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## Molecular methods for identification of antigenic types of canine parvovirus

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**Abstract**

Canine parvovirus and Feline panleukopenia virus are pathogens which cause acute haemorrhagic enteritis and myocarditis and is a contagious disease with high morbidity and mortality. In this study, a total of 90 faecal samples from live dogs, and tissue samples from 16 dead dogs showing symptoms of CPV and 7 samples from dead cats reported with severe clinical symptoms of FPLV were subjected to Haemagglutination assay, Conventional and Differential PCR. Our results indicates the evidence of CPV-2a and CPV-2b variants circulating among dogs population in Tamil Nadu.

**Keywords:** Canine parvovirus, Dogs, Feline panleukopenia virus, Cats, Differential PCR, Haemagglutination assay

**Introduction**

Canine Parvovirus (CPV) is said to have derived from Feline Panleukopenia Virus (FPLV) with several amino acid mutation (Parrish, 1990 and Osterhans *et al.*, 1980) [23] but other evidence suggested that CPV and FPLV might have evolved from common viral ancestors in wild life (Allison *et al.*, 2013) [1]. The virus was first named as CPV-2 to distinguish it from the unrelated Canine Minute Virus (CMV), (Binn *et al.*, 1970) [2]. In 1978, the original CPV-2 was first identified in canines in the United States and then it quickly became widespread with outbreaks in many countries. The novel antigenic variants viz., CPV-2a, 2b, 2c has been reported worldwide with evolution of the virus.

Initially, FPLV could replicate within the thymus but not within the intestinal tract of dogs and was therefore not shed along with the faeces (Truyen and Parrish, 1992; Truyen *et al.*, 1995) [29, 31] and also original CPV-2 did not infect cats (Truyen *et al.*, 1996) [32]. The highly contagious and principal etiological agent of haemorrhagic enteritis in dogs has been identified as CPV-2. CPV-2 is very similar to FPLV, it has been argued that FPLV had mutated into CPV-2 (Decaro and Bounavoglia, 2012) [11].

However, the CPV-2a, 2b and 2c variants that are naturally isolated from cats could experimentally infect cats by several workers (Truyen *et al.*, 1996 and Nakamura *et al.*, 2001) [32, 17]. Moreover, CPV-2 variants caused up to approximately 80% of cases of FPLV in unvaccinated population (Ikeda *et al.*, 2000) [13]. In addition, the phenomenon of asymptomatic infection by CPV-2 variants in cats has been characterised by Clegg *et al.*, (2012) [6]. Several reports indicated that the original CPV-2 is no longer circulating while the new variants CPV-2a and 2b are variously distributed in canine and feline populations worldwide with no difference in the pathogenesis (Parrish *et al.*, 1988; Mochizuki *et al.*, 1993; Pereira *et al.*, 2000 and Costa *et al.*, 2005) [22, 16, 24].

The purpose of this study was to check the specificity of already published differential PCR primers for detection of variants of canine parvoviruses.

**Materials and Methods**

A total of 90 faecal samples from dogs showing clinical signs like vomiting, foul smelling blood tinged diarrhoea, dehydration, sunken eyes, depression, weakness, etc., (Pollock and Carmichael, 1982; Shashidhara *et al.*, 2009) [25, 28], were from outpatient ward, Madras Veterinary College, Chennai, 16 tissue samples from post-mortem dogs and 7 tissue samples from post-mortem cats were collected from Department of Pathology, Madras Veterinary College, Chennai, Tamil Nadu, during the period from October 2016 to February 2018. The faecal samples were collected by using sterile rectal swabs in phosphate buffered saline (PBS, pH 7.2),

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and stored in -20 °C until further processing. The tissue samples of post mortem cats and dogs were homogenised (Decaro *et al.*, 2006) [9] in phosphate buffered saline (PBS, pH7.2) and the homogenates were frozen and thawed thrice, subsequently clarified by centrifuging at 10000 rpm for 15 minutes. 200 µl of the supernatant was treated with 1 ml of proteinase K buffer and 4 µl of proteinase K (10mg/ml). The DNA was extracted by Phenol: Chloroform: Iso-amyl alcohol method (Walter *et al.*, 2000) [33]. The final DNA pellet was dissolved in 30 µl of nuclease free water and stored at -20 °C.

### Haemagglutination test (HA)

Initial screening of CPV and FPLV samples were done by haemagglutination assay (HA). This test was performed as described by Carmichael *et al.*, (1980) [8]. The samples were serially diluted twofold in PBS (0.2 M) in V- bottom plates. First, 50 µl of PBS was added to each well of the plate. In the first column, 50 µl of sample (faecal suspension) was added. The sample was mixed five times, and 50 µl was transferred to the next well. Each sample was diluted from 1:2 through 1:4,096. Then 50 µl PBS was added to each well. The HA test was performed using porcine erythrocytes (1%) to each well (Senda *et al.*, 1986) [26]. The plate was shaken for 30 seconds. The plates were incubated at 37 °C for 30 minutes and incubated at 4 °C overnight. The results were scored and the HA titre is expressed as a reciprocal of the highest antigen dilution showing complete agglutination. The CPV and FPLV vaccine virus was used as positive control and the faecal samples from healthy dogs and cats were used as negative control.

### Conventional Polymerase Chain Reaction

The extracted DNA from 90 faecal samples and 16 tissue

samples were subjected to conventional PCR using the already published primers CPV F1 and CPV R1 (Mizak and Rzezutka, 1999) [15]. The PCR reaction mixture consisted of 2 µl of the template DNA, 1 µl each of forward and reverse primer (25pmol/µl), 12.5 µl Mastermix (2X) containing Taq DNA polymerase to make the final reaction of 25 µl using nuclease free water, and kept in a thermocycler with 35 cycles of denaturation at 94 °C for 60s, annealing at 55 °C for 60s, elongation at 72 °C for 150s and a final elongation at 72 °C for 10 minutes (Mizak and Rzezutka., 1999) [15]. PCR products were electrophoresed in 1% agarose gel with ethidium bromide at 5 volts/cm along with 100bp and 1Kb Gene Ruler ladder as molecular weight marker (Genie, Bangalore) and it was visualized and photographed using gel documentation system (Mega Bio-print, France).

### Differential PCR

For identifying various antigenic types, CPV-2, 2a, 2b, 2c individual PCR reactions was set up using the already published differential primers (Kumar *et al.*, 2011) [14] in this study. The PCR reaction mixture consisted of 2 µl of the template DNA, 1.0 µl each of forward and reverse primer (25pmol/µl), 12.5 µl Mastermix(2X) containing TaqDNA polymerase to make the final reaction of 25µl using nuclease free water, and kept in a thermocycler with 30 cycles. For the differential PCRs the conditions were unaltered and were same as that of conventional PCR except for the variation in annealing temperature (Table1). PCR products were electrophoresed in 1% agarose gel with ethidium bromide at 5 volts/cm along with 100 bp and 1 Kb Gene Ruler ladder as molecular weight marker (Ge Nei, Bangalore) and it was visualized and photographed using gel documentation system (Mega Bio-print, France)

**Table 1:** Primers for Conventional and Differential PCR

S no	Primer	Sequence	Product Size (bp)	Annealing temperature (°C)	Gene Region
1.	CPV-FP	5'AGCTATGAGATCTGAGACAT-3'	1198	55	VP <sub>2</sub>
	CPV-RP	5'AGTATGTTAATATAATTCTAGGTGC-3'			
2.	CPV-2-FP	5'CTGCTTACTCAGCCACCAACT-3'	719	59	VP <sub>2</sub>
	CPV-2-RP	AGGTGTTCTCCTGTTGTTG-3'			
3.	CPV-2a FP	5'AGAGCATTGGGCTTACCAACC-3'	379	60	VP <sub>2</sub>
	CPV-2a RP	5'ATCTCCTGTATCTTGATGTGCT-3'			
4.	CPV-2b FP	5'TGTATTGCTACCAACAGATCCA-3'	178	59	VP <sub>2</sub>
	CPV-2b RP	5'TGGTGCATTACATGAAGTCTGG-3'			
5.	CPV-2c FP	5'GTGGTCTGGGGGTGTGG-3'	470	60	VP <sub>2</sub>
	CPV-2c RP	5'AGCTGCTGGAGTAAATGGCA-3'			

## Results and Discussion

### Haemagglutination Assay

Out of 106 samples of CPV from dogs and 7 samples of FPLV from cats, 60 samples were positive for CPV and all 7 samples were positive for FPLV by HA test with titres ranging between

1: 16 to 1: 1024. The agglutination results at 4°C for CPV 2a ranged from 16 to 512 and CPV-2b ranged from 16 to 1024 and FPLV ranged from 16 to 512. The number of positive samples in HA test may be due to non-specific agglutination reaction in faeces. The HA activity with CPV-2a, CPV-2b and FPLV occurred at 4°C (high titre up to 1:1024) and at 37°C (low titre 1:16). Temperature dependence was regarded as positive when the value was more than 1:16 (Senda *et al.*, 1988) [27].

The main source of the infection seems to be, the faeces of infected dogs because more than 10<sup>9</sup> virus particle per gram of

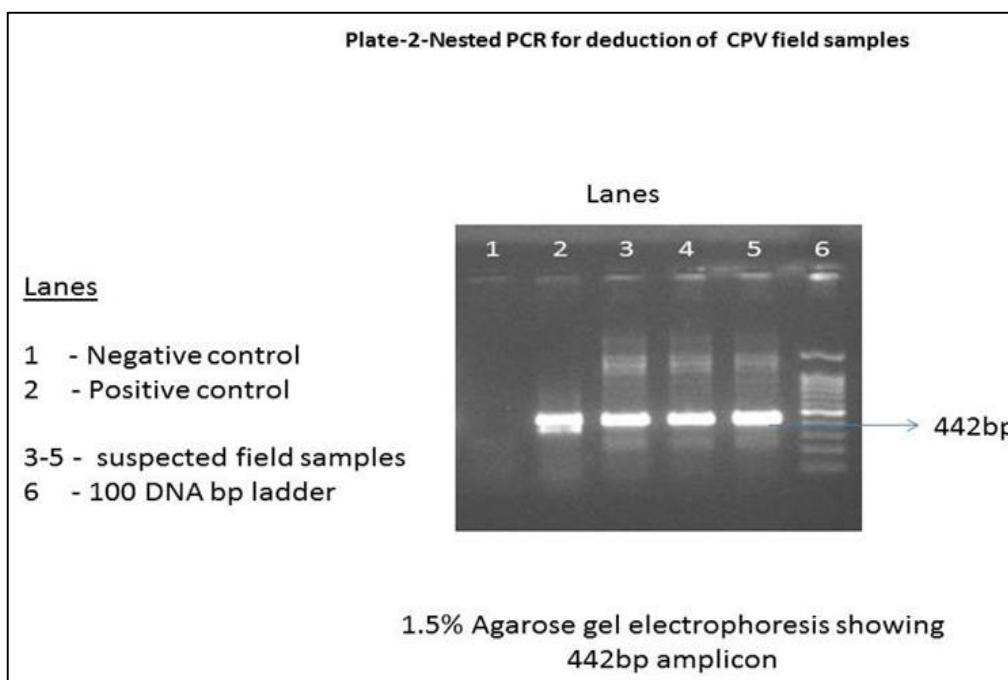
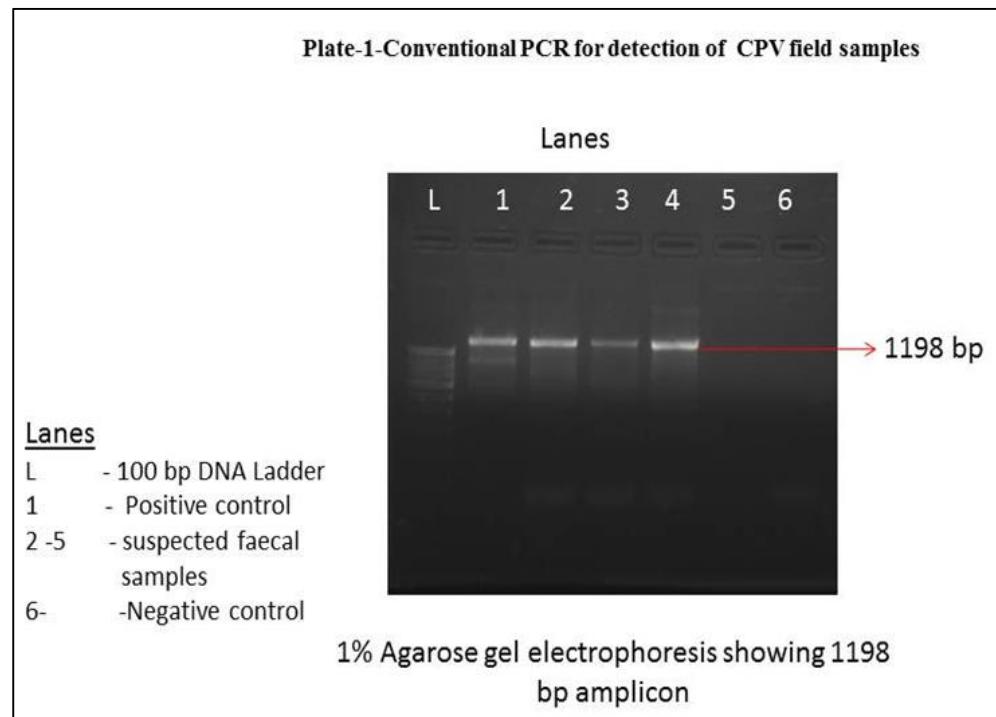
faeces can be shed during the acute phase of the enteric disease. Therefore, faeces are accepted as a suitable material to detect the virus in the enteric form of the disease in live dogs (Carmichael and Binn, 1981) [4] and intestinal rectal tissue in dead dogs and dead cats. HA and HI tests are simple, inexpensive and easy to perform but require RBCs of porcine origin afresh every time. Moreover, the presence of non-specific agglutinin in faeces makes HA test less reliable for CPV diagnosis as reported by Parthiban *et al.*, (2012) [21]. But according to Mochizuki *et al.*, (1993) [16], and Pereira *et al.*, (2000) [24], haemagglutination test for virus diagnosis did not allow distinguish between wild and vaccine strain for virus diagnosis.

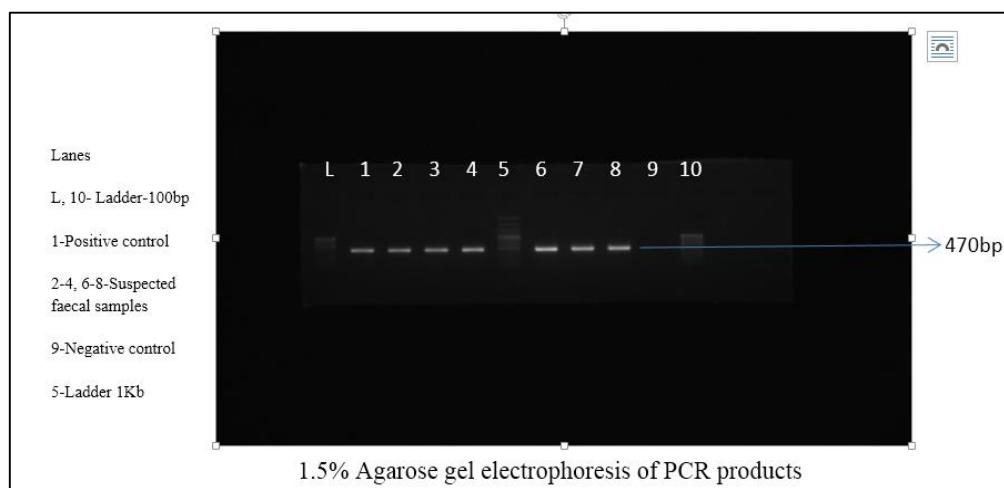
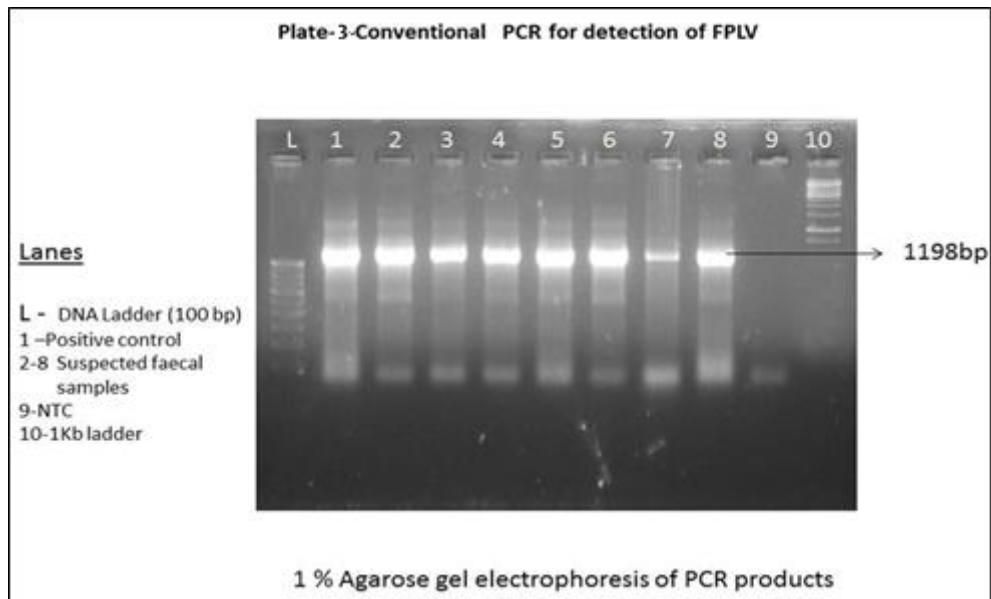
### Polymerase Chain Reaction

On the other hand, PCR is a highly specific assay which avoid non-specific reactions because samples were analysed at

nucleic acid level. In this study, out of 90 samples 73 were positive by conventional PCR and 82 positive by nested PCR, yielding a product size of 1198bp and 442bp respectively. These samples were subjected to antigenic characterisation by differential PCR of CPV with already published primers (Table-1) for identification of the CPV variants such as CPV-2, 2a, 2b and 2c. All the 73 samples were amplified by 2c primers and the expected amplicon size of 470bp was observed. In positive control there was amplification of template DNA whereas, in the negative control no amplification of template DNA was visualised on agarose gel electrophoresis. These samples were further subjected to sequencing to confirm the antigenic variants, it was found that the samples which were amplified with 2c primers with band

size 470bp were of CPV-2a and 2b and both were of 99% homology with other CPV isolates available in the gene. The amplification of template DNA extracted from commercial vaccine was not observed with CPV-2 primers (Table-1) of band size 719bp. When subjected to sequencing to confirm the antigenic variant, it was found that CPV-2 with 97% homology with other CPV isolates available in the gene. Hence, the PCR amplification using differential primers revealed that the already published primers were not specific to differentiate the CPV variants. Therefore, after PCR amplification with conventional PCR, followed by sequencing or high end amplification using Minor groove binding assay by real time PCR may be used as confirmatory assay for identification of antigenic types of canine parvoviruses



**Plate-4 - Differential PCR-CPV-2c**

Manubindi@gmail.com NCBI Blast:Nucleotide Sequi... https://blast.ncbi.nlm.nih.gov/Blast.cgi Apps Prestained Protein Star Hard-ShellB 96-Well PC Probiotic "Bird-Powder" Simple Calculations: Fer... Lactic Acid Bacteria: Mi... Fluid Pressure

Sequences producing significant alignments:

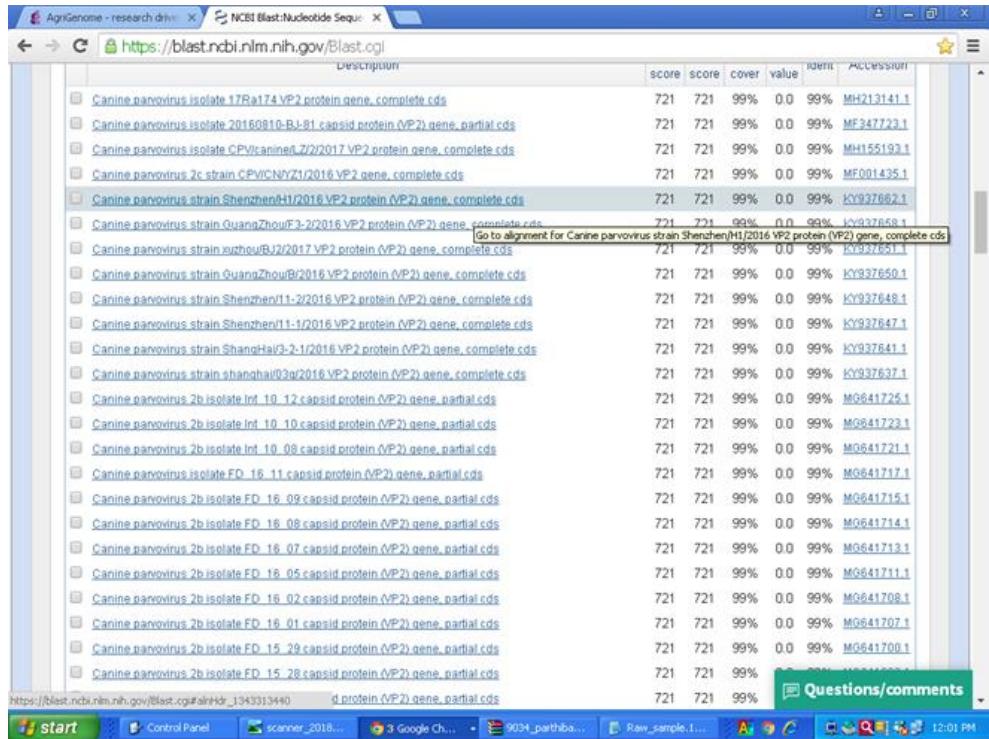
Select: All None Selected 0

Alignments Download GenBank Graphics Distance tree of results

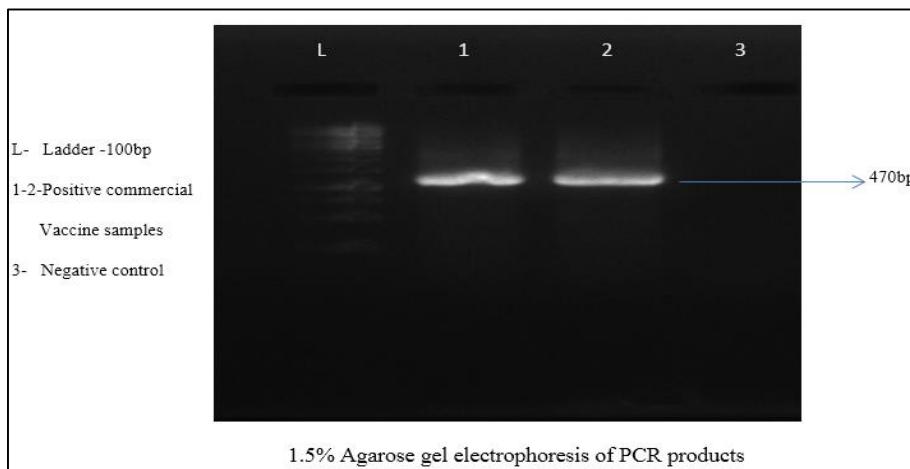
Description	Max score	Total score	Query cover	E value	Ident	Accession
Canine panovirus 2a isolate RJ-7 new CPV-2a/2016/nd VP2 gene_ complete cds	682	682	94%	0.0	99%	KO469435.1
Canine panovirus 2a isolate RJ-2 new CPV-2a/2016/nd VP2 gene_ complete cds	682	682	94%	0.0	99%	KO469434.1
Canine panovirus 2a isolate KN-5 new CPV-2a/2015/nd VP2 gene_ complete cds	682	682	94%	0.0	99%	KO469433.1
Canine panovirus 2a isolate Alk1 new CPV-2a/2012/nd VP2 gene_ complete cds	682	682	94%	0.0	99%	KO469431.1
Canine panovirus 2a isolate KN-1 new CPV-2a/2015/nd VP2 gene_ complete cds	682	682	94%	0.0	99%	KO425922.1
Canine panovirus 2a isolate Kee-2 VP2 gene_ complete cds	682	682	94%	0.0	99%	KO219738.1
Canine panovirus 2a isolate Kee-2 VP2 gene_ complete cds	682	682	94%	0.0	99%	KO219737.1
Canine panovirus 2a isolate TkM-Orissa VP2 gene_ complete cds	682	682	94%	0.0	99%	KO219736.1
Canine panovirus 2a isolate Kee-1 VP2 gene_ complete cds	682	682	94%	0.0	99%	KO219732.1
Canine panovirus 2a isolate YAD23 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983491.1
Canine panovirus 2a isolate YAD22 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983490.1
Canine panovirus 2a isolate YAD21 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983489.1
Canine panovirus 2a isolate YAD18 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983488.1
Canine panovirus 2a isolate YAD15 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983487.1
Canine panovirus 2a isolate YAD14 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983486.1
Canine panovirus 2a isolate YAD13 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983485.1
Canine panovirus 2a isolate YAD12 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983484.1
Canine panovirus 2a isolate YAD11 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983483.1
Canine panovirus 2a isolate YAD99 structural protein gene_ complete cds	682	682	94%	0.0	99%	KU983482.1

0317\_492 (1).zip 0317\_491 (2).zip Show all downloads...

**Fig 1: NCBI Blast Sequence–CPV-2a**



**Fig 2:** NCBI blast sequence-CPV-2b



**Plate 5:** Differential PCR – Commercial vaccines - CPV-2c

	Description	Max score	Total score	Query cover	E value	Ident	Accession
<input type="checkbox"/>	<a href="#">Canine parvovirus 2 strain 388/05-3 capsid protein (VP2) gene, complete cds</a>	1797	1797	94%	0.0	97%	<a href="#">FJ222824.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate VD2 VP2 gene, partial cds</a>	1792	1792	94%	0.0	97%	<a href="#">KP406929.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus strain 2010-BJ-A72 capsid protein (VP2) gene, partial cds</a>	1792	1792	94%	0.0	97%	<a href="#">KF803602.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate vac4 VP2 gene, complete cds</a>	1792	1792	94%	0.0	97%	<a href="#">JN625222.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus 2 Isolate CPV-Cv VP2 gene, complete cds</a>	1792	1792	94%	0.0	97%	<a href="#">FJ432718.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate CPV-128 coat protein VP1, complete cds</a>	1792	1792	94%	0.0	97%	<a href="#">U22186.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus 2 isolate H-25 capsid protein VP2 gene, complete cds</a>	1786	1786	94%	0.0	97%	<a href="#">KF539801.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate vac1 VP2 gene, complete cds</a>	1786	1786	94%	0.0	97%	<a href="#">JN625219.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate CPV-5 us_79 non-structural protein 1, non-structural protein 2, capsid protein 1, s</a>	1786	1786	94%	0.0	97%	<a href="#">EU659116.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate RD-80 coat protein VP1, complete cds</a>	1786	1786	94%	0.0	97%	<a href="#">U22192.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus gene for capsid protein, partial cds, isolate CP49</a>	1786	1786	94%	0.0	97%	<a href="#">D26081.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate CPV-d Cornell #320) capsid protein genes VP1 and VP2, complete cds</a>	1786	1786	94%	0.0	97%	<a href="#">M23255.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus NS-1 protein and capsid proteins VP-1 and VP-2 genes, complete cds</a>	1786	1786	94%	0.0	97%	<a href="#">M38245.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus isolate RD/PV HeB10-3 VP2 protein gene, complete cds</a>	1781	1781	94%	0.0	97%	<a href="#">KJ194463.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus 2 isolate H-2 capsid protein VP2 gene, complete cds</a>	1781	1781	94%	0.0	97%	<a href="#">KF539803.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus 2 isolate HB1 capsid protein (VP2) gene, partial cds</a>	1781	1781	94%	0.0	97%	<a href="#">GU392236.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus 2 strain HB3 capsid protein (VP2) gene, complete cds</a>	1781	1781	94%	0.0	97%	<a href="#">GU392240.1</a>
<input type="checkbox"/>	<a href="#">Canine parvovirus 2 strain HB6 capsid protein (VP2) gene, complete cds</a>	1781	1781	94%	0.0	97%	<a href="#">GU392239.1</a>
<input type="checkbox"/>	<a href="#">canine parvovirus VP2 gene, partial, isolate CPV-Pudel</a>	1781	1781	94%	0.0	97%	<a href="#">AJ002981.1</a>
<input type="checkbox"/>	<a href="#">canine parvovirus VP2 gene, partial, isolate CPV-Chow Chow</a>	1781	1781	94%	0.0	97%	<a href="#">AJ002971.1</a>

**Fig 3:** NCBI blast sequence-Commercial vaccine

Sequence analysis indicated that CPV-2a is the most common variant prevalent during the period from October 2016 to February 2018, although CPV-2b variant was also detected sporadically and it is noteworthy that the variant CPV-2c was not detected in our samples. Dogs and cats are normally vaccinated by the age of 2 months and the present commercial vaccines contain only CPV-2 strain and in all disease outbreaks only CPV-2a and 2b strains were observed. Hence, cross neutralization assay is to be carried out in order to find out the efficacy of the current vaccine. These findings can be used for molecular comparison of vaccine and field strains and it may be helpful in further vaccine development studies, Decaro *et al.*, (2007) [12] and Nandi *et al.*, (2010) [19]. Nevertheless, continuous evolution of CPV requires diagnostic assays based on a single detecting monoclonal antibody be evaluated periodically for their continuous sensitivity against new strains of CPV. Similarly, nucleic acid based tests need to be evaluated continuously to ensure that mutations have not occurred in primer/probe binding regions (Hong *et al.*, 2007) [12]. The usual CPV strains in a region or a country should be identified before selecting a candidate strain of vaccine for controlling the disease in an effective manner ie., by vaccines containing strains matching the antigenic features of the field strains circulating in the local canine population or polyvalent vaccines, could represent an alternative strategy to improve the effectiveness of prophylaxis for CPV-2. (Cavalli *et al.* 2008) [5].

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