glu-426 mutant (Nakamura et al., 2004), so that we proposed to designate such mutant as type 2c (Decaro et al., 2005a, 2006b). Previously, another CPV mutant had been reported in leopard cats that was referred to as CPV-2c (Ikeda et al., 2000). Such a mutant was found to display the substitution Gly300Asp in an epitope antigenically less strategic with respect to the Glu-426 mutant, allowing for the designation of the latter virus as true CPV-2c.

The present study reports a severe outbreak of CPV-2c infection occurring in a breeding kennel in Spain.

Materials and Methods

Clinical case

In January 2006, a severe outbreak of haemorrhagic enteritis occurred in a breeding kennel of basset hounds located in Tarragona, Catalonia, Spain. The kennel housed 80 adult dogs and 25 pups. All the adult dogs had been immunized regularly using multivalent vaccines containing the original type CPV-2. The outbreak involved a litter of seven 40-day-old pups, all of which displayed haemorrhagic diarrhoea and vomiting and underwent a fatal outcome within 3–5 days after the onset of the clinical signs. At that age, the pups had not been yet vaccinated against CPV-2.

Necropsy showed haemorrhagic enteritis in all seven pups, in the absence of remarkable lesions in other organs. A faecal sample was collected from one of the dead pups and subjected to virological examinations.

Sample preparation

The faecal sample was homogenized (10% w/v) in phosphate buffered saline (PBS, pH 7.2) and subsequently clarified by centrifuging at 1500 ***g*** for 15 min. The supernatant was then used for the diagnostic tests.

Table 1. Amino acid variations in the VP2 protein of different CPV types

	Amino acid variations at residue[a]							
	87	101	297	300	305	375	426	555
Nucleotide position	3045–3047	3087–3089	3675–3677	3684–3686	3699–3701	3909–3911	4062–4064	4449–4451
Codon observed	ATG (Met) TTG (Leu)	ATT (Ile) ACT (Thr)	TCT (Ser) GCT (Ala)	GCT (Ala) GGT (Gly)	GAT (Asp) TAT (Tyr)	AAT (Asn) GAT (Asp)	AAT (Asn) GAT (Asp) GAA (Glu)	GTA (Val) ATA (Ile)
CPV-2	Met	Ile	Ser	Ala	Asp	Asn	Asn	Val
CPV-2a	Leu	Thr	Ser	Gly	Tyr	Asp	Asn	Ile
CPV-2b	Leu	Thr	Ser	Gly	Tyr	Asp	Asp	Val
New CPV-2b	Leu	Thr	Ala	Gly	Tyr	Asp	Asp	Val
New CPV-2a	Leu	Thr	Ala	Gly	Tyr	Asp	Asn	Val
Asp-300 (CPV-2a/CPV-2b)	Leu	Thr	Ala	Asp	Tyr	Asp	Asn Asp	Val
CPV-2c	Leu	Thr	Ala	Gly	Tyr	Asp	Glu	Val

[a]Positions are referred to the amino acid and nucleotide sequences of strain CPV-b (GenBank accession no. M38245).

CPV-2 detection

Haemagglutination test

Two-fold dilutions in PBS of the supernatant of the faecal homogenate were subjected to haemagglutination (HA) test. Fifty microlitres of each dilution was mixed to equal amounts of a suspension containing 0.8% pig erythrocytes and 1% foetal calf serum (FCS) in a 96-well V-plate. Results were read after 4 h at +4°C and expressed as the reciprocal of the highest sample dilution able to produce HA.

Virus isolation

The supernatant of the faecal homogenate was treated with antibiotics (penicillin 5000 IU/ml, streptomycin 2500 μg/ml, amphotericin B 10 μg/ml) at 37°C for 30 min and inoculated onto freshly trypsinized A-72 cells grown in Dulbecco's minimal essential medium containing 5% FCS. After an incubation period of 5 days at 37°C, the inoculated cells were trypsinized and layered onto wells with glass slides. After additional 5 days of incubation, the cells were tested by an immunofluorescence (IF) assay using a dog anti-serum for CPV-2 and a rabbit anti-dog IgG conjugated with fluorescein isothiocyanate (Sigma Aldrich srl, Milan, Italy).

Real-time PCR assay with TaqMan probe

Viral DNA was extracted from the faecal homogenate by boiling for 10 min and chilling on ice (Schunck et al., 1995; Uwatoko et al., 1995). To reduce residual inhibitors of DNA polymerase activity to ineffective concentrations, the DNA extract was diluted 1 : 10 in distilled water (Decaro et al., 2005c).

The DNA extract was tested by a TaqMan assay able to recognize all the CPV-2 strains (Decaro et al., 2005c). The assay is internally controlled by using as exogenous DNA the nucleic acid extracted from ovine herpesvirus type 2 (Decaro et al., 2003). The reaction of real-time PCR (25 μl) contained 12.5 μl of master mix (Bio-Rad Laboratories Srl, Milan, Italy), 600 nM of primers CPV-For (5′-AAACAGGAATTAACTATACTA-ATATATTTA-3′) and CPV-Rev (5′-AAATTTGACC-ATTTGGATAAACT-3′), 200 nM of probe CPV-Pb (5′-FAM-TGGTCCTTTAACTGCATTAAATAATGTACC-TAMRA) and 10 μl of standard (plasmid) or template DNA. The following thermal protocol was used: activation of iTaq DNA polymerase at 95°C for 10 min and 40 cycles consisting of denaturation at 95°C for 15 s, primer annealing at 52°C for 30 s and extension at 60°C for 1 min.

CPV-2 characterization

Haemagglutination inhibition (HI) test

The antigenic characterization of the isolated strain of CPV-2 was performed using a panel of five MAbs (A4E3, B4A2, C1D1, B4E1 and 21C3) and eight HA units of virus, as described previously (Buonavoglia et al., 2001; Nakamura et al., 2004; Desario et al., 2005). The CPV-2 strain was typed as CPV-2 (original type), CPV-2a, CPV-2b or CPV-2c on the basis of MAb reactivity.

Real-time PCR assay with MGB probes

The MGB probe assays specific for types 2a/2b and 2b/2c (Decaro et al., 2006b) were carried out in a total volume of 25 μl containing 10 μl of template or standard DNA, 12.5 μl of IQ^{TM} Supermix (Bio-Rad Laboratories Srl), 900 nM of primers CPVa/b-For (5′-AGGAAGATATCCAGAAGGAG-ATTGGA-3′) and CPVa/b-Rev (5′-CCAATTGGATCTGT-TGGTAGCAATACA-3′) (type 2a/2b assay) or CPVb/c-For (5′-GAAGATATCCAGAAGGAGATTGGATTCA-3′) and CPVb/c-Rev (5′-ATGCAGTTAAAGGACCATAAGTATT-AAATATATTAGTATAGTTAATTC-3′) (type 2b/2c assay), 200 nM of probes CPVa-Pb (5′-VIC–CTTCCTGTAA-CAAATGATA-MGB-3′) and CPVb1-Pb (5′-FAM–CTTCCTGTAACAGATGATA-MGB-3′) (type 2a/2b assay) or CPVb2-Pb (5′-FAM–CCTGTAACAGATGATAAT-MGB-3′) and CPVc-Pb (5′-VIC–CCTGTAACAGAAGA-TAAT-MGB-3′) (type 2b/2c assay). Standard DNAs for types 2a, 2b and 2c were obtained from field faecal samples which had been found to contain high CPV-2 DNA titres by means of the TaqMan assay, as previously described (Decaro et al., 2005c). The thermal cycle protocol used was the following: activation of iTaq DNA polymerase at 95°C for 10 min and 45 cycles consisting of denaturation at 95°C for 30 s and primer annealing-extension at 60°C for 1 min.

Sequence analysis

For sequence analysis, the CPV-2 positive sample was amplified by a PCR assay with primer pair 555for/555rev (Buonavoglia et al., 2001), amplifying a fragment of the VP2 gene that contains informative residues for CPV-2 typing. The PCR products were purified on Ultrafree-DA columns (Amicon, Millipore Corporation, Billerica, MA, USA) and subjected to direct sequencing (Genome Express, Meylan, France). For sequence comparison, the nucleotide sequences of type 2, 2a, 2b and 2c CPVs were retrieved from the GenBank database. The strains and accession numbers used for sequence analysis were the following: CPV-2: CPV-b, M38245 and CPV-Norden, M19296; CPV-2a: CPV-15, M24003 and CPV-31, M24000; CPV-2b: CPV-39, M74849 and CPV-133, M74852; CPV-2c (Glu-426 mutant): 56/00, AY380577. Alignments and sequence analysis were performed using the BioEdit software package, version 7.0.1 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html).

Screening for pathogens of dogs associated to infectious enteritis

The sample collected from the died dog was also tested by (RT-)PCR or real-time (RT-)PCR for detection of other common viral pathogens that are responsible for enteritis in dogs, such as reoviruses (Leary et al., 2002; Decaro et al., 2005d), rotaviruses (Gouvea et al., 1994), caliciviruses (Hashimoto et al., 1999; Marsilio et al., 2005), canine adenoviruses (Hu et al., 2001), canine distemper virus (Elia et al., 2006), canid herpesvirus type 1 (Schulze and Baumgartner, 1998) and canine coronavirus (CCoV; Decaro et al., 2004a). Bacteriological investigations were also carried out using standardized methods.

Results

CPV-2 detection

The faecal sample tested positive by the HA test, giving a HA titre of 1 : 1024. The CPV-2 strain was isolated successfully on A-72 cells at the first passage, as shown by the nuclear fluorescence observed in the inoculated cells by the IF test specific for CPV-2 antigen. The TaqMan assay detected the CPV-2 DNA in the specimen at high titre (2.28×10^{10} copies/mg of faeces).

CPV-2 characterization

On the basis of the reactivity to the panel of MAbs, the isolated strain was characterized as type 2c. By real-time PCR with MGB probes, no signal was registered in the type 2a/2b assay, whereas VIC fluorescence was generated in the type 2b/2c assay, which recognized the parvovirus strain as type 2c, confirming the results of MAb analysis.

Sequence analysis of the fragment amplified with primers 555for and 555rev showed a 100% nucleotide identity to strain 56/00 (Buonavoglia et al., 2001), assigning definitively the isolated strain to CPV-2c.

Simultaneous detection of other pathogens of dogs

By means of real-time RT-PCR assays specific for CCoV types I and II (Decaro et al., 2005f), both genotypes were detected in the faecal sample. The molecular assays gave negative results for the other viral pathogens. Bacteriological investigations failed to detect other important pathogens of the dog.

Discussion

The present study represents the first report on the detection of CPV-2c in Spain. Whether the virus is widespread in this country or its presence is sporadic could be assessed only by extensive collection and analysis of faecal samples from dogs with diarrhoea housed in Spanish shelters, breeding kennels and pet shops.

An intriguing finding is represented by the simultaneous detection in the faeces of CCoV type I and type II, as mixed infections are associated to a more severe clinical course of the disease and frequent fatal outcome of the infected dogs. In previous studies, both genotypes were detected simultaneously in the faecal samples of most dogs infected with CCoV (Pratelli et al., 2004; Decaro et al., 2005f). Moreover, it has been shown that CCoV can exacerbate the clinical course of concurrent infections caused by CPV-2, by damaging the intestinal villi and enhancing the mitotic activity of the crypt cells where CPV replicates (Evermann et al., 1980; Yasoshima et al., 1983; Pratelli et al., 1999).

The occurrence of canine parvovirosis in 40-day-old pups born to vaccinated bitches may indicate a failure of the maternally derived antibodies (MDA) in protection against CPV-2. The involvement of the entire litter in terms of morbility and mortality could be related to a poor protection against the CPV variants by MDA for the original type 2, rather than to a failure in the transfer of MDA from the bitch to its offspring (Decaro et al., 2004b). Because of the physicochemical properties of CPV-2 (high resistance in the environment with long persistence in kennels and shelters), a good vaccine should prevent the disease as well as the viral shedding by dogs eventually infected. Dogs with HI MDA titres $\geq$1 : 80 are considered protected against disease and viral shedding after challenge with virulent CPV-2 (Pollock and Carmichael, 1982). However, more recently it has been observed that pups with HI MDA titres up to 1 : 160, conventionally considered protective from CPV infection (Pollock and Carmichael, 1982), were infected by CPV-2b and shed virus in their faeces (Decaro et al., 2005e; Elia et al., 2005). Consequently, the minimal MDA level required for protection from CPV infection has to be reconsidered (Decaro et al., 2005e). The present report stresses the need to update the CPV vaccines, by replacing the original type 2 ('ghost' virus) with the CPV variants currently circulating (Martella et al., 2005a; Truyen, 2006).

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