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Review

# Canine parvovirus—A review of epidemiological and diagnostic aspects, with emphasis on type 2c

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ABSTRACT

Canine parvovirus type 2 (CPV-2) emerged in late 1970s causing severe epizootics in kennels and dog shelters worldwide. Soon after its emergence, CPV-2 underwent genetic evolution giving rise consecutively to two antigenic variants, CPV-2a and CPV-2b that replaced progressively the original type. In 2000, a new antigenic variant, CPV-2c, was detected in Italy and rapidly spread to several countries. In comparison to the original type CPV-2, the antigenic variants display increased pathogenicity in dogs and extended host range, being able to infect and cause disease in cats. Epidemiological survey indicate that the newest type CPV-2c is becoming prevalent in different geographic regions and is often associated to severe disease in adult dogs and also in dogs that have completed the vaccination protocols. However, the primary cause of failure of CPV vaccination is interference by maternally derived immunity. Diagnosis of CPV infection by traditional methods has been shown to be poorly sensitive, especially in the late stages of infections. New diagnostic approaches based on molecular methods have been developed for sensitive detection of CPV in clinical samples and rapid characterisation of the viral type. Continuous surveillance will help assess whether there is a real need to update currently available vaccines and diagnostic tests.



## Contents



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## 1. An overview on canine parvovirus

### 1.1. Taxonomy and virus structure

The family *Parvoviridae* comprises two subfamilies, *Parvovirinae* and *Densovirinae*, infecting vertebrates and insects, respectively. Currently, five genera are included in the subfamily *Parvovirinae*, namely *Parvovirus*, *Erythrovirus*, *Dependovirus*, *Amdovirus* and *Bocavirus*. Canine parvovirus (CPV) belongs to genus *Parvovirus* and has been included in the unique species *Feline panleukopenia virus* together with feline panleukopenia virus (FPV), mink enteritis virus (MEV) and raccoon parvovirus (RPV) (Tattersall et al., 2005). CPV is genetically and antigenically unrelated to canine minute virus (CnMV), formerly known as canine parvovirus type 1 (CPV-1), which is responsible for neonatal death in dogs and is now included in the genus *Bocavirus* together with bovine parvovirus and human bocavirus (Tattersall et al., 2005).

Parvoviruses are small (diameter of 25 nm), nonenveloped viruses infecting vertebrates and insects. The parvovirus virion consists of a spherical capsid, which is composed by three proteins and contains a linear, single-strand DNA molecule (Muzyczka and Berns, 2001). By means of X-ray crystallography, the parvovirus capsid has been found to be formed by 60 copies of a combination of VP1, VP2 and VP3. VP1 contains the full-length VP2 sequence plus an additional N-terminal domain. VP2, the most abundant structural protein, accounts for 90% of the viral capsid, representing the major determinant of host range and virus-host interactions, and is cleaved to VP3 by host proteases. The 60 capsid protein subunits are made up of a core eight-stranded, antiparallel β-barrel domain with loop insertions between the strands forming large spike-like protrusions at or surrounding the icosahedral threefold axes. Other characteristic features of the parvoviral capsid include a 15-Å canyon-like depression about the fivefold axes and a dimple-like depression at the icosahedral twofold axes. The parvoviral genome consists of a ∼5000-nucleotide DNA molecule containing two large open reading frames (ORFs) and smaller or overlapping genes, encoding for two nonstructural (NS1 and NS2) and two structural (VP1 and VP2) proteins through alternative splicing of the same mRNAs.

Virus replication takes place in the cell nuclei and requires rapidly dividing cells of fetuses and newborns or of hematopoietic and intestinal tissues of young and adult animals. Their replication in vivo is rarely associated with the appearance of nuclear inclusion bodies, whereas the cytopathic effect induced in vitro is not always evident. All parvoviruses are highly stable in the environment, as they are extremely resistant to pH and temperature changes and to treatment with lipid solvents, trypsin and most disinfectants. Virions can be inactivated by formalin, sodium hypochlorite, beta propiolactone, hydroxylamine, oxidizing agents and ultraviolet irradiation. Several parvoviruses are able to agglutinate erythrocytes of different mammal and bird species, and some diagnostic tests for parvovirus infections rely on this hemagglutination activity (Muzyczka and Berns, 2001).

### 1.2. Origin and evolution of canine parvovirus type 2

CPV emerged as dog pathogen in the late 1970s as host variant of FPV, likely through adaptation of an FPV-like parvovirus of wild carnivores. Although there is no definitive evidence, this hypothesis is supported by the active circulation of intermediate viruses between FPV and CPV in wild carnivores and by the inability of FPV to infect dogs (Truyen, 2006). The original viral strain, designated as CPV-2 to distinguish the novel virus from the previously-known CPV-1, caused severe, fatal epizootics of hemorrhagic gastroenteritis and subacute myocarditis in kennels and shelters worldwide (Truyen, 2006). There are at least six or seven amino acid (aa) changes between FPV and CPV-2 (Table 1), mostly accumulated in the VP2 domain interacting with the host-cell transferrin receptor (TfR). These changes may have contributed to the gain of affinity for canine TfR observed during the shift from FPV or FPV-like parvovirus to CPV-2 (Shackelton et al., 2005). Another important difference between these two carnivore parvoviruses is that CPV evolves much more rapidly than FPV (Battilani et al., 2006; Decaro et al., 2008c, 2009b), showing genomic substitution rates similar to those of RNA viruses, with values of about $10^{-4}$ substitutions per site per year (Shackelton et al., 2005).

Active virus circulation and initial vaccination programmes helped develop herd immunity in canine populations, which greatly reduced mortality and further spreading of the virus. However, host-immunity pressure may have also contributed to the progressive emergence of CPV-2 antigenic variants. In the 1980s, two antigenic variants, distinguishable using monoclonal antibodies (MAbs), emerged within few years and were termed CPV types 2a (CPV-2a) and 2b (CPV-2b). Currently, the antigenic variants have completely replaced the original type 2, which is still used in most commercial vaccines, and are variously distributed in the canine population worldwide. CPV-2a and CPV-2b differ from the original strain CPV-2 in five or six aa residues of the VP2 capsid protein (Table 1). In contrast, only two residues differentiated CPV-2a from CPV-2b, i.e., N426D and I555V (Truyen, 2006). Residue 426 is situated in epitope A, over the threefold spike of the capsid, and a role of antigenic escape has been

**Table 1**
Amino acid variations in the VP2 protein of feline and canine parvoviruses.[a]

| Aa residue | 80 | 87 | 93 | 101[b] | 103 | 232 | 297 | 300 | 305 | 323 | 375 | 426[c] | 555 | 564 | 568 |
|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|---|
| Nt position | 3024–3026 | 3045–3047 | 3063–3065 | 3087–3089 | 3093–3095 | 3480–3482 | 3675–3677 | 3684–3686 | 3699–3701 | 3753–3755 | 3909–3911 | 4062–4064 | 4449–4451 | 4476–4478 | 4488–4490 |
| Codon observed | AAA (Lys)<br>AGA (Arg) | ATG (Met)<br>TTG (Leu) | AAA (Lys)<br>AAC (Asn)<br>AAT (Asn) | ATT (Ile)<br>ACT (Thr) | GUA (Val)<br>GCA (Ala) | GTA (Val)<br>ATA (Ile) | TCT (Ser)<br>GCT (Ala) | GCT (Ala)<br>GGT (Gly) | GAT (Asp)<br>TAT (Tyr) | GAC (Asp)<br>AAC (Asn) | AAT (Asn)<br>GAT (Asp) | AAT (Asn)<br>GAT (Asp)<br>GAA (Glu) | GTA (Val)<br>ATA (Ile) | AAT (Asn)<br>AGT (Ser) | GCT (Ala)<br>GGT (Gly) |
| FPV | Lys | Met | Lys | Ile | Val | Val | Ser | Ala | Asp | Asp | Asp | Asn | Val | Asn | Ala |
| CPV-2 | Arg | Met | Asn | Ile | Ala | Ile | Ser | Ala | Asp | Asn | Asn | Asn | Val | Ser | Gly |
| CPV-2a | Arg | Leu | Asn | Thr | Ala | Ile | Ser | Gly | Tyr | Asn | Asp | Asn | Ile | Ser | Gly |
| CPV-2b | Arg | Leu | Asn | Thr | Ala | Ile | Ser | Gly | Tyr | Asn | Asp | Asp | Val | Ser | Gly |
| New CPV-2a | Arg | Leu | Asn | Thr | Ala | Ile | Ala | Gly | Tyr | Asn | Asp | Asn | Val | Ser | Gly |
| New CPV-2b | Arg | Leu | Asn | Thr | Ala | Ile | Ala | Gly | Tyr | Asn | Asp | Asp | Val | Ser | Gly |
| Asp-300 (2a/2b) | Arg | Leu | Asn | Thr | Ala | Ile | Ala | Asp | Tyr | Asn | Asp | Asn (2a)<br>Asp (2b) | Val | Ser | Gly |
| CPV-2c | Arg | Leu | Asn | Thr | Ala | Ile | Ala | Gly | Tyr | Asn | Asp | Glu | Val | Ser | Gly |

[a] Positions are referred to the amino acid and nucleotide sequences of strain CPV-b (accession no. M38245).
[b] Codon affected by SNPs used to design type-specific probes differentiating CPV-2 from CPV-2a/2b/2c.
[c] Codon affected by SNPs used to design type-specific probes differentiating CPV-2a from CPV-2b and CPV-2b from CPV-2c.

assigned to the same residue of the VP2 of the parvovirus minute virus of mice. At residue 555, old type 2a CPVs displayed an isoleucine residue differentiating this variant from CPV2/2b strains both exhibiting V555. With the subsequent introduction of the change I555V, the recent CPV-2a strains differ from CPV-2b/2c only at position 426 of the VP2 protein (Table 1) (Martella et al., 2006; Decaro et al., 2009b).

Other mutations occurring during the last decades and being fixed in type 2a/2b CPVs include S297A and T440A. Residue 297 is located in the middle of epitope B and substitutions at this position may be responsible for changes in antigenicity of CPV variants (Truyen, 2006). Change T440A has been reported worldwide (Kapil et al., 2007; Kang et al., 2008; Decaro et al., 2009b), but its implications are not clearly understood. The majority of these changes affects the VP2 region comprised between residues 267 and 498, which forms the GH loop located between the βG and βH strands and is affected by the greatest variability among parvoviruses due to its exposure on the capsid surface.

### 1.3. Pathology

CPV recognizes as target tissues for viral replication the intestinal crypts and the lymphoid organs, but the virus can spread to all tissues (Pollock, 1982), including the brain (Elia et al., 2007; Decaro et al., 2009a). After penetration through the oronasal route, the virus replicates in gastroenteric associated lymphoid tissues and is disseminated by infected leukocytes to the germinal epithelium of the crypts of the small intestine, causing diarrhea. Infection of leukocytes, mainly circulating and tissue-associated lymphocytes, induces acute lymphopenia (often associated with neutropenia) (Pollock, 1982). In 2–3-week-old seronegative pups, CPV is also able to replicate in cardiac cells inducing a fatal myocarditis. However, this form is no longer observed as almost all young pups are protected by maternally derived antibodies (MDA) (Greene and Decaro, in press).

The most characteristic clinical form induced by CPV is represented by hemorrhagic enteritis, the extent of which is often dependent on the MDA titers of the infected pups at the moment of infection. Clinical signs occur after an incubation period of 3–7 days and consist of anorexia, depression, vomiting and mucoid or bloody diarrhea, frequently dehydration and fever. Leukopenia is a constant finding, with white blood cell (WBC) counts dropping below 2000–3000 cells/μL of blood. However, total WBC counts may be even within normal ranges due to concomitant virus-induced lymphopenia, and neutrophilia consequent to infections by opportunistic bacteria. Concurrent pulmonary infections may lead to the onset of respiratory distress. Subclinical and inapparent infections are frequently detected, mainly in pups with intermediate MDA titers and in adult dogs (Decaro et al., 2005a). The mortality rates can be high (up to 70%) in pups, but are usually less than 1% in adult dogs.

Hemorrhagic enteritis of the small intestine and enlargement of mesenteric lymph nodes and Peyer's patches are the main gross lesions observed in dogs that

die as a consequence of CPV infection. Histopathologically, the small intestine is affected by multifocal crypt necrosis and intranuclear inclusion bodies, whereas extensive depletion of lymphocytes is seen in Peyer's patches, lymph nodes, spleen and thymus (Greene and Decaro, in press).

### 1.4. Prophylaxis

Prophylaxis of CPV infection relies mainly on extensive vaccination. Since inactivated vaccines are able to induce only short-term immunity, modified live virus (MLV) vaccines are widely used. These vaccines, prepared by using either the original type CPV-2 or its variant CPV-2b, are highly effective, being able to protect dogs against parvoviral disease as well as infection, and almost completely safe, as postvaccinal reactions are very rarely observed. A recent study showed that most dogs developing parvovirus-like diarrhea after vaccination were infected by the field virus alone or with the attenuated vaccine virus (Decaro et al., 2007b).

The primary causes of failure of CPV vaccination are interfering levels of MDA that are transmitted by bitches to their offspring through colostrum and, at a lesser extent, milk. Thus, in order to avoid the interference with active immunisation, vaccines should be administered to pups only after waning of MDA (Greene and Decaro, in press). Different strategies have been proposed to overcome the MDA interference, including high-titer vaccines (Burtonboy et al., 1991) and intranasal vaccination (Martella et al., 2005). The Vaccination Guidelines Group of the World Small Animal Veterinary Association also recommends delaying finish of primary CPV vaccination course to 14–16 weeks of age to ensure protection even in pups with long-lasting MDA. In addition, there are some concerns about the complete efficacy of type 2-based vaccines against the antigenic variants (Kapil et al., 2007; Decaro et al., 2008a, 2009a).

## 2. Canine parvovirus type 2c

### 2.1. Emergence and taxonomical issues

In 2000, a new antigenic type of CPV was detected in Italy (Buonavoglia et al., 2001). The fecal samples of two dogs with hemorrhagic enteritis tested positive for CPV by hemagglutination (HA) and both strains were characterised as CPV-2b on the basis of their reactivity to monoclonal antibodies (MAbs) and the results of genotyping polymerase chain reaction (PCR). However, by sequence analysis of a large fragment of the VP2 gene, the two CPV-2b strains were found to contain two unexpected amino acid variations, S297A and D426E, with respect to classical type 2b CPVs. While the change at residue 297 is shared by most type 2a and 2b CPVs circulating currently (Truyen, 2006), the substitution D426E, due to a single nucleotide change at the third codon position, had not been observed previously and is situated in epitope A, over the three-fold spike of the capsid. Subsequently, viruses sharing this unusual mutation, firstly named Glu426 mutant, were detected at high frequency in Italy (Decaro et al., 2006b, 2007a) and were found to have a worldwide distribution (Nakamura et al., 2004; Decaro et al., 2006c, 2007a, 2011b; Hong et al., 2007; Kapil et al., 2007; Perez et al., 2007; Vieira et al., 2008; Touihri et al., 2009; Nandi et al., 2010; Ntafis et al., 2010; Filipov et al., 2011).

The widespread distribution of the Glu-426 mutant posed a taxonomical issue as this mutant was proposed as a new variant CPV-2c (Decaro et al., 2006b). However, the same designation had been previously assigned to CPV strains detected in domestic and wild felids in southern Asia. Those mutant strains displayed the novel substitution irrespective of the antigenic type (2a or 2b), so that had been referred to as CPV-2a(c) or CPV-2b(c) on the basis of the aa encountered at residue 426 (Asn or Asp) of the VP2 protein (Ikeda et al., 2000). The mutation at residue 300 was associated to the further adaptation of the CPV variants to the feline host (Ikeda et al., 2000), as indicated by the appearance of the same change in a CPV mutant after consecutive passages in feline cells in vitro. Although this change has been recently reported in a dog in Korea (Kang et al., 2008), it was absent from all CPV variant strains sequenced in several studies conducted in different parts of the world (Meers et al., 2007; Ohshima et al., 2008; Decaro et al., 2009b), thus restricting circulation of those mutants to South-East Asia. In contrast, the Glu-426 mutant has spread very efficiently to the canine population in Italy and has been reported in other parts of world. Moreover, although mutation G300D is located in the middle of antigenic site B and controls the binding of many antibodies to the capsid, the changeD426E has been previously taken into account for classification of the variants 2a and 2b (Truyen, 2006). On the basis of the above considerations, the Glu-426 mutant has been tentatively designated as true antigenic variant 2c, whereas the Asp-300 CPVs should be regarded as mutants of CPV-2a and CPV-2b (Decaro et al., 2009b).

### 2.2. Antigenic type distribution in the world

The recent development of real-time PCR assays based on minor groove binder (MGB) probe technology has led to a considerable simplification of the diagnostic approaches for CPV characterisation. Type-specific MGB probe assays were employed to characterise 414 CPV-positive samples collected in Italy during the decade 1995–2005, finding 268 (64.73%) type 2a, 49 (11.83%) type 2b and 97 (23.43%) type 2c CPVs. The retrospective analysis has revealed that CPV-2c was not present in Italy before 2000. The frequency of the variants underwent a rapid fluctuation during years 1995–2005, with CPV-2c replacing very rapidly CPV-2b, that has been detected more rarely in Italy in the last years (Decaro et al., 2006b). More recently, the new variant 2c was detected in several countries. In an epidemiological survey for CPV, Nakamura et al. (2004) found a CPV-2c strain in Vietnam. In 2005, such a variant was isolated from an outbreak of fatal gastroenteritis occurred in a breeding kennel of basset hounds located in Catalonia, Spain (Decaro et al., 2006c). The results of two different European epidemiological surveys (Decaro et al., 2007a, 2011b) showed that CPV-2c is now predominant in Italy, Germany and Spain and is also widely distributed in Portugal, France and Belgium, where CPV-2b or CPV-2a are more frequently

**Table 2**
Distribution of the canine parvovirus variants in the world.[a]

| Continent/country | Number of strains detected | | |
|---|---|---|---|
| | CPV-2a | CPV-2b | CPV-2c |
| Europe | | | |
| Italy | 56 | 6 | 62 |
| Portugal | 0 | 16 | 15 |
| Spain | 3 | 1 | 9 |
| France | 0 | 9 | 7 |
| UK | 117 | 182 | 1 |
| Belgium | 17 | 0 | 9 |
| Germany | 13 | 18 | 21 |
| Greece | 81 | 1 | 2 |
| Switzerland | 1 | 0 | 0 |
| Czech Republic | 1 | 1 | 0 |
| Romania | 2 | 0 | 0 |
| Hungary | 27 | 0 | 0 |
| Bulgaria | 31 | 9 | 1 |
| Slovenia | 1 | 0 | 0 |
| Africa | | | |
| Tunisia | 15 | 21 | 14 |
| North America | | | |
| USA | 1 | 36 | 30 |
| South America | | | |
| Uruguay | 1 | 0 | 24 |
| Argentina | 9 | 4 | 14 |
| Brazil | 37 | 0 | 0 |
| Asia | | | |
| India | 37 | 4 | 0 |
| India | [b] | [b] | 3 |
| Taiwan | 2 | 34 | 0 |
| Korea | 119 | 7 | 0 |
| Japan | 4 | 21 | 0 |
| China | 27 | 5 | 0 |
| Thailand | 19 | 7 | 0 |
| Oceania | | | |
| Australia | 41 | 1 | 0 |

[a] Adapted from Greene and Decaro (in press).
[b] Sixteen samples were positive for CPV-2a or CPV-2b, which one was not determined.

detected (Table 2). Sporadic isolation of CPV-2c has been obtained also from the United Kingdom, Greece and Bulgaria, where there was a higher frequency of CPV-2a/2b detection (Decaro et al., 2007a; Ntafis et al., 2010; Filipov et al., 2011). The oldest CPV-2c strain was isolated in 1996 (Decaro et al., 2007a), thus providing evidence that this variant had been circulating in Germany for 4 years before its first detection in Italy in 2000 (Buonavoglia et al., 2001). VP2 sequence analysis of 150 CPV strains obtained from a large cross-sectional sample of dogs presenting with severe diarrhea to veterinarians in the United Kingdom over a 2-year period revealed that 43% of viruses were CPV-2a, and 57% CPV-2b, with no type 2c found (Clegg et al., 2011).

Outside Europe, all three variants are well represented in Tunisia (Touihri et al., 2009). Type 2b and 2c isolates predominate in North America (Hong et al., 2007; Kapil et al., 2007), whereas CPV-2c is more widespread in South America (Perez et al., 2007; Calderon et al., 2009) with the exception of Brazil, where all circulating strains were characterised as CPV-2a (Castro et al., 2011). CPV-2a is the predominant variant in Asia (Kang et al., 2008; Ohshima et al., 2008; Nandi et al., 2010) and Australia (Meers et al., 2007), although few CPV-2c strains have been detected in India (Nandi et al., 2010) (Table 2).

### 2.3. Host range and pathogenic potential

There are at least six or seven aa changes between FPV and CPV-2, and at least five or six aa changes between the variants CPV-2a/b and the original type 2 (Table 1). These few aa differences in the VP2 sequences of FPV, CPV-2 and CPV-2a/b account for important antigenic and biological changes (e.g., the host range shift), making CPV an important model to study virus evolution (Hueffer and Parrish, 2003).

In vitro, while FPV replicates efficiently only in feline cell lines, CPV can infect with the same efficiency cells of canine and feline origin. In vivo, FPV replicates in dogs in the thymus and bone marrow without being shed in the feces, and the original canine virus, CPV-2, does not replicate at all in cats. Conversely, both the type 2a and 2b variants have re-gained the ability to replicate in vivo in the feline host (Truyen, 2006). Studies on the interactions of FPV and CPV with their cellular receptor, the transferrin receptor type 1, have revealed that FPV specifically binds the feline TfR, whereas CPV-2 and its variants can bind both the feline and the canine TfRs. Interestingly, the antigenic variants of CPV-2 bind the canine and feline TfRs less efficiently than the original type-2 (Palermo et al., 2006).

Cases of feline panleukopenia caused by CPV-2a or 2b in wild and domestic felids have been reported in different parts of the world (Truyen, 2006). As for the new variant CPV-2c, a first case was reported in a cat in Italy, but nothing was known about the clinical conditions, hematological parameters and outcome of the infection (Battilani et al., 2006). More recently, two cases of CPV-2c infection in cats were observed in the same country (Decaro et al., 2010a, 2011a). In both cases, a mild form of disease was evident with no or moderate modifications of hematological parameters; neurological disease occurred in one cat, but this was associated to a concurrent intracranial abscess (Decaro et al., 2011a).

Another biological consequence of the accumulation of aa changes in the VP2 protein is the increased pathogenicity of the CPV-2 variants (Carmichael, 2005). In comparison to the original type 2, the antigenic variants 2a and 2b have been reported to be shed in the feces at much higher titers and to cause a more severe disease. In addition, a lower virus dose seems to be required for efficient infection. Experimental infection of dogs with different levels of MDA showed that CPV-2b is shed at very high titers in the feces of the infected dogs and that even pups with MDA hemagglutination-inhibition titers of 1:160 may become infected (Decaro et al., 2005a).

The pathogenicity of CPV-2c has been investigated in two litters of German shepherds with natural infection (Decaro et al., 2005b). All pups displayed clinical signs of parvovirosis (mucoid or watery diarrhea and transient leukopenia), but none showed either hemorrhagic diarrhea or vomiting and all recovered in a few days. By real-time PCR, prolonged shedding (up to 51 days) of CPV-2c was observed. However, the benign course of the CPV-2c infection is in contrast with recent findings that are indicative of a more severe disease induced by this mutant. Spibey et al. (2008) have carried out an experimental infection of beagle dogs with a field isolate of CPV-2c. This

experimental challenge was carried out principally to confirm the ability of an existing type 2-based vaccine to prevent clinical signs and shedding. However, in this context it is of interest that all six of the infected control dogs became severely ill and displayed leukopenia from day 4 post-infection; three of them had to be euthanized and the remaining three recovered but required a supportive therapy. To date, whether there is a greater infection or virulence by one variant over the others is not well established either in experimental systems or on the basis of field observations. Indeed, it is reasonable to think that all three variants have a similar pathogenetic potential, which can increase depending on particular field conditions (age, immunological status, stressing factors, etc.).

Commonly, CPV infects 4–12-week-old pups that are prone to acquire the virus in concomitance with the wane of MDA (Greene and Decaro, in press). Adults are thought to be resistant to CPV infection due to the age-reduced susceptibility and presence of specific immunity induced by vaccination or previous (often subclinical) infections. Although CPV infection is generally restricted to young animals, it has recently become an issue also in adult dogs. Apart from personal observations (Kapil et al., 2007), there are some scientific reports of the occurrence of parvovirosis in adult dogs, mostly associated to CPV-2c infection (Cavalli et al., 2001; Decaro et al., 2008a, 2009a). In one episode, a two-year-old mixed breed dog was severely affected as a consequence of infection with a strain which was mischaracterised as CPV-2b by means of MAbs (Cavalli et al., 2001). However, molecular characterisation by PCR-RFLP and sequence analysis of the VP2 protein gene was able to correctly type the virus as 'true' CPV-2c (Martella, unpublished data). Another CPV-2c outbreak occurred in a breeding kennel in Italy, affecting 11 dogs aged between 6 months and 2.5 years and leading to the death of a 20-month-old Bernese mountain pregnant bitch (Decaro et al., 2008a). A case of disease induced by this variant was described even in a 12-year-old dog displaying fever (40.5 °C), accelerated pulse and respiratory rates, abdominal pain, vomiting, hemorrhagic diarrhea and dramatic lymphopenia. However, this dog recovered progressively despite the severity of the disease (Decaro et al., 2009a).

### 2.4. Cross-protection and perspectives of canine parvovirus vaccination

In most CPV-2c outbreaks reported in adult dogs so far, the animals had undergone the full vaccination schedules including a booster vaccination on yearly basis (Decaro et al., 2008a, 2009a). In CPV-2 variant outbreaks involving younger dogs, the vaccination protocol scheduled for the first year of age had been completed (Kapil et al., 2007; Perez et al., 2007; Calderon et al., 2009; Ntafis et al., 2010; Castro et al., 2011; Filipov et al., 2011).

Protection elicited by CPV-2 based vaccines against the field variants still represents a "vexata quaestio" as the 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. On the opposite side, 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. It has been shown that there is a one-way cross-reactivity between the antigenic variants and the original CPV-2. Sera raised against CPV-2 displayed lower virus neutralizing (VN) titers against the heterologous virus (CPV-2b) in comparison to those obtained when sera raised against CPV-2b were run against the original CPV-2 (Pratelli et al., 2001). Similar experiments were carried out for evaluation of serological cross-reactivity between CPV-2 and its antigenic variants in sera of dogs and rabbits (Cavalli et al., 2008). Animals administered CPV-2 showed significant lower VN antibody titers against the heterologous viruses with respect to the homologous virus. These findings were confirmed by cross-hemagglutination inhibition studies conducted in dogs (Ohshima et al., 2008) and guinea pigs (Kang et al., 2008).

Despite this measurable difference in strain recognition, other studies have shown that 'old-type' vaccines are able to protect dogs against next challenge with CPV-2c in experimental conditions (Spibey et al., 2008; Siedek et al., 2011). However, in those experiments the challenge was carried out in controlled conditions few weeks after vaccination, when antibodies commonly reach the highest levels (Decaro et al., 2005a). Nothing is know about the protection induced by the original type against CPV-2c (as well as against the other variants) after a long period interval between vaccination and challenge, when the type-2 antibody titers could be not high enough to efficiently prevent infection and disease caused by a field strain. In addition, in one study dogs vaccinated with CPV-2 were clinically protected from challenge with virulent variant virus, but shed the challenge virus, thus proving to be susceptible to infection (Siedek et al., 2011). Taking into account the concern that the antigenic differences between the original type 2 and its variants may decrease the effectiveness of the CPV-2 based vaccines, a more extensive use of vaccines prepared with the strains circulating in the field (type 2b-based) has been suggested (Pratelli et al., 2001; Martella et al., 2005; Cavalli et al., 2008; Decaro et al., 2008a, 2009a; Ohshima et al., 2008; Calderon et al., 2009; Ntafis et al., 2010; Castro et al., 2011). Nevertheless, even considering all case reports of vaccination failures, there is no definitive evidence for the absence of cross-protection from clinical disease between old and variant CPVs. Thus, the primary cause of widespread virus circulation despite extensive vaccination is still represented either by interference of MDA in vaccinated pups or by poor efficiency of immune system in old dogs.

As a consequence of the emergence of the CPV-2 variants in the feline population, a similar debate has arisen about the real efficacy of traditional FPV-based vaccines against the infections caused by CPV-2a, CPV-2b and CPV-2c. In a recent study (Gamoh et al., 2005), an FPV-based vaccine was able to cross-protect against the subsequent infection with a virulent CPV-2b strain. However, in this study only two vaccinated cats were used that were challenged in a very short period after the

administration of the second vaccine dose. Therefore, even acknowledging that additional studies are required to address this issue, the development of multivalent vaccines containing FPV in combination with a CPV variant strain should be seriously considered (Decaro et al., 2010a, 2011a).

## 3. Diagnosis of canine parvovirus infection

A rapid diagnosis of CPV infection is especially important in kennels and shelters in order to isolate infected dogs and prevent secondary infections of susceptible contact animals. Clinical diagnosis is indecisive and several other viral pathogens may cause diarrhea in dogs, such as coronaviruses, adenoviruses, morbilliviruses, rotaviruses, reoviruses, noroviruses (Greene and Decaro, in press). Thus, a suspect clinical case should always be confirmed by laboratory tests. Several methods have been developed for the laboratory diagnosis of CPV infection, which is usually carried out on the feces (or intestinal contents if the animal is dead) of affected dogs. In the late stages of infection, EDTA-blood samples have been proven to be more useful for the diagnosis as CPV viremia is exceptionally long lasting (Decaro et al., 2005a,b).

### 3.1. Traditional methods

Tests detecting viral antigens by means of antibody-based methods are suitable for CPV diagnosis in the veterinary practice and represent the only tests available in the field (Greene and Decaro, in press). However, their sensitivity, like other traditional diagnostic methods, has been proven inferior to molecular assays. An immunochromatographic (IC) test was compared to molecular techniques, showing that the relative sensitivity of the test did not exceed 50% with respect to the nucleic acid-based methods, whereas the specificity was 100%. The poor sensitivity of the IC test was associated with the low amounts of virus shed in the feces during the late stages of infection and/or the early presence of high CPV antibody titers in the gut lumen that may sequestrate most viral particles (Desario et al., 2005). A more recent study compared the performances of three different commercially available, antibody-based tests for rapid detection of CPV antigens with PCR and immunoelectron microscopy, confirming the high specificity and low sensitivity of the antigen-detection kits (Schmitz et al., 2009).

Taking advantage on the close genetic and antigenic relationship among carnivore parvoviruses, in-house test systems developed for CPV diagnosis are able to detect also FPV (Neuerer et al., 2008). Surprisingly, some concerns have been expressed about the ability of in-hospital rapid parvovirus tests to recognise efficiently the new variant CPV-2c. Those concerns took into account the circumstantial evidence that the increase in rapid test failure paralleled the emergence and spreading of CPV-2c (Kapil et al., 2007). Almost simultaneously, it was recommended to test the MAbs contained in the in-house assays against the additional mutations detected in strains currently circulating (Hong et al., 2007). However, by IC testing of specimens representative of the three CPV variants including 100 samples containing the recently identified CPV-2c, the detection rate of this variant was similar to those of CPV-2a/CPV-2b, thus dissipating any previous concerns about the hypothesized but never demonstrated less efficiency of the test in detecting the new variant (Decaro et al., 2010b).

Alternative techniques, such as hemagglutination (HA) and virus isolation (VI), can be carried out only in specialized laboratories. Fresh erythrocytes are required for HA testing causing problems related to the management and housing of donor pigs. Other species' erythrocytes, e.g., cat or rhesus monkey red cells, may be used, but they are either difficult to obtain in the quantities required, or are expensive. Nevertheless, for a clear reading of the HA test, good quality erythrocytes should be ensured since the test is affected by an altered coefficient of erythrocyte sedimentation which may occur in case of stress or disease of the donor pig (Desario et al., 2005). Furthermore, CPV-2 strains lacking HA activity have been reported (Cavalli et al., 2001). Nevertheless, the HA test carried out in a 96-well plate format allows rapid processing of many samples. Results are read after only 4 h. VI requires the availability of cell cultures that can be propagated only in laboratories with specialized personnel and a cell culture capability. Moreover, VI is time-consuming; it requires a long incubation period (5–10 days) and additional testing by immunofluorescence or HA in order to detect viral antigens. The main disadvantage of HA and VI, however, is the low sensitivity, most likely due to antibodies in the intestinal lumen of the infected dogs which may bind virions and prevent both HA and viral attachment to cell receptors (Desario et al., 2005). It has been demonstrated in natural (Decaro et al., 2005b) and experimental (Decaro et al., 2005a) infections, that CPV is detectable by HA and/or VI only for few days post-infection, whereas those techniques may give false-negative results despite high amounts of viral DNA detected by real-time PCR analysis. A simpler HA protocol, designated as slide agglutination test, has been also proposed to detect all CPV variants in fecal and intestinal samples, but this test does not seem to overcome the limitations of the classical methods (Marulappa and Kapil, 2009).

Electron microscopy identification of parvovirus-like morphology is poorly sensitive, although it is possible to concentrate viral particles by means of specific antibodies in the immunoelectron microscopy technique. Viral antigens can be also detected by means of immunohistochemistry carried out on intestinal, brain or tongue sections (Greene and Decaro, in press).

### 3.2. Molecular methods

While molecular methods are generally not affected by the host immune response, they are generally time-consuming, labour-intensive, and need the expertise of specialists. A nucleic acid hybridization assay was available since early 1990s (Remond et al., 1992). Subsequently, several PCR assays were developed that displayed increased sensitivity and specificity in comparison with traditional methods (Desario et al., 2005). A loop-mediated isothermal amplification assay has been also proposed in

recent years as an alternative to PCR-based methods (Cho et al., 2006). However, none of these nucleic-acid detection methods were designed to be quantitative, although they are time consuming and contain a certain risk of carryover contamination, especially when a high sample throughput is required.

A real-time PCR assay based on the TaqMan technology was developed for rapid, specific and sensitive detection of CPV DNA (Decaro et al., 2005d). Real-time PCR has several advantages over conventional PCR, allowing a large increase in throughput and enabling simultaneous processing of several samples. Real-time PCR is run in a 96-well format, and many of the steps in the assay are automated. Because of the inexpensive and quick method used for DNA preparation, based on boiling of fecal homogenates, the total time requested for analysis of 20–30 samples was about 6 and 3 h for conventional and real-time PCR, respectively (Decaro et al., 2005d). The high sensitivity and reproducibility of the real-time PCR assay may allow for identification of dogs shedding CPV at low titers in their feces, helping to adopt adequate measures of prophylaxis to prevent CPV infection, especially in kennels and shelters, where this virus is often responsible for dramatic epizootics. The established TaqMan assay was successfully employed in pathogenesis studies carried out during natural (Decaro et al., 2005b) and experimental (Decaro et al., 2005a) infections and was also used to evaluate virus distribution in different tissues (Decaro et al., 2007c). A TaqMan real-time RT-PCR assay developed for detection of CPV RNA transcripts demonstrated the presence of replicating virus in CNS (Elia et al., 2007). A SYBR Green-based real-time PCR assay was proposed as an alternative method to the TaqMan technology, displaying the same detection limit (10 copies of viral DNA) (Kumar and Nandi, 2010).

## 4. Virus characterisation

With the exception of discrimination between vaccine and field viruses, virus strain determination may have a limited interest for practitioners, taking into account that prognosis and treatment of CPV enteritis (mainly supportive) disregard the involved variant. However, virus characterisation is of particular interest under an epidemiological point of view. The methods used for characterisation of the CPV strains can be performed only in highly specialised laboratories. While diagnosis of CPV infection is easily feasible using innovative techniques, such as PCR and real-time PCR, the identification of the viral type is sometimes indecisive and more than one method is often required for the definitive prediction of the antigen specificity.

### 4.1. Traditional methods

For several decades, the only method available for characterisation of CPV strains has been hemagglutination inhibition (HI) using MAbs. A panel of four MAbs (A4E3, B4A2, C1D1 and B4E1) was developed for antigenic characterisation of FPV, CPV-2, CPV-2a and CPV-2b, through assessing the HI reactivity. Types 2a and 2b differ for a lack of reactivity of MAb B4A2 to CPV-2b; however, this MAb is not able to recognise even CPV-2c. Recently, a MAb was developed which can differentiate the new variant 2c from type 2b CPVs (Nakamura et al., 2004).

However, MAb analysis can be applied only on fecal samples with optimal HA activity or with CPV isolates obtained in cell cultures. Only specimens with HA titers$\geq$1:64 can be characterised, but several samples with high CPV DNA titers test negative or poorly positive by HA (Decaro et al., 2005d; Desario et al., 2005). Moreover, only few HA-negative samples are successfully amplified on cell cultures in order to increase their HA titer.

### 4.2. Molecular methods

A genotyping PCR system was developed by Pereira et al. (2000). Due to the limited number of nucleotide variations between types 2a and 2b, the type 2b-specific primers were selected taking advantage of two single nucleotide polymorphisms (SNPs), A4062 T and G4449A, that determine the replacement of asparagine by aspartic acid at position 426 and of isoleucine by valine at position 555, in types 2a and 2b, respectively (Table 1). Each primer was selected to have one such mutation at the very 3′ end, as nucleotide mismatches that occur at the 3′ end of a primer are highly detrimental to primer extension and strongly decrease PCR amplification. However, these mismatches, albeit present at the very 3′ end of the primers, were not sufficient to completely prevent the amplification of the other CPV types (Martella, personal observation). Moreover, currently circulating type 2a strains have the mutation I555 V, due to the nucleotide change G4449A (Martella et al., 2006). Therefore, the PCR-based genotyping system developed by Pereira and colleagues (Pereira et al., 2000) is no longer able to discriminate between type 2a and type 2b strains, as almost all the novel type 2a strains (555-Val) will go mischaracterized as type 2b. Finally, by the type-specific PCR assays, type 2c CPVs are not detectable, since the substitution D426E is due to a change (T $\rightarrow$ A) in the third codon position, at nucleotide 4064, so that this mutant is erroneously recognised as type 2b by this PCR strategy (Buonavoglia et al., 2001).

To address this point, a PCR-RFLP assay using enzyme *Mbo*II was developed which was proven to be useful for discrimination between types 2b and 2c (Buonavoglia et al., 2001). However, RFLP analysis is not able to differentiate CPV-2b from CPV-2a, since both types remain undigested after *Mbo*II digestion. In addition, CPV-2a strains have been detected in Hungary that were erroneously recognized as CPV-2c by this method due to a constant point mutation in the VP2 gene introducing an *Mbo*II restriction site (Demeter et al., 2010). Therefore, only sequence analysis could predict definitively the viral type (type 2a or 2b) for samples negative by HA and not digested or giving misleading results by PCR-RFLP, leading to a remarkable increase of costs and time losses.

Sequence analysis can give ample information for CPV typing since the fragment amplified by conventional PCR using primer pair 555for/555rev (Buonavoglia et al., 2001) encodes for at least one informative aa (residue 426) of the

VP2 protein, thus allowing differentiation between CPV-2a, CPV-2b and CPV-2c, but not between CPV-2 (old type) and CPV-2a.

In the effort to speed up routine analysis, two real-time PCR assays, based on the MGB probe technology, were designed in order to recognize SNPs (Table 1) existing between types 2a/2b (A4062T) and between types 2b/2c (T4064A) at codon 426 of the VP2 protein (Decaro et al., 2005c, 2006b). Both assays were found to be highly sensitive and reproducible. In comparison with traditional typing methods, the MGB probe assays are more labour- and time-saving. In fact, the 96-well format and the automation of some steps allow for a simultaneous processing of several samples and ensure a direct characterisation of the CPV variants within a couple of hours, considering that the assays can be run in parallel in the same plate.

Recently, CPV-2c strains uncharacterised by the MGB probe assays have been reported (Decaro et al., 2009b). Such strains displayed a non-coding mutation in the probe binding region which prevented the correct hybridization of the type-specific probe. These findings highlight the need to update the CPV-typing methods based on SNPs as additional mutations may hinder the correct strain characterisation, reinforcing previous suggestions to keep using diagnostic molecular tools targeting more conserved regions to avoid false-negative results (Decaro et al., 2006b). Accordingly, the untypeable samples were found to be CPV positive using a TaqMan assay able to detect all CPV types and FLPV as well (Decaro et al., 2005d, 2008b). Similar concerns have been recently expressed by American scientists that detected type 2c strains with the change A1318G, which is located near the critical codon 426 (Kapil et al., 2007). Albeit present within the target region of the MGB assay, that mutation should not prevent the correct strain characterisation being not included in the primer/probe binding sites. Indeed, a US strain displaying the change A1318G (Hong et al., 2007) was submitted to the 2b/2c assay, being correctly recognised by the type 2c-specific probe (Decaro et al., 2009b).

### 4.3. Discrimination between vaccine and field strains

Diagnosis of CPV infection may be ambiguous when carried out on fecal samples from dogs presenting with diarrhea few days after vaccination. In fact, the modified-live virus contained in the vaccines is able to replicate in the intestinal mucosa of vaccinated dogs, despite the unnatural route of administration (intramuscular or subcutaneous instead of oronasal), and to be shed in the feces, albeit at low titers and for a shorter time period with respect to field strains (Decaro et al., 2006a). In such a circumstance, the detection of CPV-2 or its nucleic acid by conventional assays (ICT, HA, VI or PCR) in the feces of vaccinated dogs could provide false-positive results, leading to a misdiagnosis of the disease probably caused by other enteric pathogens of dogs. Moreover, it would be important to rule out vaccine-induced disease due to regaining of virulence of the vaccine virus. CPV vaccines are exceptionally safe and cases of vaccine-induced disease have been only anecdotally reported but never clearly demonstrated. However, considered that reversion to virulence of CPV vaccines can theoretically occur, virus strain determination is required at least when gastroenteritis occurs few days after CPV vaccination.

CPV characterisation using traditional techniques is often inconclusive, since a simultaneous infection by the type 2-based vaccine and wild-type virus may mislead the results of HI with MAbs, PCR-RFLP and sequence analysis.

To address this point, a PCR-based approach has been proposed by Senda et al. (1995), taking advantage of two SNPs, A3045T and C3685G, that determine the replacement of methionine by leucine at position 87 and of alanine by glycine at position 300, in old and wild-type strains, respectively (Table 1). However, in our experience, such mutations were not sufficient to prevent completely amplification of the old-type virus (vaccine) (personal observation). Moreover, samples containing both vaccine and wild-type strains are amplified successfully, so that the PCR-based strategy will not be able to detect the simultaneous presence of the two viruses in the feces. The MGB probe assays developed for the rapid characterisation of the CPV strains (Decaro et al., 2005c, 2006b) do not discriminate between the old type CPV-2 and type 2a. Even sequence analysis of the VP2 protein gene can be inconclusive or misleading as, unless sequencing of several clones is carried out, PCR may selectively, or more efficiently, amplify either of the viruses, so that the other one remains undetected by subsequent sequence analysis.

To overcome the limitation of existing methods, an MGB probe assay (Decaro et al., 2006a) was established which is able to discriminate between type 2 (vaccine) and variant (field) strains on the basis of the SNP T3088C responsible for the change from isoleucine to threonine at VP2 residue 101 (Table 1). Thus, all samples collected from vaccinated dogs and characterised as CPV-2a should be tested by the novel MGB probe assay in order to assess whether they are true type 2a (field) strains or vaccine (old type) virus.

Few companies in Europe have licensed type 2b vaccines. Although at the moment such vaccines are not used widely, some problems may arise when analysing samples collected from dogs administered a type 2b vaccine. Using the type 2/variants MGB probe assay (Decaro et al., 2006b), type 2b vaccine viruses shed in the feces would be recognized by the probe specific for the CPV variants, being characterised as field strains instead of vaccine strains. Similarly, the type 2a/2b assay is not able to distinguish between type 2b vaccinal and field strains, since the same nucleotides are encountered in the binding region of the type 2b-specific probe (Decaro et al., 2006b). Consequently, additional two MGB probe assays were designed to precisely differentiate CPV-2b strains that circulate in the field from those contained in commercially available vaccines (Decaro et al., 2006d). To the authors' knowledge, the developed assays are able to detect all vaccine strains available at least in the European market. If additional vaccines containing different CPV strains are licensed in the future, further tests should be established to ensure vaccine strain determination.

The established assays can be useful to address controversies arising between dog owners, veterinarians

and vaccine companies when diarrhea occurs within few days after CPV vaccine administration. The molecular assays discriminating between vaccine and field viruses were successfully applied to 29 fecal samples collected from dogs displaying acute gastroenteritis shortly after CPV vaccination (Decaro et al., 2007b). The results showed that, in most cases, a CPV field strain or a different dog pathogen was being incubated at the moment of vaccination, thus ruling out any reversion to virulence of the vaccine viruses.

## 5. Conclusions

While FPV since its first identification in 1920 has not undergone significant changes in the antigenic and biological properties (Decaro et al., 2008c), CPV has progressively evolved under partly positive selection (Shackelton et al., 2005; Decaro et al., 2009b), showing genomic substitution rates similar to those of RNA viruses, with values of about $10^{-4}$ substitutions per site per year (Shackelton et al., 2005). Such an evolution has lead to the emergence of antigenic variants that seem to be more virulent than the original type. In contrast, despite some anecdotal reports claiming a higher pathogenicity of CPV-2c, there is no evidence for a different virulence between the variants. Thus, to what extent antigenic variants identified by mouse monoclonal antibodies represent clinically relevant immunological events is still to be determined. At the same time, concerns about the real efficacy of CPV-2 vaccines against the antigenic variants are mainly based on in vitro cross-neutralization assays that might be even negligible in vivo. Indeed, apart from well-documented case reports, experimental data and field observations seem to suggest that the great majority of infections still occur in the puppies about the time when MDA wane and the animals become susceptible to any strain of virus.

In conclusion, continuous evolution of CPV through accumulation of point mutations in the viral genome presents two different, but equally important, implications: (i) the emergence of new antigenic variants displaying different biological and antigenic properties may require an extensive revision of the vaccination programmes and an update of the viral strains contained in the commercially available products; (ii) the sophisticate molecular methods able to detect and characterise the CPV strains, that are based on perfect matching between viral DNA and assay oligonucleotides, may be affected by the onset of point mutations and should be therefore updated as well. Thus, a continuous epidemiological surveillance is needed to detect new CPV variants potentially escaping the host immune system and detection methods.

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