Research Article

Phylogenetic Analysis of VP2 Gene in Canine Parvovirus Isolates in India and their Molecular Implications

Pavana Jyothi Vanjavaka[1], Mouradam Veerasami[2], Mohana Subramanian Bhaskaran[2]*, Vijay A.K.B. Gundi[1]*

[1]1MBIG Research Laboratory, Department of Biotechnology, Vikrama Simhapuri University, Nellore – 524 324, Andhra Pradesh, India; [2]Cisgen Biotech Discoveries Private Limited, IITM Research park, Tharamani-600113, Chennai, Tamilnadu, India.

Abstract | Canine parvovirus (CPV) is a contagious and pathogenic virus in puppies and dogs. It was first reported in 1978; however, it was later replaced by three antigenic variants at different periodic intervals that now circulate globally with random strains. This particular study aimed to determine the genetic changes in the VP2 gene, which are responsible for creating different antigenic variants, and to understand their impact on pathogenicity. In contrast, molecular characterization has become an essential tool in comprehending the evolution of viruses. For studying the molecular level changes, 28 field isolates were collected from various regions in India and subjected to PCR for amplification, with sequencing of a partial region of the VP2 gene. Among these isolates, 27 samples were positive for CPV, of which one was CPV 2b while the remaining were CPV 2a, and one isolate was CPV negative. The obtained sequences were submitted to NCBI to get gene accession numbers, and the phylogenetic tree was created to deduce the relationship between the different isolates of dogs, with a distance matrix derived from sequence variations in the genomes.

Keywords | Canine parvovirus, Hemorrhagic enteritis, VP2 gene, Maternal antibodies, CPV variants, Immunisation

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***Correspondence** | Mohana Subramanian Bhaskaran and Vijay A.K.B Gundi, Cisgen Biotech Discoveries Private Limited, IITM Research park, Tharamani-600113,Chennai,Tamilnadu, India; 1MBIG Research Laboratory, Department of Biotechnology, Vikrama Simhapuri University, Nellore – 524 324, Andhra Pradesh, India; **Email:** bhaskaran.mohana@gmail.com, gundi.vijay@gmail.com
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INTRODUCTION

Canine parvovirus is known to cause acute hemorrhagic gastroenteritis and myocarditis in puppies over 3-4 months old, leading to high mortality rates among this vulnerable population (MacLachlan, 2016). Puppies receive maternal antibodies immediately after birth, providing passive immunity that gradually diminishes over time. Consequently, a series of vaccinations are required for their protection; the first shot should be administered first three weeks of after birth. As puppies grow older, they need further vaccinations, spaced 3-4 weeks apart, to bolster their immunity effectively. The canine parvovirus is a small, single standard DNA with the range of 4-5Kb. It is classified with in the family Parvoviridae and the genus Protoparvovirus (Cavalli *et al*., 2008). The virus genome has two open reading frames (ORFs), One of which codes for two nonstructural proteins (NS 1 and NS 2), and the other codes for two structural proteins (VP1 and VP2). VP2 is a significant capsid protein required for host immune response (Reed *et al*., 1988) and is crucial in detecting CPV-2. The viral DNA is replicated through a rolling hairpin mechanism on both ends of the genome using palindromic hairpins of around 150 bases (Parrish,

1999; Wang *et al*., 1998). The capsid contains 60subunits of VP1 (5-6 copies) and VP2 (54-55 copies) protein. The two structural proteins and nonstructural proteins are synthesized mRNAs through alternate splicing (Agbandje *et al*., 1993; Parrish and Kawaoka, 2005; Tsao *et al*., 1991; Wang *et al*., 2005). The VP2 protein can be cleaved near its N-terminus by host proteases to produce another structural protein, VP3. The capsid proteins have a highly conserved central core composed of an eight-stranded, anti-parallel β barrel with flexible loops between the β-strands that interact to form most capsid surfaces. The genetic variations in the VP2 gene can be well characterized by sequencing and individual analysis of the samples.

To comprehend the evolution of the parvovirus and implementing preventive measures to halt its transmission are crucial steps and this can be achieved by monitoring outbreaks and quickly diagnosing cases, followed by providing appropriate treatment. The emergence of all CPV variants with different epidemiologic and antigenic properties can be studied using numerous *in vivo* and *in vitro* tests. The in vitro tests for diagnosis of parvovirus methods like Hemagglutination tests and enzyme-linked Immunosorbent Assay (ELISA) are familiar tests, though not always sufficiently sensitive, but are used for routine diagnosis (Decaro and Buonavoglia, 2012). At the molecular level, methods like Polymerase Chain Reaction (PCR), Rapid mini sequence technique (Decaro *et al*., 2005), Whole genome sequencing, NGS, and Real-Time PCR for quantification are done, which are highly sensitive and more specific (Nandi and Kumar, 2010). Moreover, the available CPV genomes sequenced so far are similar except in the VP2 region. The VP2 gene must be amplified and the PCR product sequenced to determine the link between antigenic variations. This process reveals several significant and relevant amino acids that indicate the genetic and antigenic differences between the vaccination strains, their variants, and the original CPV-2 (Hueffer *et al*., 2003). However, in the present study, 28 fecal samples were collected from various regions of India as per Table 1. The collected samples were characterized by the nucleotide sequence of the partial VP2 gene at the conserved region to find out the type of strain and occurrence of mutations across India. The reported deduced amino acids pattern is depicted in the phylogenetic tree to represent the evolutionary relationship in the CPV variants.

MATERIALS AND METHODS

A total of 28 fecal swab samples were collected from dogs with clinical signs such as hemorrhagic diarrhea and vomiting from veterinary hospitals located across India between 2016 and 2018. Samples were transported to the lab and stored at -80 °C until further processing. 300µL of sample was used for genomic DNA isolation using the DNAzole reagent (Invitrogen) followed as per manufacturer instructions. The extracted DNA was then stored at -20°C until further use. Subsequently, the samples were subjected to PCR amplification. A 100µl reaction was prepared for each sample using an amplicon PCR master mix. The PCR program was set with an initial denaturation step of 94°C for 3 minutes, 40 cycles of 94°C for 45 seconds, 50°C for 45 seconds, 72°C for 1 minute, and a final extension of 72°C for 15 minutes. The primer pair, CPV Ext For and CPV Ext Rev (CPV Ext Rev-5´-GGCAAACAAATAGAGCATTGG-3´ and CPV Ext For-5´-CCCAAATTTGACCATTTGGAT-3'), was used (V *et al*., 2016). These primers were created from the conserved regions of the VP2 gene and were, therefore, 100% homologous with the VP2 DNA sequence of all the CPV types. Although the VP2 protein is a significant capsid protein that plays a vital role in determining the virus's antigenic properties, pathogenicity, and host range.

Table 1: Sample IDs, GenBank accession number and corresponding sample results.

S. No	Sample ID	Gene bank accession number	PCR result
1	CPV-29	OR659056	Positive
2	CH-1/14(7512)	OR659057	Positive
3	CPV-13/1	OR659058	Positive
4	CPV 17050	OR659059	Positive
5	SB 21-2-14	OR659060	Positive
6	ST 21-2-14	OR659061	Positive
7	CPV-122432	OR659062	Positive
8	CPV 2/14	OR659063	Positive
9	CPV 22	OR659064	Positive
10	CPV 23	OR659065	Positive
11	CPV 115869	OR659066	Positive
12	CPV 128626	OR659067	Positive
13	CPV-24	OR659068	Positive
14	CPV-25	OR659069	Positive
15	CPV-26	OR659070	Positive
16	CPV-129025	OR659071	Positive
17	CPV-129032	OR659072	Positive
18	CPV-128445	OR659073	Positive
19	CPV-128652	OR659074	Positive
20	CPV 28	OR659075	Positive
21	CPV-128702	OR659076	Positive
22	CPV-128819	OR659077	Positive
23	Maha_2a	OR659078	Positive
24	Karur_2a	OR659079	Positive
25	Gujarat_2a	OR659080	Positive
26	Chennai_2a	OR659081	Positive
27	Hyd_2b	OR752446	Positive

To identify CPV-2 variants, scientists examine amino acid sequence changes in the VP2 protein at residue 426. However, other VP2 residues may also have important amino acid modifications. The amplified products were subjected to agarose gel electrophoresis using 20µL of each sample along with 6x loading dye in 1.5% agarose gel with 40 mM Tris-acetate 1 mM EDTA (TAE) running buffer and stained with ethidium bromide solution (Sigma-Aldrich, USA) and the separated bands were visualized under UV light using the Gel Doc system. Positive bands were observed at a length of 576 bp (Figure 1). Out of the above 28 samples, one sample was CPV negative, and the remaining 27 were CPV positive. The 27 PCR products were gel purified using RBC HiYield Gel/PCR DNA Extraction Kit following the manufacturer's instructions. All the gel eluted samples were sequenced by using the ABI Prism®BigDyeTM Terminator Cycle Sequencing (Applied Biosciences, USA) as per manufacturer's instructions. The obtained data was converted to FASTA format and BLASTed with VP2 reference sequences available in NCBI and made phylogenetic analysis to understand evolutionary relationship. The generated sequence data were submitted to the NCBI under the accession number mentioned in the below Table 1.

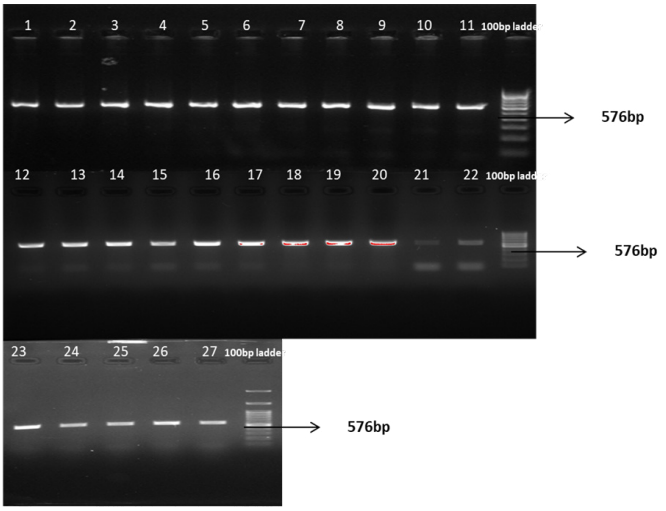

Figure 1: Fecal samples subjected to PCR and gel electrophoresis picture. Sample details are mentioned in the **Table 1** (SNo-1to 26 are 2a and 27 is 2b).

Phylogenetic analysis

Phylogenetic analysis is a vital tool for studying the diversity of species and gaining insights into mutation occurrences. In our study, we used this tool to differentiate between sequences. We also utilized BLASTn to determine the percentage of homology with other GenBank sequences. The obtained sequences were then translated into amino acid sequences using Clustal software. After that, by using Mega 11 software, a phylogenetic tree was generated to visualize the relationships between the 27 CPV isolates with references, which were taken from NCBI. Based on the deduced amino acid analysis, most of the isolates found in India are 2a, with only one being 2b. In the obtained outbreaks, there were no positive isolates for CPV 2 or the recently evolved strain of CPV 2c. The variant CPV 2a was analyzed using reference sequences S. No: 28-33, CPV 2b was analyzed using S. No: 34-39, and the variant CPV 2c from S. No: 40-42, references. Vaccine strains were also used as reference sequences downloaded from the gene bank and analyzed for the changes that occurred in 2a and 2b. In the previous studies, the changes were observed from CPV 2 to CPV 2a as signature mutations at 87(Met→Leu), 101(Ile→Thr), 300(Ala→Gly), 305(Asp→Tyr), and 426(Asp→Asn). In contrast, in 2b reversion at 426(Asn→Asp), and finally in 2c, only one amino acid change was observed at 426(Asp-Glu) across the globe between 1979 and 2000. Furthermore, among all antigenic variations of CPV 2a, 2b, and 2c, reversion at position 555(Ile→Val) was obtained from CPV/FPV given in Table 2. However, these partial sequences contained regions spanning the amino acids 426 in VP2 and are used to distinguish CPV types 2a, 2b, and 2c. The VP2 gene sequences were evaluated by comparing them to each other, as well as CPV VP2 gene sequences in GenBank using Maximum Neighborhood (Mega 11 Software) as references. Phylogenetic tree generated in Figure 2.

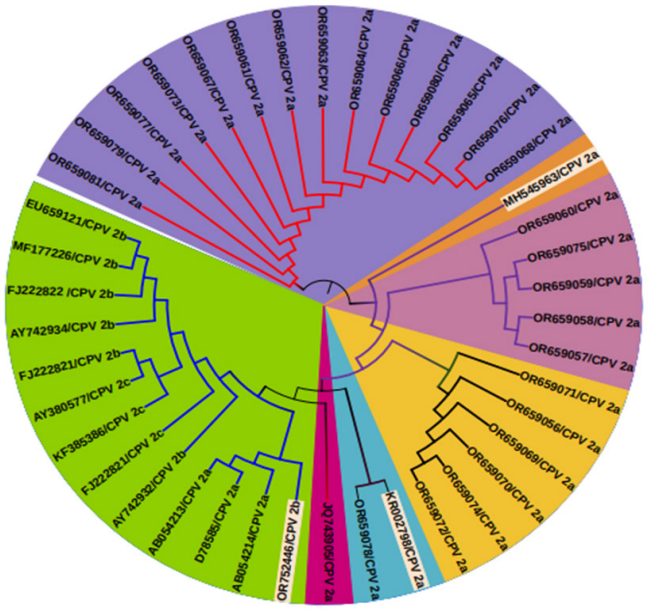

Figure 2: Maximum neighborhood joining tree depicting the correlation between the partial nucleotide sequences of CPV isolates, which we have collected from various regions of India and analysed from obtained Sanger sequences.

As shown in Table 3, the obtained partial VP2 sequences distinguished between CPV 2a and 2b; no other variants were found in the collected isolates. Despite the Presence of CPV 2a and 2b, several other deduced amino acids were discovered in the current study.

Table 2: Signature mutations occurred at the VP2 region.

Variants	87	101	297	300	305	323	426	555
FPV→CPV 2	K→N					D→N		
CPV→CPV 2a	M→L	I→T	S→A	A→G	D→Y		D→N	I→V
CPV→CPV 2b							N→D	
CPV→CPV 2c							D→E	

Table 3: Correlation of deduced amino acid sequences at different residues of Canine parvovirus isolates and their references.

S. No	ID Name	Geno-type	297	300	305	324	356	426	429	440
1	OR659056	CPV 2a	A	G	Y	I	P	N	V	T
2	OR659057	CPV 2a	A	G	Y	I	P	N	V	A
3	OR659058	CPV 2a	A	G	Y	I	P	N	V	A
4	OR659059	CPV 2a	A	G	Y	I	P	N	I	A
5	OR659060	CPV 2a	A	G	Y	I	P	N	V	A
6	OR659061	CPV 2a	A	G	Y	I	P	N	V	A
7	OR659062	CPV 2a	A	G	Y	I	P	N	V	A
8	OR659063	CPV 2a	A	G	Y	I	P	N	V	A
9	OR659064	CPV 2a	A	G	Y	I	P	N	V	A
10	OR659065	CPV 2a	A	G	Y	I	P	N	V	A
11	OR659066	CPV 2a	A	G	Y	I	P	N	V	A
12	OR659067	CPV 2a	A	G	Y	I	P	N	V	A
13	OR659068	CPV 2a	A	G	Y	I	P	N	V	A
14	OR659069	CPV 2a	A	G	Y	I	P	N	V	T
15	OR659070	CPV 2a	A	G	Y	I	P	N	V	T
16	OR659071	CPV 2a	A	G	Y	I	P	N	V	A
17	OR659072	CPV 2a	A	G	Y	I	P	N	V	A
18	OR659073	CPV 2a	A	G	Y	I	P	N	V	A
19	OR659074	CPV 2a	A	G	Y	I	P	N	V	T
20	OR659075	CPV 2a	A	G	Y	I	P	N	I	A
21	OR659076	CPV 2a	A	G	Y	I	P	N	V	A
22	OR659077	CPV 2a	A	G	Y	I	P	N	V	A
23	OR659078	CPV 2a	A	G	Y	I	P	N	V	T
24	OR659079	CPV 2a	A	G	Y	I	P	N	V	A
25	OR659080	CPV 2a	A	G	Y	I	P	N	V	A
26	OR659081	CPV 2a	A	G	Y	I	P	N	V	A
27	OR752446	CPV 2b	A	G	Y	Y	H	D	V	A
28	AB054214	CPV 2a	A	G	Y	Y	P	N	V	A
29	AB054213	CPV 2a	A	G	Y	Y	P	N	V	A
30	D78585	CPV 2a	A	G	Y	Y	P	N	V	A
31	JQ743905	CPV 2a	A	G	Y	I	P	N	V	T
32	KR002798	CPV 2a	A	G	Y	I	P	N	V	T
33	MH545963	CPV 2a	A	G	Y	I	P	N	V	A
34	FJ222822	CPV 2b	A	G	Y	Y	P	D	V	T
35	FJ222823	CPV 2b	A	G	Y	Y	P	D	V	T
36	AY742932	CPV 2b	A	G	Y	Y	P	D	V	A
37	AY742934	CPV 2b	A	G	Y	Y	P	D	V	A
38	EU659121	CPV 2b	A	G	Y	Y	P	D	V	A
39	MF177226	CPV 2b	A	G	Y	Y	P	D	V	A
40	AY380577	CPV 2c	A	G	Y	Y	P	E	V	T
41	KF385386	CPV 2c	A	G	Y	Y	P	E	V	T
42	FJ222821	CPV 2c	A	G	Y	Y	P	E	V	T

*G, glycine; Y, Tyrosine; I, Isoleucine; T, Threonine; N, Asparagine; D, Aspartic acid; V, Valine; P, Proline; H, Histidine.

Maximum Neighborhood was used to construct evolutionary relationships between CPV strains from various geological areas of India. As previously stated, the primers were designed at conserved regions from approximately 280 to 472 amino acid positions for amplification to cover all CPV variations. A total of 28 isolates were found to be CPV positive, with 26 being CPV 2a (Table 1 S. No. 1 to 26) positive, one isolate was CPV 2b (Table 1 S. No. 27) positive and one was CPV negative.

In this study, we discovered the prevalence of CPV 2a and 2b in various regions of India. According to the findings, 2a is predominant in most locations in India, and 2b and 2c are not found in the collected regions. The sequence analysis of current experiment revealed that Alanine at position 297, Glycine at position 300, and Tyrosine at position 305 are common mutations in both CPV 2a and 2b. Other than this, additional changes were observed at different positions in 2a and 2b. Arsinine at position 426, Isoleucine at 429 positions, Alanine at 440 for 2a and Tyrosine at 324 positions, Histidine at 356, and Aspargine at position 426 for 2b details are given in the table-3. In the previous study, the identified common variations at residue S297A, A300G, D305Y, D426N, N426D, and Y440A are prone in CPV 2a and 2b and designated as new CPV 2a and new CPV 2b as well as considered as molecular signature (Battilani *et al*., 2019; Martella *et al*., 2005). All these signature mutations are in the GH loop; this large loop comprises 267–498 residues and is located between the βG and βH strands in the major capsid protein. The amino acid substitution Valine→Isoleucine at 429 (S. No-4 and 20) was found only in two 2a isolates, and the change refers directly to the virus's surface (Chinchkar *et al*., 2006). Similarly, other amino acids Try324Ile/ Ile324Try (Battilani *et al*., 2019) position was identified in 2b (S. No: 27) close to the binding of canine transferrin receptors, resulting in changes in the host range of canine parvovirus. It can determine the connection between the severity of clinical symptoms. An additional mutation at 356 positions Pro→His (S. No-27) was observed in 2b, and this particular change was previously observed in 2a and 2c in Italy (Battilani *et al*., 2019) and China. Remarkably, this unique amino acid substitution is significantly less common and needs further investigation.

Other than that, we found one more amino acid substitution, Alanine→Threonine at 440 (Isolate S. No-1, 13, 14, 19 and 23) in type 2a isolates, which had been

found in references of various regions worldwide (Geng *et al*., 2015; Mukhopadhyay *et al*., 2014; Nandi *et al*., 2009; Nookala *et al*., 2016; Tuteja *et al*., 2022), particularly in China. The 440 amino acid is located at the top of the GH Loop (3-fold spike) of the VP2 protein, which is the primary antigenic site of the virus (Parrish *et al*., 1991). The evolution of amino acids 324, and 440 was mentioned by (Battilani *et al*., 2002). Presence of above listed mutations (Table 3) is at high levels in India, and other countries like Northern America, Italy, and Asian countries (Nandi *et al*., 2009). In this study, 81, 101, 267, and 555 are unknown because the PCR-selected partial VP2 gene does not cover the regions. There have been reports of amino acid substitutions in the VP2 protein's variable GH loop, which includes 267-498aa (Battilani *et al*., 2002; Gallo Calderón *et al*., 2012; Truyen, 2006). The integration of CPV-2a, CPV-2b, and CPV-2c at different ratios in different countries demonstrates no evolutionary advantage of one type over the other and that this coexistence did not evolve due to immunoselection pressure from vaccines (Battilani *et al*., 2002). This study reveals that canine parvo vaccines used in India based on CPV2, CPV 2a, and CPV2b, as mentioned in the reference sequences, are distinct from field isolates obtained from Indian subcontinents, which 93% of isolates belong to CPV 2a and in that 27% isolates are new 2a and one of the isolates new 2b (S. No-27). As a result, ongoing prevalence oversight and gene sequencing will be unable to find mutations and provide insight into the distribution of various antigenic variants of CPV globally. Because of this study geographically, the dynamics of the transmission, diversity of the virus have strived to alter the genotype due to many factors, including vaccine failure, interference from maternal antibodies, and reversion in virulence, vaccination response to the evolutionary strains, and non-responders to the vaccines.

CONCLUSIONS AND RECOMMENDATIONS

In conclusion, our study analyzed field isolates in comparison to reference sequences, including vaccine strains. We observed several noteworthy patterns, including changes in variants and the predominance of specific strains across different geographic regions. Notably, CPV-2a remains prevalent in India, with only one isolate identified as CPV-2b (refer to Table 3). Our evaluation of isolates using the conserved region of the VP2 gene and stringent PCR primer conditions suggests that these methods can effectively monitor variant prevalence and detect the emergence of new variants. As discussed earlier, while many mutations identified in our study have been reported previously, some unique substitutions warrant further investigation. The coexistence of CPV-2a and CPV-2b at varying levels of prevalence in India may be attributed to geographical and host-dependent factors. These rates may also vary depending on viral lineage, geographic location, and time period studied. Although host range jumps are expected to be rare, understanding the mechanisms underlying host receptor interactions can aid in anticipating and potentially preventing the emergence of new viruses. Furthermore, investigating the causes of mutations and their impact on antigenicity, particularly in the context of vaccine failure cases worldwide, is crucial. Additional research encompassing a wider geographical range is warranted. Such studies could provide valuable insights into the role of CPV-2 variants in the pathogenesis of parvoviral disease in dogs and inform the development of effective disease control strategies. In light of these findings, further exploration involving samples from diverse geographical areas is recommended. This research could significantly contribute to our understanding of CPV-2 variants' role in parvoviral disease pathogenesis and aid in formulating appropriate control measures.

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NOVELTY STATEMENT

The study unveils significant insights into the prevalence and genetic variations of canine parvovirus (CPV) in India. Through VP2 gene sequence analysis, we identified and shared mutations in CPV-2a and CPV-2b, including specific amino acid substitutions designated as molecular signatures. Mutations at positions 429 and 324 are particularly intriguing, with implications for virus surface properties and host receptor binding. Moreover, a unique mutation at position 356 in CPV-2b identified and it warrants further investigation. These genetic findings highlight the dynamic nature of CPV transmission, influenced by factors like vaccine failure and evolutionary pressures.

AUTHOR'S CONTRIBUTION

All the authors are contributed equally like study design, Sample collection from veterinary hospitals, lab work, Phylogenetic analysis, from drafting data to final manuscript.

Conflict of interest

The authors have declared no conflict of interest.

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