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Study of canine parvovirus evolution: comparative analysis of full-length VP2 gene sequences from Argentina and international field strains

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Abstract The continuous emergence of new strains of canine parvovirus (CPV), poorly protected by current vaccination, is a concern among breeders, veterinarians, and dog owners around the world. Therefore, the understanding of the genetic variation in emerging CPV strains is crucial for the design of disease control strategies, including vaccines. In this paper, we obtained the sequences of the full-length gene encoding for the main capsid protein (VP2) of 11 canine parvovirus type 2 (CPV-2) Argentine representative field strains, selected from a total of 75 positive samples studied in our laboratory in the last 9 years. A comparative sequence analysis was performed on 9 CPV-2c, one CPV-2a, and one CPV-2b Argentine strains with respect to international strains reported in the GenBank database. In agreement with previous reports, a high degree of identity was found among CPV-2c Argentine strains (99.6–100% and 99.7–100% at nucleotide and amino acid levels, respectively). However, the appearance of a new substitution in the 440 position (T440A) in four CPV-2c Argentine strains obtained after the year 2009 gives support to the variability observed for this position located within the VP2, three-fold spike. This is the first report on the genetic characterization of the full-length VP2 gene of emerging CPV strains in South America and shows that all the Argentine CPV-2c isolates cluster together with European and North American CPV-2c strains.

Keywords *Canine parvovirus* · VP2 full-length gene · CPV-2c variants · Sequence analysis

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Introduction

Canine parvovirus (CPV) is the causative agent of a severe and, sometimes, fatal gastroenteritic syndrome affecting domestic and wild canids. The CPV virion is non-enveloped, icosahedral shaped particles of 26 nm in diameter [1]. The genomic DNA is a single-stranded negative-sense molecule of approximately 5.2 kb [2] encoding for two structural proteins VP1 and VP2, formed by alternative splicing from the same RNA, and two nonstructural proteins (NS1 and NS2) [3, 4]. VP2 is the major capsid protein containing the main antigenic determinants and also playing an important role in determining virus pathogenicity. Another relevant property of VP2 is its ability to self-assemble, forming immunocompetent virus-like particles (VLPs) [5].

The VP1 protein represents only the 10% of the total protein mass of the virion.

One of the main biological properties of CPV is its continuous evolution, which seems to be related to its high rate of nucleotide substitution, which is closer to that of RNA viruses than to that of double-stranded DNA viruses [6]. This feature is reflected in the appearance of new variants through the years, since the first identification of CPV in 1978 [7–9].

The original type 2 was gradually replaced by new genetic and antigenic variants, such as type 2a (CPV-2a) and type 2b (CPV-2b) [2]. The newest antigenic variant,

Table 1 Age, gender, breed, vaccination records, and clinical signs of dogs infected with CPV

Strain	Year	Age (months)	Sex	Breed	Vaccination status	Clinical signs	CPV strain	Origin	aa at 440
Arg 5	2003	135	M	NA	V	Y	CPV-2b	Buenos Aires	T
Arg 9	2003	5	M	MB	NV	+	CPV-2a	Buenos Aires	T
Arg 32	2008	1	M	P	NV	+	CPV-2c	Buenos Aires	T
Arg 35	2008	4	M	FT	V(C)	+	CPV-2c	Tucuman	T
Arg 44	2009	3	M	B	V(C)	+	CPV-2c	Buenos Aires	T
Arg 48	2009	6	F	LR	V(C)	+	CPV-2c	Buenos Aires	A
Arg 60	2009	2	F	Bea	NV	+	CPV-2c	Buenos Aires	A
Arg 64	2010	NA	NA	SFT	NA	NA	CPV-2c	Bahía Blanca	T
Arg 66	2010	5	F	Bea	V(C)	+	CPV-2c	Buenos Aires	A
Arg 67	2010	NA	F	MS	V(C)	+	CPV-2c	Buenos Aires	A
Arg 68	2010	3	M	MS	V(C)	+	CPV-2c	Mar del Plata	T

F female, *M* male, *MB* mixed breed, *LR* labrador retriever, *MS* miniature schnauzer; *FT* fox terrier, *SFT*, smooth fox terrier, *P* poodle, *B* boxer, *Bea* beagle, *V* vaccinated, *NV* no vaccinated, *NA* no information available, *C* complete vaccination according to its age

carrying the amino acid (aa) substitution Asp426Glu, located at the major antigenic region over the three-fold spike of the CPV capsid, was first reported in Italy in the year 2001. This mutant, designated CPV-2c, has been detected later in several countries including Argentina [10–24].

Currently, the mentioned antigenic variants have replaced completely the original type 2 strain (which is only found in commercial live attenuated vaccines) and are distributed in the canine population worldwide [12, 25–34].

The high sanitary impact of CPV at international level makes it important to perform the precise identification of new emerging strains and to understand its evolution in the field [35, 36].

In this paper, we extend our study of the CPV evolution [23, 24] focusing in the analysis of 11 VP2 gene full-length sequences of representative strains, selected from 75 positive fecal samples of dogs showing clinical signs of hemorrhagic gastroenteritis, received between the years 2002 and 2010.

Materials and methods

Clinical specimens and PCR detection of CPV sequences

Rectal swabs samples obtained from domestic dogs living in different provinces of Argentina were processed for diagnosis of CPV.

DNA was extracted directly from rectal swabs and from a commercial live attenuated vaccine (Nobivac DHP Intervet, Boxmeer, the Netherlands), in a lysis buffer containing 50 mM Tris–HCl pH 8, 100 mM NaCl, 25 mM sucrose, 10 mM EDTA, and 1% SDS. After lysis, the extracts were digested with proteinase K (Invitrogen®, Carlsbad, CA) at 56°C for 30 min, and the DNA was extracted with phenol–chloroform. Confirmation of the presence of CPV in clinical specimens was performed by amplification of a 583-base pair (bp) fragment of the VP2 gene [10] in 75 from a total of 118 analyzed rectal swab samples, received between the years 2002 and 2010 [23, 24].

Eleven representative samples were selected for this study. The selection criterion was based on the maintenance of the heterogeneity of the analyzed population, therefore involving individuals of different ages, vaccination status, breed, year of isolation, etc. Information about the animals, such as clinical symptoms, age, gender, breed, and vaccination status, is provided in Table 1.

PCR amplification of the VP2 gene of CPV-2

Selected CPV-positive field samples and a commercial live attenuated vaccine (Nobivac DHP Intervet, Boxmeer, the Netherlands) were further screened by amplification of the VP2 full-length gene with the VP2FLf: 5′-GTGCAGGA CAAGTAAAA-3′ and 555rev 5′-GGTGCTAGTTGATA TGTAATAAACA-3′ primer pair.

Amplifications were performed using a *Taq* recombinant DNA polymerase (Invitrogen®, Carlsbad, CA) in an MJ Research cycler (PTC-100, Sierra Point, CA).

PCR was performed with an initial activation of *Taq* polymerase at 94°C for 10 min, followed by 40 cycles of denaturation step at 94°C for 30 s, annealing at 50.5°C for 60 s, and extension at 72°C for 2 min.

The reaction mixture (50 µl) consisted of PCR buffer 1×, MgCl2 1.5 mM, 0.2 mM of dNTPs, 50 pmol of primers, 2 U of *Taq* recombinant DNA polymerase, and 2 µl of template DNA. A final extension step was performed at 72°C for 5 min.

PCR products were separated by electrophoresis through a 1% agarose gel and visualized under UV light after ethidium bromide staining.

Cloning and sequencing of the amplified DNA

The amplified DNA fragments containing the sequences from the VP2 gene were cloned into the pGEM-T Easy vector® (Promega, Madison, WI). For each DNA fragment, two recombinant plasmids were sequenced in both directions using universal T7 and SP6 primers, as well as an internal primer by an automated dideoxy-mediated chain termination method (Macrogen Inc, Korea). Electropherograms were visually checked to avoid misreading of peak dyes, and sequences were edited.

The nucleotide (nt) sequence accession numbers in the NCBI database of the viruses used in this study are as follows: prototype CPV-2 strain CPV-b (M38245); prototype CPV-2a strain CPV-15 (M24003); prototype CPV-2b strain CPV-39 (M74849), prototype CPV-2c strain (FJ222821.1).

Vaccine strains Intervet vaccine06 (strain CPV-2) (FJ011098.1); Merial Vaccine06 (strain CPV-2) (FJ011097.1); CPV-2b strain SAH capsid protein (VP2) Fort Dodge vaccine (FJ222822.1).

International strains Italy CPV-2a, 2002 (FJ005252.1); Italy CPV-2a, 2005 (FJ005253.1); Italy CPV-2a, 2008 (FJ005256.1); Thailand CPV-2a, 2008 (GQ379049.1); Thailand CPV-2a, 2009 (GQ379048.1); China CPV-2a, 2001 (GU569946.1); China CPV-2a, 2006–2008 (GQ169540.1); China CPV-2a, 2008 (FJ432717.1); Brasil CPV-2a, 1995 (DQ340432.1); Germany CPV-2b,1997 (FJ005260.1); Italy CPV-2b, 2004 (FJ005262.1), Italy CPV-2b, 2005 (FJ005263.1), China CPV-2b, 2002 (GU569944.1); Germany CPV-2c, 1997 (FJ005196.1); Germany CPV-2c, 1998 (FJ005203.1); Germany CPV-2c, 1999 (FJ005204.1); Italy CPV-2c, 2004 (FJ005210.1); Italy CPV-2c, 2005 (FJ005213.1); Italy CPV-2c, 2006 (FJ005215.1); Italy CPV-2c, 2007 (FJ005233.1); Italy CPV-2c, 2008 (FJ005245.1); Spain CPV-2c, 2006 (FJ005214.1); Spain CPV-2c, 2008 (FJ005246.1); USA CPV-2c, 2007 (FJ005235.1); USA CPV-2c, 2007 (FJ005236.1); Belgium CPV-2c, 2008 (FJ005 247.1); Greece CPV-2c, 2008 (GQ865518.1); Greece CPV-2c, 2009 (GQ865519.1); China CPV-2c, 2008–2009 (GU380305.1).

The nt. sequences obtained of VP2 gene for Argentine strains were submitted to the GenBank and accession numbers are as follows: Arg 5 (JF414817), Arg 9 (JF346754), Arg 32 (JF414818), Arg 35 (JF414819), Arg 44 (JF414820), Arg 48 (JF414821), Arg 60 (JF414823), Arg 64 (JF414822), Arg 66 (JF414824), Arg 67 (JF414825) and Arg 68 (JF414826).

VP2 sequence and phylogenetic analysis

Alignments were obtained using the ClustalW algorithm. A phylogenetic tree was constructed covering the complete VP2 coding region, using the neighbor-joining (NJ) method as implemented in MEGA, version 5 [37]. Phylogenetic tree reliability was estimated with 1,000 bootstrap replications.

Selection pressure analysis

Maximum Likelihood analysis of natural selection codon-by-codon was performed using the HyPhy software package [38], implemented in the MEGA 5 program. dN and dS estimates were produced using the joint maximum likelihood reconstructions of ancestral states under a Muse–Gaut model [39] of codon substitution and general time-reversible model of nucleotide substitution. For estimating ML values, a tree topology was automatically computed. A cut-off *P*-value of 0.05 was used.

Results

PCR amplification of the full-length VP2 gene

The selected representative samples, originated in previous studies [23, 24], included one characterized as CPV-2a, one as CPV-2b, and 9 as CPV-2c.

A fragment of 1834-bp covering the full-length sequence of the VP2 gene was successfully amplified from the eleven samples as well as from a vaccine sample, which was used as positive control (data not shown).

Sequence analysis of the full-length VP2 gene

The amplicons obtained were cloned and sequenced. The resulting sequences were aligned by ClustalW method.

Figure 1 shows the alignment of the deduced aa sequences of CPV reference international strains (type 2, 2a, 2b, and 2c) together with seven representative Argentine strains (CPV-2a, CPV-2b, and CPV-2c).

An extensive analysis, comparing local sequences with respect to 17 type 2c (Germany, $n = 3$; Italy, $n = 6$, Spain, $n = 2$. USA, $n = 2$ Belgium, $n = 1$; Greece, $n = 2$; China, $n = 1$); 9 type 2a (Italy, $n = 3$; Thailand, $n = 2$; China, $n = 3$; Brazil, $n = 1$), 4 type 2b (Germany, $n = 1$; Italy, $n = 2$; China, $n = 1$), and one type 2 (reference strain), plus 3 vaccine strains (Merial, Lyon, France; Duramune Fort Dodge Animal Health, IA, USA; and Intervet, Boxmeer, The Netherlands) showed a high degree of aa conservation among CPV strains (97.3–100%).

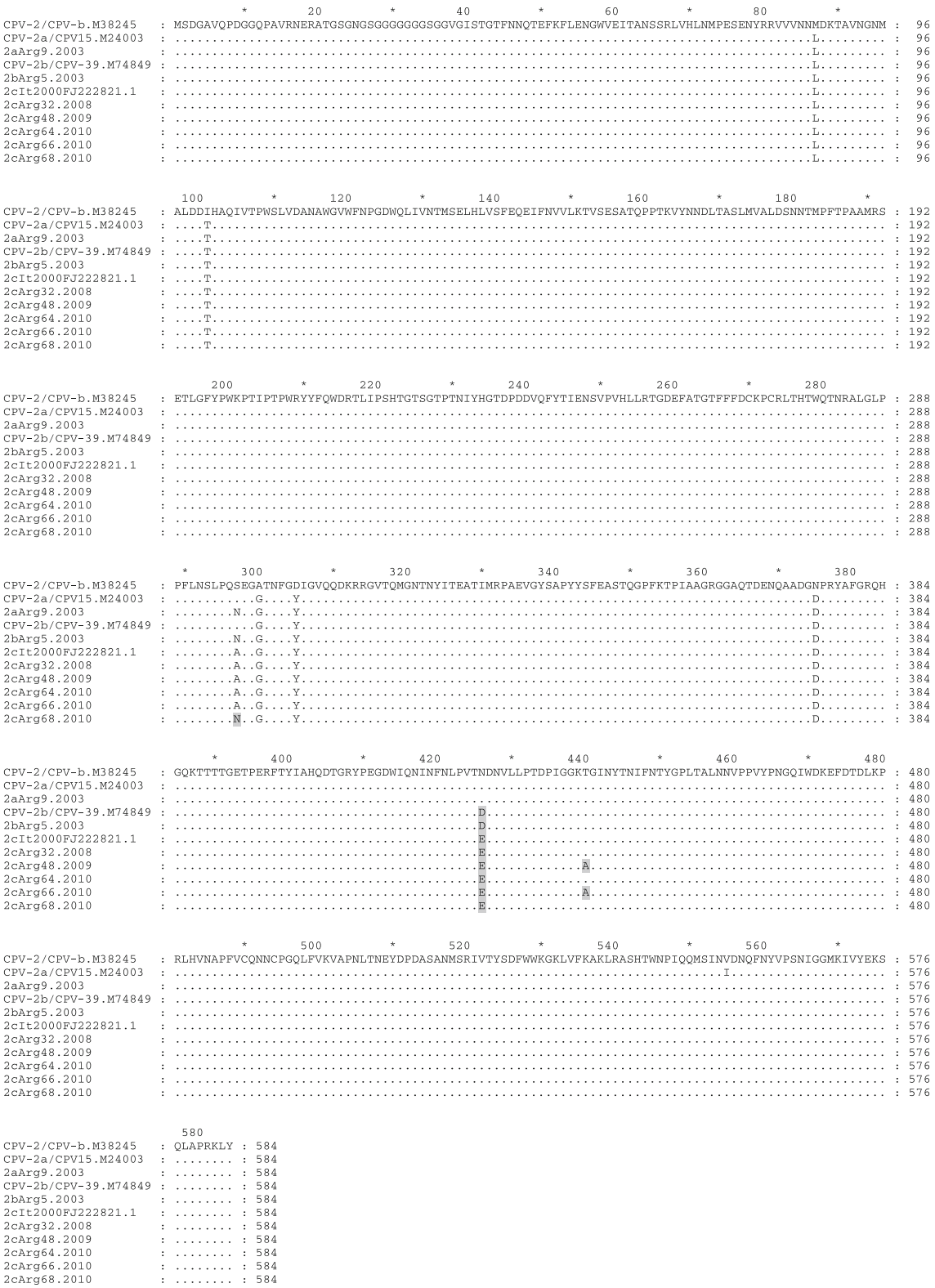

Fig. 1 Alignment of the deduced amino acid sequences of the full-length VP2 gene amplified by PCR from clinical samples. The sequences of two local field variants, one identified as CPV-2a (Arg 9) and the other as CPV-2b (Arg 5), and five representative CPV-2c variants (Arg 32, 48, 64, 66, 68) are shown aligned. Sequence accession numbers are described in section "Materials and methods". Amino acid positions 426 and 440 are *gray shaded*, as well as the S297N of Arg 68. Strains Arg 35 and Arg 44 showed to be identical at amino acid level to Arg 32 and Arg 64. The same case for strains Arg 60 and Arg 67 with respect to Arg 48 and Arg 66. Reference strains are: CPV-2 (CPV-b, M38245); CPV-2a (CPV-15, M24003); CPV-2b (CPV-39, M74849), and CPV-2c (FJ222821.1)

International strains showed aa changes with respect to the original CPV-2 (M87L, I101T, A300G, D305Y, N375D) [35], which were shared by the Argentine strains (Fig. 1).

The only differences found with respect to these positions were I101A in the Chinese strain 2aChi06/08 (GQ169540.1) and N375, which was retained by 2aChi2001 (GU569946.1) and 2bChi2002 (GU569944.1) (data not shown).

Truyen, et al. (2000) observed the consistent appearance of an aa change at position 297 (S297A), which is now a predominant mutation in Europe found in both CPV-2a and CPV-2b viruses [36]. Surprisingly, Argentine samples showed the same change S297A in eight strains characterized as CPV-2c, while it was not found in samples characterized as CPV-2a or CPV-2b, which showed the substitution S297N, also found in one CPV-2c strain (Arg 68, Fig. 1) [23].

Another important substitution affecting Argentine CPV-2c strains and already reported in other variants is the substitution T440A. This change was found in four Argentine CPV-2c strains, as well as in several international strains [3, 19, 35, 40, 41].

In the 555 position, a V residue was found in all CPV strains, except in the prototype isolate CPV-15, which retained I555 [35].

Phylogenetic analysis

A phylogenetic tree, including Argentine and international strains, was built. As shown in Fig. 2, wild-type viruses

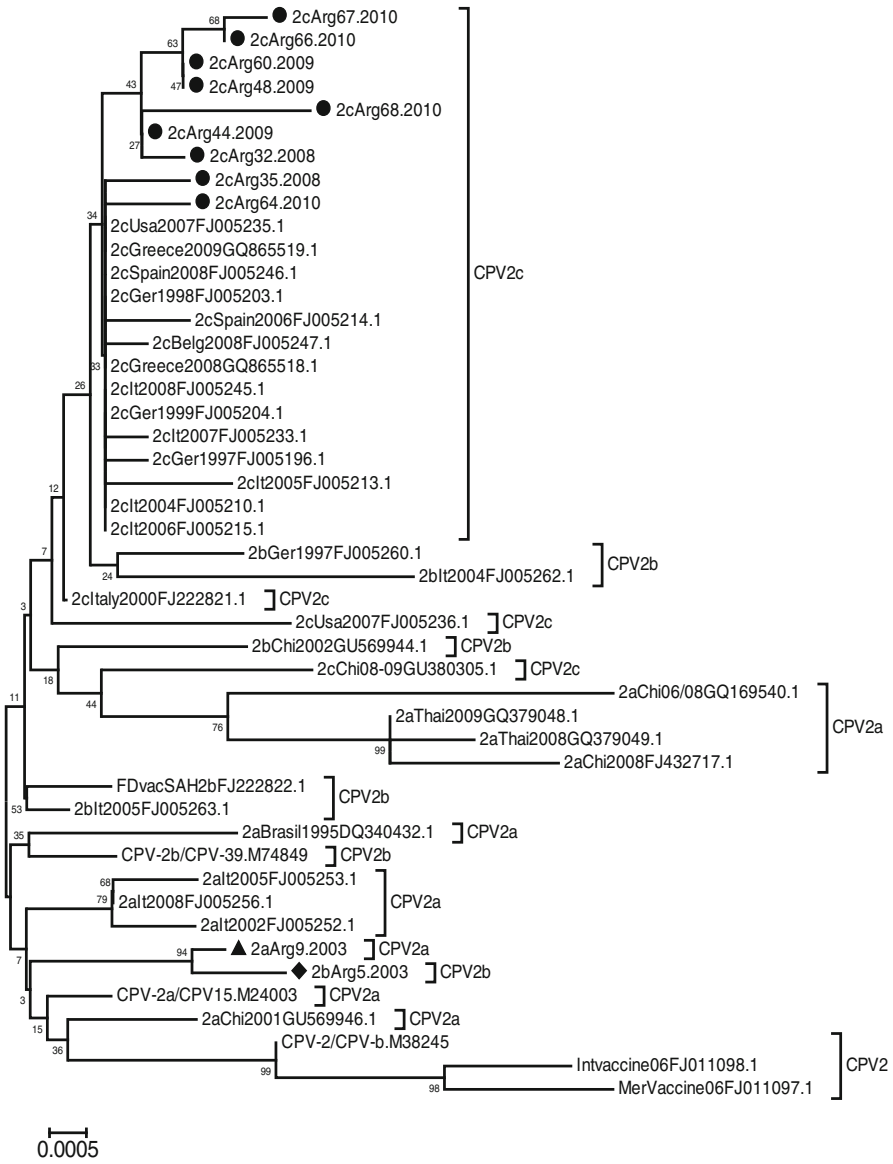

Fig. 2 Neighbor-joining tree based on the full-length VP2 gene sequences (1755 nt) of local and worldwide strains of CPV. GenBank accession numbers for the reference strains used in the phylogenetic tree construction are listed in the text. Statistical support was provided by bootstrapping over 1,000 replicates

from Argentina and the rest of the world are phylogenetically separated from vaccine CPV-2 strains.

Analyzing sequences at the nt. level, it was observed that Argentine CPV-2c strains showed a 99.3–99.9% of identity with respect to international CPV-2c strains, whereas the aa identity was 99.7–100%.

All Argentine CVP-2c isolates clustered together, showing intra-group nucleotide identity values of over 99.6–100%, with the closest relationship (99.5–99.9% identity) with European and USA isolates of the same variant (CPV-2c) recovered between 1997 and 2009 (Fig. 2). Within this cluster, isolates Arg 48, Arg 60, Arg 66, and Arg 67 formed a subgroup, all them carrying T440A.

Although only a low number of sequences were analyzed, the results confirm the observations of Decaro et al. [35] being both Argentine CPV-2a and CPV-2b strains intermingled with CPV-2a and CPV-2b strains from abroad.

Selection pressure analysis

The role of positive and negative selection has been previously documented in CPV evolution [40, 42]. Our results are in consistent with previous studies that point at the purifying selection as the main evolutionary force.

When a significance level of 0.3 was used, we also found positions subjected to positive selection such as 195, 297, 426, and 440 (Fig. 3). Interestingly, except for the position 195, these sites undergoing positive selection are located within the GH loop of the VP2 capsid protein.

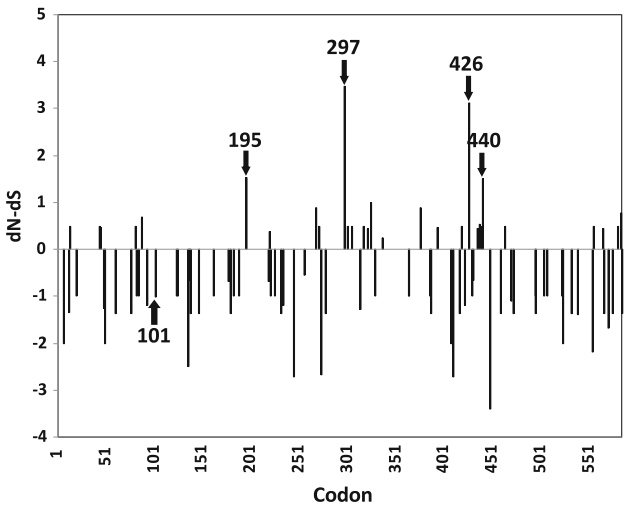

Fig. 3 *Sliding window* representing the rate of synonymous and non-synonymous nt substitutions/site from CPV-VP2 sequences. Extent of positive and negative selection on individual codons was studied. The value *above* and *below* the abscissa, indicates the number of per-site non-synonymous substitutions (d_N) over per-site synonymous ones (d_S) in an individual codon. The zero value means $d_N = d_S$. *Arrows* highlight relevant positions

Discussion

In the recent years, an increasing number of cases of clinically ill dogs showing symptoms compatible with CPV disease were reported in Argentina including vaccinated animals. This situation raised concerns among breeders, owners, and clinical practitioners about the ability of the current vaccines to protect their pups.

Therefore, in order to investigate the genetic variability of CPV strains circulating in Argentina, their evolution and their possible sanitary impact, we have studied the full-length VP2 gene sequences of 9 representative CPV-2c strains, one type 2a, and one type 2b.

The results of the aa sequence analysis showed a low overall variability among the analyzed VP2 sequences. However, the results obtained clearly demonstrate the presence of some relevant aa substitutions, located in an exposed region comprising aa 267–498 of the VP2 protein, named the large GH loop, where the greatest variability among parvo viruses was observed [36, 43].

Several aa substitutions were found within this region of VP2, involving aa A300G, D305Y, and N375D (Fig. 1). These substitutions were found both in the Argentine as well as in the international strains studied herein.

In addition, the aa substitution S297A, which was recently described in strains CPV-2a, CPV-2b, and CPV-2c around the world [3, 12, 16, 22, 35, 36, 40, 44, 45], was also found in eight of the CPV-2c Argentine strains. Surprisingly, in this aa position, the CPV-2a, the CPV-2b, and the remaining one CPV-2c Argentine strains (Arg 68/2010) showed the aa substitution (A297N) (Fig. 1), which is identical to that found in the Brazilian CPV-2a strain (BR46-95) [40], making this substitution unique for South American isolates. Although residue 297 is located within a minor antigenic site, substitutions in this position may be relevant for changes in antigenicity and perhaps pathogenicity of CPV variants [44]. Moreover, as described above, site 297 is under strong positive selection (Fig. 3 and [40]).

Regarding the regions of the VP2, outside the GH loop, two main aa substitutions (M87L, I101T) were consistently found in all strains studied herein, when comparison was performed with respect to the original CPV-2 (Fig. 1). Another consistent change was found in position 555, in which the old CPV type 2a displays an isoleucine residue (I), while all the Argentine and foreign strains showed a valine (V) [35].

Except for a few scattered aa substitutions found in different isolates, the alignment performed with local and internationals strains showed a surprisingly high level of aa

conservation in the VP2 protein (data not shown). Taking into account the high rate of nucleotide substitution reported for CPV ($10^{-3}/10^{-4}$) [6], this high degree of identity was somehow unexpected. Although we cannot provide a definitive explanation about this high level of aa conservation, it is clear that eventual non-synonymous substitutions outside the identified hot spots in VP2 could strongly affect viral fitness for replication or the self-assembly capacity of VP2, leading to alterations in viral assembly and viability [46, 47]. The concept of the negative selection as the main evolutionary force for CPV-VP2 is supported by our results and by reports from other authors [40].

On the contrary, some fixed substitutions found at positions 87, 300, 305, 375, and 555 could be apparently the result of positive selection, as well as those identified for positions 195, 297, 426, and 440 in the selection pressure analysis (Fig. 3). Amino acid position 101, although also presenting a fixed substitution (I101T), was identified as being under negative selection pressure, probably due to the observed existence of two different triplets codifying for the same aa (data not shown). An interesting observation related to the tridimensional structure of VP2 is that positions 87, 101, and 555, although outside the GH loop, are in an exposed region of the protein [48, 49].

In conclusion, the results of this work confirm and extend our previous findings, providing additional data on the evolution of CPV-2 Argentine strains, which showed the same pattern observed in other countries, giving no indication of the existence of a separate lineage (Fig. 2).

The tree topology also suggests that Argentine strain CPV-2b could have arisen upon evolutionary forces derived from strain CPV-2a, as both belong to the same cluster (99.8% nt identity). On the contrary, most 2c Argentine strains, showed higher identity (99.3–99.8%), with the European 2b strains than with 2b Argentine strain CPV-2b Arg5 (99.3–99.4% identity), suggesting that CPV-2c Argentine isolates could have derived from European strains rather than from Argentine 2b strains. However, the limited number of CPV-2b isolates studied herein could also account for the lower parenthood found with respect to the Argentine 2b strains (Fig. 2). It is noteworthy to mention the presence of a subgroup (Arg 48, 60, 66 and 67) within the CPV-2c Argentine group, which carry the T440A substitution and shows a geographical clustering, all of them coming from Buenos Aires city.

This is the first report on the genetic characterization of the full-length VP2 gene of emerging CPV strains in South America. Our results are in agreement with previous reports, which point the CPV-2c as a new and worldwide distributed strain. This variant, with an evident evolutionary adaptative advantage, could contribute for viral circulation even in vaccinated dogs. Although international trade and exhibitions of live dogs could not be ruled out as a source of new genetic variants, positive selection acting in specific sites could account for the evolutionary convergence observed in Argentine and overseas strains.

The understanding of CPV virus evolution leading to the appearance of new strains with changes in the biological and/or clinical behavior together with the capacity of VP2 to self-assembly-forming VLPs is of relevance for the design of a new generation of updated vaccines [44]. Moreover, although it is clear that actual vaccines are effective and vaccination should not be dropped, the development of more effective adjuvants together with the implementation of new vaccination procedures (i.e., prime and boost regimes) [50, 51] may be important steps toward a more efficacious vaccination and to a better control of CPV spreading.

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