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Molecular diversity of the VP2 of *Carnivore protoparvovirus 1* (CPV-2) of fecal samples from Bogotá

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ABSTRACT

Background: *Carnivore protoparvovirus 1*, also known as canine parvovirus type 2 (CPV-2), is the main pathogen in hemorrhagic gastroenteritis in dogs, with a high mortality rate. Three subtypes (a, b, c) have been described based on VP2 residue 426, where 2a, 2b, and 2c have asparagine, aspartic acid, and glutamic acid, respectively.

Objectives: This study examined the presence of CPV-2 variants in the fecal samples of dogs diagnosed with canine parvovirus in Bogotá.

Methods: Fecal samples were collected from 54 puppies and young dogs (< 1 year) that tested positive for the CPV through rapid antigen test detection between 2014–2018. Molecular screening was developed for VP1 because primers 555 for VP2 do not amplify, it was necessary to design a primer set for VP2 amplification of 982 nt. All samples that were amplified were sequenced by Sanger. Phylogenetics and structural analysis was carried out, focusing on residue 426.

Results: As a result 47 out of 54 samples tested positive for VP1 screening, and 34/47 samples tested positive for VP2 980 primers as subtype 2a (n = 30) or 2b (n = 4); subtype 2c was not detected. All VP2 sequences had the amino acid, T, at 440, and most Colombian sequences showed an S514A substitution, which in the structural modeling is located in an antigenic region, together with the 426 residue.

Conclusions: The 2c variant was not detected, and these findings suggest that Colombian strains of CPV-2 might be under an antigenic drift.

Keywords: Canine parvovirus; molecular characterization; nucleotide sequencing; mutation; phylogeny

INTRODUCTION

Canine parvovirus type 2 (CPV-2), which is classified into 3 subtypes (CPV-2a, CPV-2b, and CPV-2c), is from the *Carnivore protoparvovirus 1* (*Parvoviridae*, *Parvovirinae*, *Protoparvovirus*) species

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Conflict of Interest

The authors declare no conflicts of interest.

Author Contributions

Conceptualization: Santana-Clavijo NF, Brandão PE; Data curation: Reyes Romero DP, Velandia Muñoz A, Galvis-Estevez CC; Formal analysis: Galvis-Estevez CC, Taniwaki Miyagui SA, Santana-Clavijo NF, Oliveira de Souza Silva S, Brandão PE; Funding acquisition: Brandão PE; Investigation: Reyes Romero DP, Jimenez-Villegas T, Velandia Muñoz A, Santana-Clavijo NF; Methodology: Galvis-Estevez CC, Jimenez-Villegas T, Taniwaki Miyagui SA, Oliveira de Souza Silva S, Brandão PE, Santana-Clavijo NF; Project administration: Reyes Romero DP, Taniwaki Miyagui SA, Brandão PE; Resources: Brandão PE; Software: Brandão PE, Taniwaki Miyagui SA; Supervision: Brandão PE, Taniwaki Miyagui SA, Santana-Clavijo NF; Validation: Galvis-Estevez CC, Taniwaki Miyagui SA, Santana-Clavijo NF; Visualization: Santana-Clavijo NF, Galvis-Estevez CC; Writing - original draft: Galvis-Estevez CC, Jimenez-Villegas T, Reyes Romero DP, Velandia Muñoz A, Taniwaki Miyagui SA, Oliveira de Souza Silva S, Brandão PE, Santana-Clavijo NF; Writing - review & editing: Galvis-Estevez CC, Jimenez-Villegas T, Reyes Romero DP, Velandia Muñoz A, Taniwaki Miyagui SA, Oliveira de Souza Silva S, Brandão PE, Santana-Clavijo NF.

[1]. The virus is a small, non-enveloped virus with a spherical shape, icosahedral capsid, and single-stranded DNA genome of ≤ 5 kb [2]. This virus has four genes in 2 Open Reading Frames (ORFs): ORF1 encodes non-structural proteins NS1 and NS2, and ORF2 encodes capsid proteins VP1, VP2, and VP3 (cleaved from VP2 by host proteases), with a role in receptor binding and stimulation of neutralizing antibodies [3,4]. CPV-2 shows 98% genome identity to Feline Panleukopenia Virus (FPLV) and Mink Enteritis Virus and probably evolved from the FPLV. Mutations at only 3 amino acids (K93N, V103A, and D323Y) on the VP2 increase infection range from felines to canines [5,6].

Monoclonal antibody development in 1980 allowed the recognition of 2 antigenic variants—CPV-2a and CPV-2b. In 2000, a new variant (CPV-2c) was described using molecular biology techniques in Italy [7]. While CPV-2a and CPV-2b have different N426D and I555V residues of VP2, CPV-2c differs only in the N/D426E residue [8]. On the other hand, the V555 residue has been challenged because this amino acid does not present changes in the actuality variants [9].

In Colombia, CPV-2 was reported for the first time in 1980 [10]. Since then, canine parvovirus disease has been diagnosed frequently in Colombian veterinary clinics. Nevertheless, laboratory confirmation is rare [11]. Few epidemiological studies have been conducted in the country. For example, in Medellín and Bucaramanga, the CPV-2a and 2b variants were detected in 6-month-old puppies [12]. The CPV-2c variant has not been described in the country yet. This study examined the presence of CPV-2 variants in fecal samples of dogs diagnosed with canine parvovirus in Bogotá to increase the knowledge of CPV-2 in Colombia.

MATERIALS AND METHODS

Samples

Fifty-four fecal samples from puppies and young dogs under one year of age with hemorrhagic enteritis were collected from 2014 to 2018 in a private veterinary practice in Bogotá, Colombia. The dogs received at least one dose of vaccine against CPV, and others with a complete vaccination schedule (**Supplementary Table 1**) underwent a screening test using an Anigen Rapid CPV Ag (BioNote, Korea) test for antigen detection in fecal samples (**Table 1**). After collection, the fecal samples were stored at -20°C until DNA extraction.

DNA extraction and CPV detection

All fecal samples were prepared as a 20% (v/v) suspension in DEPC-treated water and clarified at $5,000 \times g$ for 15 min at 4°C . The total DNA was isolated from fecal suspensions using a guanidine-isothiocyanate protocol [13]. The integrity of the extracted DNA was confirmed by polymerase chain reaction (PCR) targeting the glyceraldehyde-3-phosphate dehydrogenase (GAPDH) gene [14]. All DNA samples were screened for CPV-VP1 with the primers, CPV/FPV diag F and CPV/FPV diag R [15]. Both PCRs were performed separately in a total volume of 25 μL , with $1 \times$ Go Taq Green Master Mix (Promega, USA), 0.5 μM each of the forward and reverse primer, ultrapure water and 2.5 μL of DNA, and with the amplification conditions described by Furtado et al [16]. A field strain of CPV-2c isolated in the A72 cell line was used as a positive control and ultrapure water as the negative control [17]. The PCR products were analyzed by electrophoresis on a 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen, USA).

Table 1. Features of the 34 positive samples for primer set 980 in this study, sequenced, and features of each sample

Sample identification	Age months	Sex	Vaccination status	Breed	Variant	Year
MN561010.1	4	M	A, B	Mongrel	2a	2018
MN561011.1	3	F	A	Mongrel	2a	2018
MN561012.1	4	F	A, B	Poodle	2a	2018
MN561013.1	12	F	A, B, C	G. Shepherd	2a	2018
MN561014.1	4	F	A, B	Samoyed	2a	2018
MN561015.1	9	F	A, B, C	Schnauzer	2a	2018
MN561016.1	9	M	A, B, C	Yorkshire T	2a	2018
MN561017.1	2	F	A	Mongrel	2a	2018
MN561018.1	7	M	A, B, C	Mongrel	2a	2018
MN561019.1	3	M	A, B	Poodle	2a	2018
MN561020.1	3	F	A, B	Mongrel	2a	2018
MN562440.1	3	M	A, B	Mongrel	2b	2014
MN562441.1	2.5	F	A	Golden R.	2b	2014
MN562442.1	3	M	A	Mongrel	2b	2018
MN562443.1	3	F	A	Mongrel	2b	2018
MN562444.1	3	F	A	Golden R.	2a	2016
MN562445.1	8	F	A, B, C	Mongrel	2a	2014
MN562446.1	2	M	A	G. Shepherd	2a	2018
MN562447.1	3	M	A	Mongrel	2a	2017
MN562448.1	3	M	A, B	Mongrel	2a	2014
MN562449.1	3	F	A, B	Mongrel	2a	2014
MN562450.1	4	M	A, B	Mongrel	2a	2014
MN562451.1	3	M	A, B	Poodle	2a	2015
MN562452.1	12	F	A, B, C	Bulldog	2a	2014
MN562453.1	4	M	A, B, C	Mongrel	2a	2017
MN562454.1	4	M	A, B	Rottweiler	2a	2014
MN562455.1	2.5	M	A	Beagle	2a	2016
MN562456.1	6	F	A, B, C	Mongrel	2a	2014
MN562457.1	2	F	A	Labrador	2a	2015
MN562458.1	4	M	A, B, C	Mongrel	2a	2017
MN562459.1	3	F	A	Poodle	2a	2018
MN562460.1	5	M	A, B, C	Golden R.	2a	2016
MN562461.1	4	M	A, B, C	Rottweiler	2a	2018
MN562462.1	2.5	M	A	Beagle	2a	2014

Vaccines are represented as below: A: CPV; B: CPV, CDV, CAcV; C: CPV, CDV, CAcV, RABV.

CPV, canine parvovirus; CDV, canine distemper virus; CAcV, canine adenovirus; RABV, rabies virus.

Primer design and partial VP2 amplification

For amplification of the partial VP2 sequence, primers were manually designed using 83 complete genomes and 305 partial VP2 sequences retrieved from the Genbank, which were aligned using the Clustal W tool included in BioEdit 7.2.5 [18]. The CPV primers for VP2 980Fw (5'-ACCAGCTGAGGTTGGTTATAG-3', position 3780-3801 based on NC_001539) and CPV VP2 980Rv (5'-CTACTAACTATGATCTAAATGTTC-3', position 4762-4739) primers were analyzed using the OligoAnalyzer 3.1 software (<https://www.idtdna.com/calc/analyser>), and the specificity was checked on BLAST-N suite/GenBank (www.ncbi.nlm.nih.gov/BLAST).

PCR reaction was performed in a total volume of 25 µL that contained 1 × Go Taq Green Master Mix (Promega), 0.5 µM of each primer, ultrapure water, and 2.5 µL of DNA. The PCR conditions included initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 seconds, annealing at 55.5°C for 20 seconds, extension at 72°C for 1 min, and a final extension at 72°C for 10 min. Finally, the PCR products were visualized by electrophoresis on 1.5% agarose gel stained with SYBR Safe DNA Gel Stain (Invitrogen). Reactions with 982 bp fragments were considered positive.

DNA sequencing, phylogenetic and structural analysis

VP2 amplicons were purified with Illustra ExoProStar 1-Step (GE Healthcare, UK) and sequenced bidirectionally using a BigDye Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, USA), and ABI-3500 Genetic Analyzer (Applied Biosystems) following the manufacturer's instructions. Sequences with Phred quality scores ≥ 20 (<http://asparagin.cenargen.embrapa.br/phph/>) were assembled with the CAP contig assembly program and aligned with homologous sequences retrieved from GenBank using CLUSTAL W, both implemented in Bioedit 7.2.5 [17]. The amino acid alignment was used to build a maximum likelihood tree (Dayhoff and JTT models) with 1,000 bootstrap replicates using MEGA X [19], rooted with Aleutian Mink Disease Virus as an outgroup (**Fig. 1**).

The identity of the nucleotide and amino acids sequences were calculated using the Bioedit Sequence Alignment Editor version 7.2.5 program [18]. Partial VP2 amino acid sequences were analyzed on residue 426 to determine the CPV variant (CPV-2a, 2b, or 2c) [7]. The VP2 monomer and pentamer structure of CPV-2a were constructed on the SWISS-MODEL system [20] using the Canine Parvovirus Empty Capsid (strain D) VP2 (GenBank access PDB 2CAS_2) as homology reference Chain A. The mutagenesis function in PyMOL was used to generate the homology model A514S mutant. Each loop and residue important for this study was highlighted in a 5-fold axis and 3-fold axis using the PyMOL software v.4.6.0 (**Fig. 2**). All sequences obtained in this study were submitted to GenBank (MN562440 to MN562462 and MN561010-MN561020).

RESULTS

CPV detection

Approximately 87.0% (47/54) of fecal samples tested positive for VP1 screening (**Supplementary Table 1**), 53.3% (26/47) of which were purebred dogs. The positive samples average age was 4.8 months, and the distribution between females and males was 40.4% and 59.6%, respectively. All samples were from puppies that were vaccinated at least once for CPV (34% [16/47]), with 2 doses (31.9% [15/47]), and 3 doses (34.1% [16/47]) (**Table 1**). The integrity of DNA of all samples was confirmed by amplification of the GAPDH gene.

Phylogenetic and structural analysis of partial VP2

Of the positive VP1 samples, 72.3% (34/47) were positive for VP2 reaction and were sequenced. Approximately 88.2% (30/34) were CPV-2a and 11.8% (4/34) were CPV-2b, based on the 426-residue being either N or D, respectively (**Table 2**).

The sequences obtained were segregated in 2 clusters within reference sequences for subtypes 2a and 2b, close to the sequences from Medellín 2013 (KX891194 2b and KX891195 2a). The formation of a cluster for each subtype of CPV was observed. The first at the top of the tree was the cluster of subtypes 2c (yellow). The formation of a cluster of 2b, where four sequences (blue) are located, was observed. A 2a cluster was observed with sequences from different regions (red). The formation of another 2a cluster, where 32 (black) the sequences of Colombia (3 cities) are located, was noted. Finally, the cluster with the sequences of feline panleukopenia was found (**Fig. 1**).

The VP2 sequences from Colombia (Bucaramanga, Medellín, and Bogotá—this work) showed 98.3%–100% amino acid identities. In addition to an A514S change in all CPV-2a due

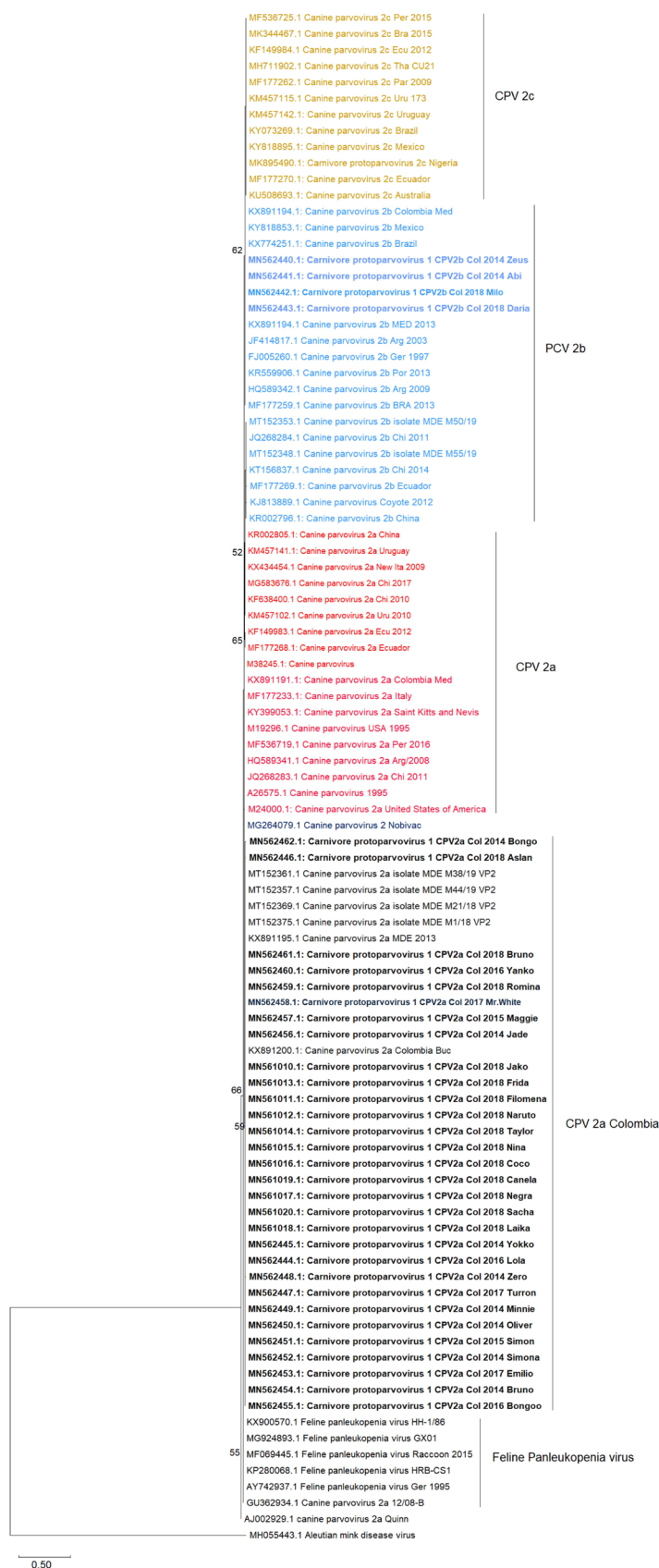


Fig. 1. Maximum likelihood tree of *Carnivore protoparvovirus* strains detected in dogs from Bogotá based on the amino acid of the VP2 structural protein (regarding MH476590.1), while the root is represented by MH055443.1 (ADV-1). The yellow dark sequences represent subtype 2c. The blue sequences represent the sequences of subtype 2b located at 4 Colombian sequences in bold. Red represents the sequences of subtype 2a, and the sequences in black and bold represent the cluster 2a in Colombia, in which the sequences obtained in this study are located. CPV, canine parvovirus; PCV, porcine circovirus.

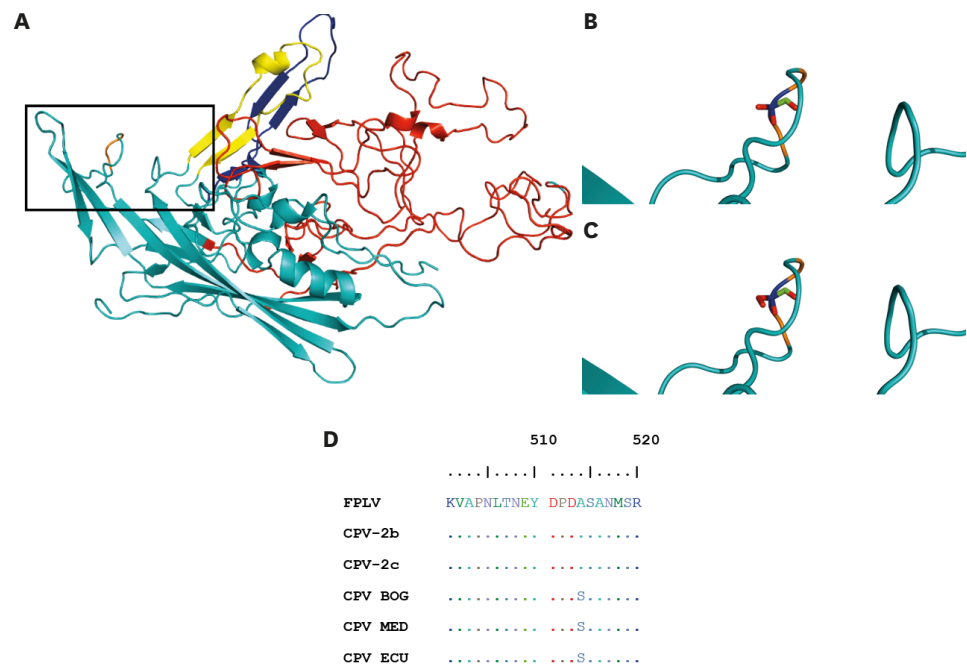


Fig. 2. Ribbon diagram and sequence of VP2 empty capsid (PDB ID 2CAS2). A. Loop 1 in yellow, loop 2 in blue, and loop 3 and 4 in red; Residue 513 to 515 are colored in orange, which outside loops zone. B and C. Comparative of residue 514 from VP2 in an augmented view of the ribbon diagram, B, showing the substitution from alanine (A) to C, serine (S) in some Colombian samples of CPV-2a. D. Amino acid sequence alignment generated using BioEdit, comparing Feline panleukopenia, CPV-2b, CPV-2c and CPV-2a from Colombia and Ecuador where mutation A514S was found. CPV, canine parvovirus.

Table 2. Differences in amino acid residues in VP2 of CPV-2 variants and some samples included in this study

Variant/Reference	Amino acid residue				
	375	426	428	440	514
Feline panleukopenia 1995 AY742937.1	D	N	N	T	A
CPV-2 M10989	N	N	N	T	A
CPV-2a MF177233	D	N	N	T	A
CPV-2b Ecuador MF177269.1	D	D	N	S	A
CPV-2c Ecuador MF177270.1	D	E	N	T	A
CPV-2a Med MT152374.1	D	N	N	T	S
CPV-2a BUC KX891206.1	D	N	N	T	A
CPV-2a MED KX891193.1	D	N	D	T	S
CPV-2a BOG MN561017.1	D	N	N	T	S
CPV-2a BOG MN561018.1	D	N	N	T	S
CPV-2a BOG MN561019.1	D	N	N	T	S
CPV-2a BOG MN561020.1	D	N	N	T	S
CPV-2b MED MT152348.1	D	D	N	T	A
CPV-2b BOG MN562440.1	D	D	N	T	A
CPV-2b BOG MN562441.1	D	D	N	T	A
CPV-2b BOG MN562442.1	D	D	N	T	A
CPV-2b BOG MN562443.1	D	D	N	T	A

The letters in residue 426 represent the Aspartic acid (D) and Glutamic acid (E) amino acids, while the letter N represent Asparagine. This residue served for classified CPV subtypes. CPV-2, canine parvovirus type 2.

to a transversion (G to T), all samples were found with T440 (**Table 2**). VP2 3-dimensional reconstruction showed that amino acid 514 is located close to the high antigenic zone at the 5-fold axis (**Fig. 3**), while the 3-fold subunits, with a higher antigenicity, contain aa 426, 440, and 555 [21].

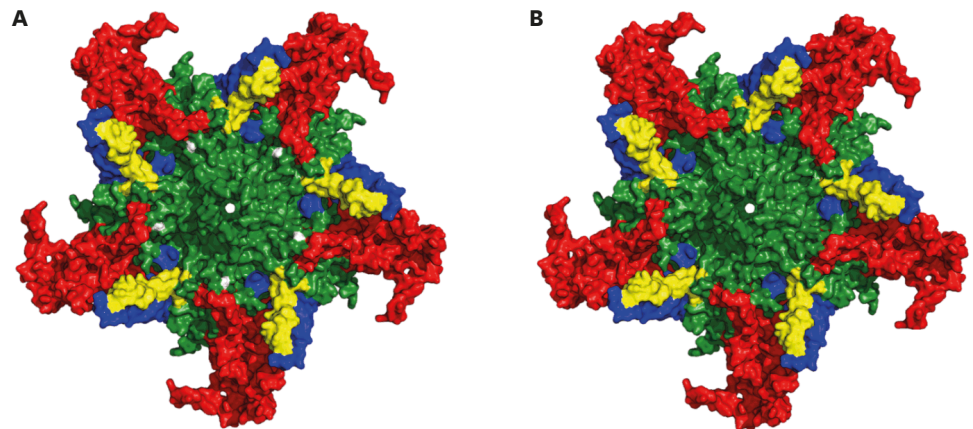


Fig. 3. Comparison between pentamers. Loop 1 in yellow, Loop 2 in blue, and Loop 3-4 in red. A. Pentamer without a 514 mutation. B. Pentamer with a 514 Colombian mutation. Mutation 514 present in the surface valley (canyon) where it has been postulated as a site of union to the receptor.

DISCUSSION

Vaccination is the primary strategy to protect against CPV. Any failures by the presence of maternal antibodies or circulation of different CPV variants could cause an inappropriate immune response, leading to the presentation of severe hemorrhagic gastroenteritis [17,22]. In the present study, PCR screening showed that all dogs testing positive had been vaccinated with at least one dose of the CPV vaccine.

In Colombia, the vaccination protocol includes 2 or 3 doses (WSAVA Vaccination Guidelines [23]), and vaccines with CPV-2b were licensed by the Colombian Agricultural Institute (ICA). Interestingly, VP2 sequencing showed the circulation of the CPV-2a variant (88.23%), including dogs with 3 vaccine doses (**Table 1**). Therefore, immunization failure could be caused by the lack of cross-immunity by different antigenic variants. Moreover, the four puppies diagnosed with CPV-2b had an incomplete vaccination protocol and were less than 3 months of age (**Supplementary Table 1**).

The prevalence of CPV in puppies or young dogs can vary depending on age, breed, and sex [24,25], in addition to the different primers set used in each study. The higher positive rates than other reports from Colombia [26] is due to differences in sampling. The samples used in this study previously tested positive in a rapid Anigen Rapid CPV Ag (BioNote) test because the main purpose of this study was the molecular characterization.

These samples were not amplified with primers 555for/rev reported by [7]. Therefore, it was necessary to design novel primers set CPV VP2 920Fw/Rv. When performing alignment of the whole genome sequences, mismatching was observed in Asian sequences in the hybridization site of the 555rev primer, which is located after the stop codon of the VP2 coding sequence, was observed in Asian sequences. A conserved region in 3' UTR of all 83 complete CPV genome sequences was used to design the VP2 980Rv primer to have a better perspective of Colombian samples amplification. Sequencing showed that the 555rev primer region is different in all samples and could explain the failure in amplification using this set of primers.

The phylogenetic tree revealed several clusters, with the formation of a specific cluster for Colombian CPV-2a, including the sequences reported by Duque et al. [12] and Giraldo-

Ramirez et al. [26]. This lineage showed the same common ancestor of CPV-2a sequences from Argentina, Brazil, USA, Italy, China, and Australia. On the other hand, the Colombian CPV-2b sequences clustered together with sequences from South America, North America, and Europe; this group shows the same common ancestor. Finally, the CPV-2c was not detected, and no cases have been reported in Colombia [12,26]. The low values in the Bootstrap between nodes did not diverge much, partly due to the minute genetic variability, which is consistent with other studies [27,28]. This suggests new classifications for CPV-2 and FPV according to their molecular basis [29]. Chung et al. [30] proposed a new classification, i.e., CPV-2 I, II, and III, where CPV-2 I is derived from American strains and CPV-2II and III are from Italian strains with a central origin of the United States. This classification was not carried out in this study because the amino acids analyzed were insufficient [30].

Residue T440 did not show any changes in the Bogota sequences, nor in the Antioquia and Bucaramanga sequences until 2017 [12]. On the other hand, a recent work carried out at the Department of Antioquia showed mutation T440A [26]. On the other hand, the Ecuadorian sequences showed a mutation T440S in 2013 [24]. The importance of this residue lies in the viral capsid (loop 4); this region can impact the antigenic responses, leading to positive selection, resulting in failure of the vaccination response [26,31,32]. This change has been reported in different geographical areas, such as Italy, the USA, and China [27,28,33].

The A514S mutation observed in this study was also reported in Colombian sequences in 2017 and 2020 [12,26]. In those studies, they specified the possibility of a new variant present in Colombia. When mutation 514 was first reported, it was present in 16/22 samples of CPV-2a [12]; these sequences were from 2013 to 2016. In recent studies, this mutation was present in all samples. Giraldo-Ramirez et al. reported that 27/0 samples had the mutation in CPV-2a [26], which was in this study at 30/0 samples, both of which dated from 2018 to 2019 and from 2014 to 2018, respectively. Furthermore, none of the CPV-2b samples in any of the 3 studies had this mutation. This mutation was also reported in 2017 in Ecuador [34]. This mutation can suggest a region the position evolutionary of CPV-2a, and this region is located in the surface valley (canyon) region in a 5-fold axis symmetry of VP2 [35], in the close areas where neutralizing antibodies act [36]. This area becomes important because it has been postulated as a site of union to the receptor [21,35,37-39], which may lead to positive selection and immune evasion. This fact is important for new vaccination strategies because evidence suggests that the differences between the antigenic types of CPV and neutralization test have clinical importance [40].

In this study, all samples came from vaccinated puppies. On the other hand, the samples were collected from puppies with a positive immunochromatography test, and it was not possible to determine the efficacy of the vaccine. Nonetheless, 60% of puppies seroconverted after vaccination with ages between 6 to 8 weeks, and only 10% of puppies with 2 and 3 re-vaccinated had not seroconverted [29,41]. The aim is not to evaluate the efficiency of the vaccine, but it is important to highlight the necessity of studies in this field.

SUPPLEMENTARY MATERIAL

Supplementary Table 1

Features of all 54 samples and test development in this study

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