



High local genetic diversity of canine parvovirus from Ecuador



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ABSTRACT

Canine parvovirus (CPV) comprises three antigenic variants (2a, 2b, and 2c) that are distributed globally with different frequencies and levels of genetic variability. CPVs from central Ecuador were herein analyzed to characterize the strains and to provide new insights into local viral diversity, evolution, and pathogenicity. Variant prevalence was analyzed by PCR and partial sequencing for 53 CPV-positive samples collected during 2011 and 2012. The full-length VP2 gene was sequenced in 24 selected strains and a maximum-likelihood phylogenetic tree was constructed using both Ecuadorian and worldwide strains. Ecuadorian CPVs have a remarkable genetic diversity that includes the circulation of all three variants and the existence of different evolutionary groups or lineages. CPV-2c was the most prevalent variant (54.7%), confirming the spread of this variant in America. Ecuadorian CPV-2c strains clustered in two lineages, which represent the first evidence of polyphyletic CPV-2c circulating in South America. CPV-2a strains constituted 41.5% of the samples and clustered in a single lineage. The two detected CPV-2b strains (3.8%) were clearly polyphyletic and appeared related to Ecuadorian CPV-2a or foreign CPV-2b strains. Besides the substitution at residue 426 that is used to identify the variants, two amino acid changes occurred in Ecuadorian strains: Val139Ile and Thr440Ser. Ser⁴⁴⁰ occurred in a biologically relevant domain of VP2 and is here described for the first time in CPV. The associations of Ecuadorian CPV-2c and CPV-2a with clinical symptoms indicate that dull mentation, hemorrhagic gastroenteritis and hypothermia occurred more frequently in infection with CPV-2c than with CPV-2a.

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1. Introduction

Canine parvovirus (CPV) is one of the most relevant infectious agents of domestic dogs worldwide. CPV causes severe gastroenteritis with a high case fatality rate, particularly in puppies less than 3 months of age (Carmichael and Binn, 1981).

CPV is a nonenveloped virus of the autonomous *Parvoviridae* family and possesses a single-stranded DNA genome (5.2 kb). The genome has two major open reading frames: one encodes the nonstructural proteins NS1 and NS2, and the other encodes the capsid viral proteins 1 and 2 (VP1 and VP2) (Reed et al., 1988). Both capsid proteins are translated from alternatively spliced mRNAs, and the VP2 amino acid sequence (584 residues) is completely contained within VP1 (727 residues). The VPs have a highly conserved central core composed of an eight-stranded β -barrel motif and flexible loops between the strands that interact to form most of the capsid surface (Tsao et al., 1991). The most variable loop protrudes at each threefold

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axis forming a raised region on the capsid, termed the threefold spike; this is the most antigenic region of the virus and thus serves as a target for neutralizing antibodies (Agbandje et al., 1995).

CPV is considered a canine-specific variant of the feline panleukopenia virus that emerged in 1978 as a consequence of an interspecies jump from other carnivores to dogs. The new virus, referred to as CPV type 2 (CPV-2), rapidly spread and caused a global epidemic disease in dogs (Parrish, 1991; Hoelzer and Parrish, 2010). The successful cross-species viral transfer and adaptation to the new canine host involved only a few amino acid substitutions in and around the threefold spike (Truyen et al., 1996).

Soon after its emergence, CPV-2 was completely replaced by a genetic and antigenic variant known as CPV-2a, which differs at five residues in VP2 and regained the ability to infect cats and other carnivores (Parrish et al., 1985; Truyen et al., 1996). CPV-2a became the new dominant lineage and underwent further evolution, retaining several point mutations. Some of these mutations changed the antigenic properties of the capsid and reached high frequencies in viral populations. In addition to the CPV-2a antigenic type, there are two antigenic variants known as CPV-2b and CPV-2c. CPV-2b was detected in 1984 in the United States (Parrish et al., 1991), and CPV-2c was first reported in 2000 in Italy (Buonavoglia et al., 2001). Antigenic differences among the three variants are caused by changes at residue 426 (asparagine in CPV-2a, aspartate in CPV-2b, and glutamate in CPV-2c), which is located at the top of the threefold spike. Within the antigenic variants there is an important level of genetic variability as a consequence of the intrinsically high mutation rate of the CPV genome (Shackelton et al., 2005).

CPV-2a, CPV-2b, and CPV-2c are currently circulating worldwide, and their relative frequencies and genetic composition vary among countries (Decaro and Buonavoglia, 2012). In South America, genetic analyses of CPV that were carried out in Argentina, Brazil, and Uruguay revealed that the variants have different distribution patterns and are subjected to temporal variations (Pereira et al., 2007; Pérez et al., 2007; Calderón et al., 2009; Streck et al., 2009; Pinto et al., 2012). A notorious change in the epidemiology of South American CPV occurred during the 2000s when the 2c variant emerged and spread rapidly, becoming prevalent in the previous CPV-2a/2b population and producing an increase in the number of affected dogs as a consequence of apparent higher pathogenicity (Pérez et al., 2007; Streck et al., 2009; Calderón et al., 2011; Pinto et al., 2012). More recently, a divergent CPV-2a strain emerged in Uruguay that has increased in frequency to overcome the previously predominant CPV-2c strain (Pérez et al., 2012; Maya et al., 2013). This epidemiological scenario encourages additional analyses in other countries to map the geographical spread and variability of CPV variants in South America. The genetic characterization of new CPV populations is also of great importance to evaluate the need for new diagnostic tools and vaccines and to detect strains with novel biological properties that can provide useful information for CPV dynamics and

epidemiology (Decaro and Buonavoglia, 2012). In the present study, we analyze CPVs from an Andean dog population in the center of Ecuador to characterize the strains and to provide new insights into local viral diversity, evolution, and pathogenicity.

2. Materials and methods

2.1. Samples

Fifty-three rectal-swab samples were obtained from puppies that had symptoms suggestive of parvovirus infection and that tested CPV-positive using the Anigen Rapid CPV Ag Test kit (Bionote, Inc. Seoul, Korea). Information regarding age, breed, vaccination status, and clinical outcome of disease were recorded for all dogs (Supplementary Table S1). Samples were collected in a veterinary clinic from July 2011 to February 2012 and came from dogs owned by residents of different parts of the city of Guaranda, the capital city of the province of Bolívar, Ecuador (Supplementary Table S1). Guaranda is located 2668 m above sea level in a valley of the Andean mountains and has a canine population of ~12,500 dogs (Ministerio de Salud Pública de Guaranda, Departamento de Epidemiología, 2011, unpublished data).

Supplementary data associated with this article can be found, in the online version, at <http://dx.doi.org/10.1016/j.vetmic.2013.06.012>.

2.2. DNA extraction, PCR, and sequencing

Viral DNA was extracted from fecal samples as described by Schunck et al. (1995). All samples were characterized by sequencing a partial VP2 coding region that included codon 426. Twenty-four samples were selected for full-length VP2 sequencing. All PCR amplifications were performed using previously described primers and conditions (Pérez et al., 2012; Maya et al., 2013).

Amplicons were sequenced twice on both strands using an ABI prism 377 Perkin Elmer automated sequencer. Sequences were assembled and compared using Lasergene software (DNASTar, Madison, WI). The nucleotide sequences were BLAST searched and submitted to GenBank (<http://www.ncbi.nlm.nih.gov>) (Table 1).

2.3. Phylogenetic inferences

DNA sequences were aligned using MAFFT (Kato et al., 2002), and the best-fit model of nucleotide substitution (TrN + Γ) was selected under the Akaike information criterion and Bayesian information criterion as implemented in jModelTest (Posada, 2008). Maximum-likelihood trees, with approximate likelihood ratio test (aLRT) for internal nodes support, were inferred using PhyML (Guindon and Gascuel, 2003). Phylogenetic trees were visualized and edited with TreeGraph 2 (Stöver and Müller, 2010).

2.4. Statistical analysis of clinical data and CPV type association

Dogs were examined and clinical data were collected by the same attending veterinarian using a predesigned case

Table 1

Ecuadorian samples employed in evolutionary studies. The full-length VP2 coding region (1755 bp) was sequenced, and the observed amino acid changes are indicated (codons are shown in parentheses). Variants were classified according to the 426 residue: 2c (glutamate), 2a (asparagine), and 2b (aspartate).

Strain	Accession number	CPV variant	VP2: codon position and amino acid		
			139	426	440
ME1	KF149962	2c	Val (GTT)	Glu (GAA)	Thr (ACA)
ME10	KF149963	2c
ME23	KF149964	2c
ME25	KF149965	2c
ME27	KF149967	2c
ME28	KF149984	2c
ME29	KF149968	2c
ME31	KF149969	2c
ME34	KF149970	2c
ME32	KF149971	2c	Iso (ATT)
ME26	KF149966	2c	Iso (ATT)	...	Ser (TCA)
ME12	KF149978	2a	...	Asn (AAT)	Ser (TCA)
ME17	KF149973	2a	...	Asn (AAT)	Ser (TCA)
ME19	KF149977	2a	...	Asn (AAT)	Ser (TCA)
ME35	KF149976	2a	...	Asn (AAT)	Ser (TCA)
ME36	KF149979	2a	...	Asn (AAT)	Ser (TCA)
ME37	KF149980	2a	...	Asn (AAT)	Ser (TCA)
ME38	KF149981	2a	...	Asn (AAT)	Ser (TCA)
ME39	KF149982	2a	...	Asn (AAT)	Ser (TCA)
ME40	KF149983	2a	...	Asn (AAT)	Ser (TCA)
ME41	KF149975	2a	...	Asn (AAT)	Ser (TCA)
ME42	KF149974	2a	...	Asn (AAT)	Ser (TCA)
ME20	KF149985	2b	...	Asp (GAT)	Ser (TCA)
ME6	KF149972	2b	...	Asp (GAT)	...

report form. The associations of CPV type with clinical symptoms (anorexia, dull mentation, fever, hemorrhagic gastroenteritis, hypothermia, tachypnea, and vomiting) and clinical outcome (mortality) were determined by χ^2 testing using one degree of freedom at the 5% level of significance ($p \leq 0.05$).

3. Results

3.1. Prevalence of CPV types

The 53 rectal-swab samples that tested positive for CPV using the Anigen Rapid CPV Ag kit also tested positive by PCR. The samples were classified into antigenic variants CPV-2a, CPV-2b, and CPV-2c according to the codon at position 426 (AAT for asparagine, GAT for aspartate, and GAA for glutamate, respectively). Of the 53 samples, 29 were classified as CPV-2c, 22 were classified as CPV-2a, and 2 were classified as CPV-2b (Supplementary Table S1).

3.2. Phylogenetic and sequence analysis

Full-length VP2 coding sequences (1755 bp) were obtained from 11 CPV-2c strains, 11 CPV-2a strains, and 2 CPV-2b strains (Table 1).

A maximum-likelihood tree was constructed using both Ecuadorian and global strains (Fig. 1). The 11 Ecuadorian CPV-2c strains appear in two positions within the phylogenetic tree. Nine Ecuadorian CPV-2c strains cluster with CPV-2c strains of Europe and South America in a well-supported monophyletic group denoted the European/South American lineage. The nucleotide sequence of one of these Ecuadorian CPV-2c strains (ME34) is identical to the most common sequence found in the European/South

American CPV-2c lineage. These nine Ecuadorian strains vary at 12 positions (10 singletons), all of them synonymous. The remaining two Ecuadorian CPV-2c strains (ME26 and ME32) appear together at a separate location within the phylogenetic tree, and there are no similar sequences in the GenBank database. These two CPV-2c strains share an isoleucine residue at position 139 (Iso¹³⁹) that is exclusively found in these strains. ME26 has a serine residue at position 440 (Ser⁴⁴⁰) instead of a threonine, which is found in the rest of the CPV-2c strains (Table 1).

The 11 Ecuadorian CPV-2a strains form a single cluster. There are only two different nucleotide haplotypes in the CPV-2a strains that vary in two positions. Ser⁴⁴⁰ was found in all CPV-2a strains (Table 1).

The two CPV-2b strains occur at different positions in the phylogenetic tree. One CPV-2b strain (ME6) clusters with the European and North American CPV-2b strains and is 100% identical to a CPV-2b strain from France. The other CPV-2b strain (ME20) clusters with Ecuadorian CPV-2a strains and differs from one of the CPV-2a strains at only the first position of the 426 codon, the A/G transition that changes asparagine (AAT) to aspartate (GAT). The two CPV-2b strains differ in eight synonymous changes and one non-synonymous substitution at position 440; ME6 has Thr⁴⁴⁰ and ME20 has Ser⁴⁴⁰ (Table 1).

3.3. Clinical symptoms and outcome in relation to CPV-2c and CPV-2a types

Most dogs included in this study were between 2 and 4 months old (84.9%). None were younger than 2 months old or older than 6 months old. All dogs were unvaccinated or had an incomplete vaccination schedule. The breeds of 31 dogs were undefined or mixed, and 22 dogs belonged to

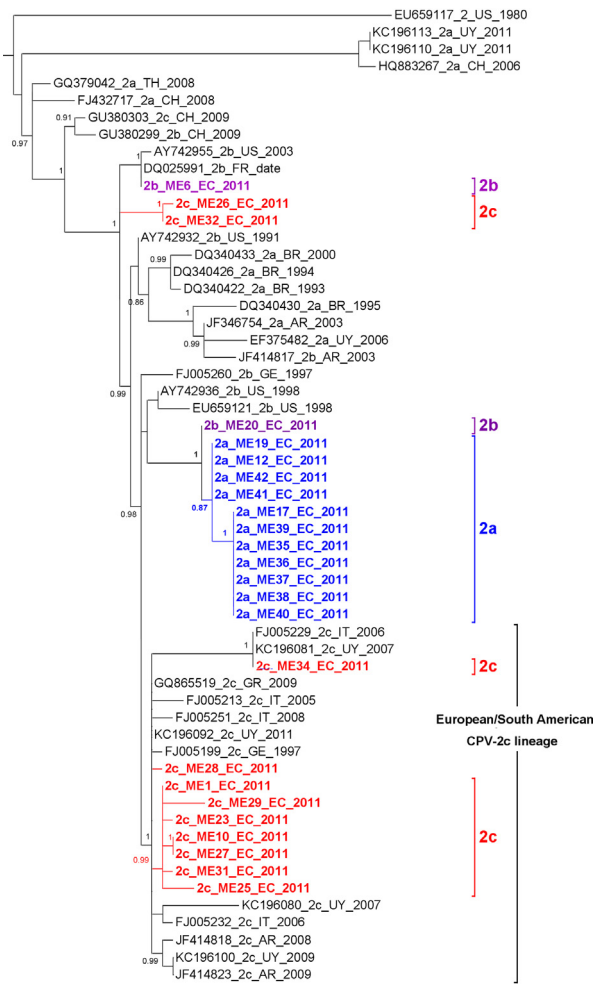


Fig. 1. Phylogenetic tree obtained using the maximum-likelihood method. Phylogenetic reconstruction was carried out using full-length VP2 nucleotide sequences from CPV-2a, 2b, and 2c. AR, Argentina; BR, Brazil; CH, China; EC, Ecuador; FR, France; GE, Germany; GR, Greece; IT, Italy; TH, Thailand; US, United States; UY, Uruguay. The sequence identifiers include the NCBI accession number, the antigenic variant, the country and the collection year. Ecuadorian strains are indicated in bold and with vertical lines on right. Mapping uncertainties for internal nodes of interest are shown as aLRT values. The tree was rooted with the oldest CPV-2 strain.

one of several breeds (Supplementary Table S1). Of these 53 dogs, 32 recovered and 21 died. Significant associations ($p < 0.05$) between some clinical symptoms (anorexia, fever, tachypnea, vomiting) and outcome with CPV type (CPV-2c or CPV-2a) were not found (Table 2). Hemorrhagic gastroenteritis was found in 63.6% (14/22) of the dogs infected with CPV-2a and in 86.2% (25/29) of the dogs infected with CPV-2c; this difference was at the limit of significance ($p = 0.0598$). The frequency of hypothermia and dull mentation was significantly higher in dogs infected with CPV-2c than with CPV-2a (Table 2).

4. Discussion

The global distribution and temporal dynamics of CPV variants have been widely studied by analyzing VP2 genetic variation in strains from diverse geographic areas and different years (Shackelton et al., 2005; Hoelzer et al., 2008; Decaro et al., 2009). Local spatial-scale analyses are comparatively scarce and, with few exceptions (Clegg et al., 2011), only characterize the strain by typing the 426 codon without obtaining the complete VP2 sequence for several strains (Hong et al., 2007; Vieira et al., 2008; Touihri et al., 2009). Here, we characterized 53 CPVs from a small city located in an Andean valley of Ecuador and determined the full-length VP2 gene sequence in several strains. This local study revealed remarkable genetic diversity that included the circulation of all three variants (2a, 2b, and 2c) and the existence of divergent evolutionary groups.

CPV-2c was the most prevalent variant in the Ecuadorian strains (54.7%). Since it emerged in the 1990s, CPV-2c has received a great deal of attention from researchers, veterinarians, and dog owners because of its tendency to spread geographically and its association with severe disease outbreaks (Decaro and Buonavoglia, 2012). In South America, CPV-2c has reached high frequencies in Argentina, Brazil, and Uruguay in the last decade (Pérez et al., 2007, 2012; Calderón et al., 2011; Pinto et al., 2012). In North America, CPV-2c was prevalent in different dog populations of the United States during the 2000s (Hong et al., 2007; Kapil et al., 2007). The high CPV-2c frequency we observed in dogs from Ecuador reveals that CPV-2c has successfully spread and has reached considerable frequencies in all countries of the American continent analyzed so far. The situation is different in the European and Asiatic continents where CPV-2c is prevalent in some countries,

Table 2
Clinical symptoms and disease outcomes of CPV-2a and CPV-2c strains.

Clinical symptoms	CPV variant		χ^2	p-Value
	2a % (n/22)	2c % (n/29)		
Anorexia	95.4 (21)	96.5 (28)	0.04	0.8415
Dull mentation	45.4 (10)	75.8 (22)	4.94	0.0261*
Fever (>39 °C)	68.1 (15)	68.9 (20)	0.01	0.9252
Hemorrhagic gastroenteritis	63.6 (14)	86.2 (25)	3.54	0.0598
Hypothermia (<37 °C)	18.1 (4)	44.8 (13)	3.99	0.0456*
Tachypnea (>40/min)	68.1 (15)	75.8 (22)	0.37	0.5427
Vomiting	95.4 (21)	96.5 (28)	0.04	0.8415
Outcome (mortality)	40.9 (9)	37.9 (11)	0.04	0.8292

* $p < 0.05$, χ^2 test.

but remains scarce or absent in others (Decaro et al., 2007, 2011, 2013).

Ecuadorian CPV-2c is composed of two divergent groups or lineages that have different recent ancestors (Fig. 1). Most Ecuadorian CPV-2c strains belong to a European/South American lineage of CPV-2c. This lineage likely originated in Europe and invaded South America during the last decade (Maya et al., 2013).

The detection of this lineage in the Ecuadorian Andean Region supports its notorious spreading and invasion capabilities in South American territories. Two CPV-2c strains were not closely related to any other CPV strains and constitute, therefore, a lineage that is exclusive to Ecuador. These findings represent the first evidence that two polyphyletic CPV-2c groups are circulating in South America and, remarkably, they co-circulate in a restricted geographic area.

CPV-2a and CPV-2b constituted 41.5% and 3.8% of the Ecuadorian strains, respectively. CPV-2a and CPV-2b variants have been circulating worldwide for more than 30 years and have been identified in Europe, Asia, Africa, Oceania, and America. CPV-2a is prevalent in several countries (Greece, India, Korea, China, and Australia) and CPV-2b has appeared with high frequencies in the United Kingdom, the United States, Taiwan, and Japan (Decaro and Buonavoglia, 2012). In South America, CPV-2a and CPV-2b have appeared in Argentina and Brazil, whereas Uruguay has reported only CPV-2a (Pereira et al., 2007; Calderón et al., 2011; Castro et al., 2011; Pinto et al., 2012; Maya et al., 2013). The Ecuadorian CPV-2a variants described here constitute a single evolutionary lineage that shares a recent common ancestor with a CPV-2b strain (Fig. 1). This lineage is not related to any CPV strain from South America that has been described so far (Gallo-Calderón et al., 2012; Pérez et al., 2012; Maya et al., 2013). The two Ecuadorian CPV-2b strains are clearly polyphyletic, supporting previous findings that CPV-2b variants have different origins (Hoelzer et al., 2008; Clegg et al., 2011). Interestingly, one CPV-2b strain (ME6) is 100% identical in the VP2 coding region to a strain from France, suggesting a common origin or a migration event. The other CPV-2b strain (ME20) only differs from Ecuadorian CPV-2a strains at the 426 codon and does not show high similarity with other CPV variants worldwide. These findings suggest that ME20 may have originated in Ecuador.

Besides the substitution at codon 426, two relevant amino acid changes occurred in Ecuadorian CPV strains: Val139Ile and Thr440Ser (Table 1). Iso¹³⁹, described here for CPV-2c strains of the Ecuadorian lineage, had been previously reported only for CPV-2a and CPV-2b strains (Pereira et al., 2007; Clegg et al., 2011). Position 139 is located inside the capsid but its biological role is unknown (Clegg et al., 2011). Ser⁴⁴⁰ is distributed among one CPV-2c strain, one CPV-2b strain, and all CPV-2a strains. Only two residues have been previously described at position 440: the original threonine residue and a more recent alanine substitution caused by a transition in the first position of the codon (ACA → GCA) (Battilani et al., 2002; Kapil et al., 2007; Pereira et al., 2007; Gallo-Calderón et al., 2012; Maya et al., 2013). The Ser⁴⁴⁰ that we identified here, which is caused by a transversion in the first position of the

codon (ACA → TCA), has been described in a single Italian strain of feline panleukopenia virus, but has not previously been reported in any CPV worldwide (Decaro et al., 2008b). Position 440 is located at the top of the main antigenic site of the virus and is one of the few VP sites that undergo positive selection (Tsao et al., 1991; Decaro et al., 2009). Its location at an important VP2 domain and its occurrence in phylogenetically different variants suggest that Ser⁴⁴⁰ has a relevant role in divergent evolution and the emergence of Ecuadorian strains.

Taken together, our findings suggest that the Ecuadorian CPV population includes strains from different origins and has signs of local differentiation through the acquisition of specific amino acid changes. The genetic heterogeneity of the CPV Ecuadorian population is intriguing because Guaranda is a relatively small city with a small dog population. Although the city is surrounded by the Andean Mountains, there is some exchange of people and dogs with other parts of the country that may have contributed to the heterogeneity of CPV strains. The existence of particular viral dynamics associated with the small dog population, the particular environmental and geographic characteristics, and the differential pathogenesis of the variant types may also play a role. Our analysis of the pathogenicity of CPV-2 variants revealed that both CPV-2c and CPV-2a were associated with severe disease and that there were not significant differences in the clinical outcome (mortality) and some clinical symptoms of dogs infected with CPV-2c versus CPV-2a (Table 2). However, hemorrhagic gastroenteritis, hypothermia, and dull mentation occurred more frequently with CPV-2c infection, supporting recent studies that found more severe clinical courses and higher mortality rates associated with CPV-2c strains (Decaro et al., 2008a).

The interesting genetic variation associated with this Ecuadorian population encourages future studies with samples taken from different time periods to further analyze the evolution of the CPV population. These additional studies could establish whether one variant eventually replaces the others and becomes predominant, as has been observed in other regions of South America (Pérez et al., 2007; Calderón et al., 2011; Pinto et al., 2012).

Conflict of interest statement

There are no conflicts of interest concerning this paper.

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