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*Original Article*

# Detection and Diagnosis of Strain Variation in Canine Parvoviral Infections in Dogs

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**Abstract**

A total of 10 clinical samples and one known control sample in the form of rectal swabs werecollected from dogs showing signs of diarrhoea. The samples were clarified, processed and subjected to haemagglutination, rapid immunochromatography kit test and polymerase chain reaction assays. Of the 10 samples screened all (100%) were positive for CPV infections. Isolation of virus from the clarified faecal samples was done in both CRFK and MDCKcell lines. With one of the positive faecal sample SDS –Page was carried out. Strain variation was detected of which 10 samples belonged to CPV 2a and one sample belonged to CPV 2b by PCR assay with CPV-2ab primers and 2b primers. Partial sequencing based on VP2 gene was carried out with one of the CPV 2a isolate and phylogenetic tree was constructed to know the homology with other Indian isolates.

**Keywords**: CPV; HA; PCR Assay; Sequencing; Phylogenetic Analysis.

## Introduction

Canine parvovirus disease is one of the most dreadful viral diseases of canines. The virus was first discovered in faeces of normal dogs in 1970 [4] and was called as Minute virus of canines (MVC) / CPV-1. This was the only canine parvovirus (CPV) until the report of a second CPV in 1978 [1] which was termed as CPV-2. CPV-1 was less pathogenic and not serologically related to CPV-2 [5]. Since its emergence in 1978, CPV-2 was well established as enteric pathogen in dogs and several wild carnivore species around the world causing high morbidity and mortality rates. The virus spread globally in a pandemic of disease during 1978,since then it remained as endemic disease in dogs throughout the world.

In India, CPV-2 is widely prevalent and is responsible for severe contagious gastroenteritis, dehydration and immune-suppression especially infecting young pups of two months age group. The virus infects intestinal epithelium leading to crypt necrosis, crypt dilatation and villous atrophy which are diagnostic of CPV infection. The monitoring and early diagnosis of CPV infection from field samples and molecular characterization has become fundamental tools to understand the virus evolution for developing effective preventive measures.

CPV-2 has mutated and spread throughout the world in dog populations and underwent genetic evolution giving rise consecutively to two antigenic variants, CPV-2a and CPV-2b that replaced progressively the original type CPV-2 [11, 17]. In 2000, a new antigenic variant, CPV-2c, was detected in Italy and rapidly spread to several countriesandthisnew variant was distinguishable from CPV 2a/2b by the substitution with Glu in position of Asn/Asp at 426 residues of the VP2 capsid protein and named as Glu mutant. [8, 18]. Additional amino acid difference was observed in both CPV-2a and CPV-2bin German CPV isolates in 1993 at position 297 (Ser to Ala) and was designated as New CPV 2a/2b [23]. The disease condition has been complicated further due to emergence ofnew of variants.

The present study was undertaken with the aim to characterize and compare the efficacy of tests for the effective diagnosis of Canine parvovirus. Preliminary screening of faecal samples infected with CPV was done by haemagglutination test with 0.8% Swine RBC followed by Polymerase chain reaction was employed for detecting the CPV from faecal samples. Molecular diagnostic techniques like PCR were the most reliable technique with high degree of sensitivity and specificity in detecting CPV from fecal samples [10, 13]. Characterization of VP2 gene of CPVfrom field cases will provide the necessary information of existence of new variants of CPV.

## Materials and Methods

A total of tenfaecal samples were collected from dogs suffering from bloody diarrhea and vomitions from various veterinary hospitals located in Hyderabad, Andhra Pradesh and one known isolate as control was obtained from Indian Immunologicals Ltd. Hyderabad.

## Methodology

The faecal samples collected in 0.2M Sorenson's PBS (pH 7.0) were clarified by centrifugation at 3000 rpm for 10 minutes at $4^0$C and the supernatant was screenedfor CPV infection by haemagglutination (HA) test using 0.8% swine RBC. The Presence of CPV antigen in fecal sample was identified by Immuno-chromatography Kit (RapiGENCanine Parvotest kit) [9, 21]. The positive samples in HA with a titre of 1:32 and above were filtered by 0.22µm membrane filters and used for the isolation of CPV in CRFK and MDCK cells lines. The CRFK and MDCK cell lines were inoculated with 0.5ml of the processed fecal sample with 2% MEM (Gibco.) with FBS and passaged for ten times. At each passage, the presence of virus was confirmed by PCR with VP2 gene primers. In CRFK cell lines mild CPE with rounding and aggregation of cells and no significant CPE in MDCK cell lines were noticed. The CPV in CRFK cell lines was purified in10-40% sucrose in TE-EDTA buffer by density gradient centrifugation method at 207000g for 2h in swing out rotor [20]. The Purified virus was subjected to polypeptide analysis in 12%resolving gel by SDS-PAGE. The Polymerase Chain Reaction (PCR) was carried for partial sequencing of VP2 gene using two sets of primers 2ab and 2b [19].

**Table 1:**

| Primer | 5' to 3' direction |
|---|---|
| 2ab-Forward | 5'GAAGAGTGGTTGTAAATAATT3' |
| 2ab-Reverse | 5'CCTATATAACCAAAGTTAGTAC3' |
| 2b-Forward | 5'CTTTAACCTTCCTGTAACAG3' |
| 2b-Reverse | 5'CATAGTTAAATTGGTTATCTAC3'. |

The PCR reaction mix was prepared with 5µl of 10x PCR buffer containing 5µl of $Mgcl_2$ (2.5mM), dNTPS (2.5mM),Taq polymerase (5units/1µl), forward and reverse primer 1µl each, 10 µl of DNA template and 27 µl of nuclease free water.Amplification was carried out with an initial denaturation of $95^0$ C for 5 minutes, followed by 30 cycles of denaturation ($94^0$ C for 30 seconds), annealing ($55^0$ C for 2 minutes), extension ($72^0$C for 2minutes) and then final extension for 4 minutes at $72^0$C. One selected VP2 gene PCR product was sequenced (MS MWG Biotechnologies Pvt. Ltd., Bangalore), analyzed and phylogenetic analysis was carried out using CLUSTAL W software.

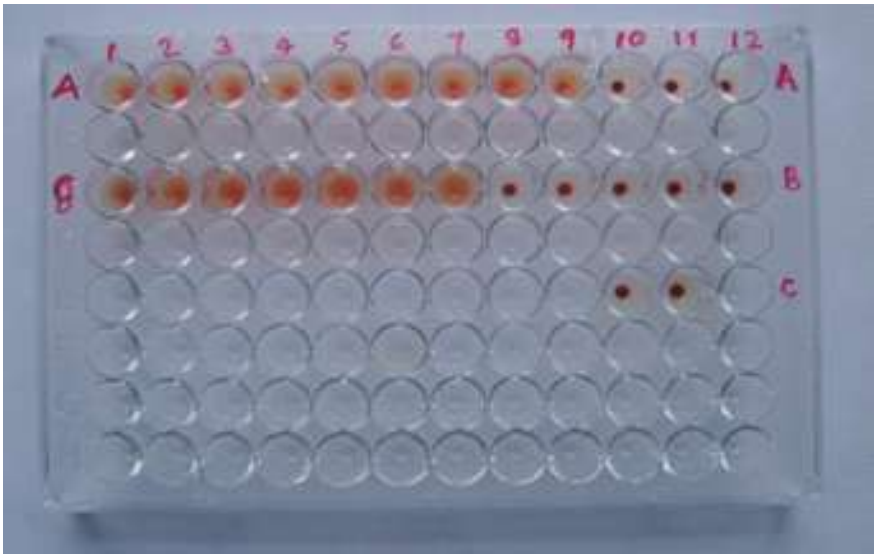
**Table 2:** List of CPV isolates employed for phylogenetic analysis based on VP2 gene sequences.

| Identification code of the isolate | Country | Accession number |
|---|---|---|
| IIL 5 | India | DQ182614 |
| IIL9 | India | DQ182615 |
| IIL10 | India | DQ182616 |
| IIL11 | India | DQ182617 |
| IIL12 | India | DQ182618 |
| IIL14 | India | DQ182619 |
| IIL15 | India | DQ182620 |
| IIL17 | India | DQ182621 |
| IIL19 | India | DQ182622 |
| IIL20 | India | DQ182623 |
| IIL24 | India | DQ182624 |
| IIL25 | India | DQ182625 |
| IIL 27 | India | DQ182626 |
| IIL 28 | India | DQ182627 |
| GR 09/09 | Greece | GQ8655519 |
| 140/05 | Italy | FJ005265 |

**Results and Discussion**

The canine parvoviral infection studies revealed that infection may occur in both vaccinated and unvaccinated pups between age group of 2-6 months. Out of the total 10 affected dogs studied, 6 pups were not vaccinated shown relatively high severity of symptoms when compared with 4 vaccinated pups. The occurrence of parvoviral infection in vaccinated pups may be due to vaccination failure or any strain variation between the vaccine strain and the etiological virus strain. On preliminary screening of the 10 fecal samples by haemagglutination test with 0.8% swine R.B.C., all samples were shown a clear agglutination reaction with a HA titres ranging between 1:32 -1:1024 (Figure 1).The diagnosis of CPV by the rapid immune-chromatography test kit method revealed only four positive out of ten samples (Fig. 2). Here the samples which shown a high HA titre of 1:512 & above were only detected in rapid immune-chromatography method and the low HA titre samples were found to be negative in rapid immune-chromatography suggesting the haemagglutination test as a simple, reliable and least cost effective test for the preliminary screening of the parvoviral infection in canines [6,15]. The rapid immune-chromatography method lacks sensitivity in detection of low parvoviral load in the fecal sample [3]. The positive fecal samples in HA test were further confirmed by PCR primers for VP2 gene.

Fig. 1: Haemagglutination test




A. sample showing a titre of 512
B. sample showing a titre of 128
C. Control

**Fig. 2:** Rapid Immuno-chromatography Test


Above – Positive Below – Negative

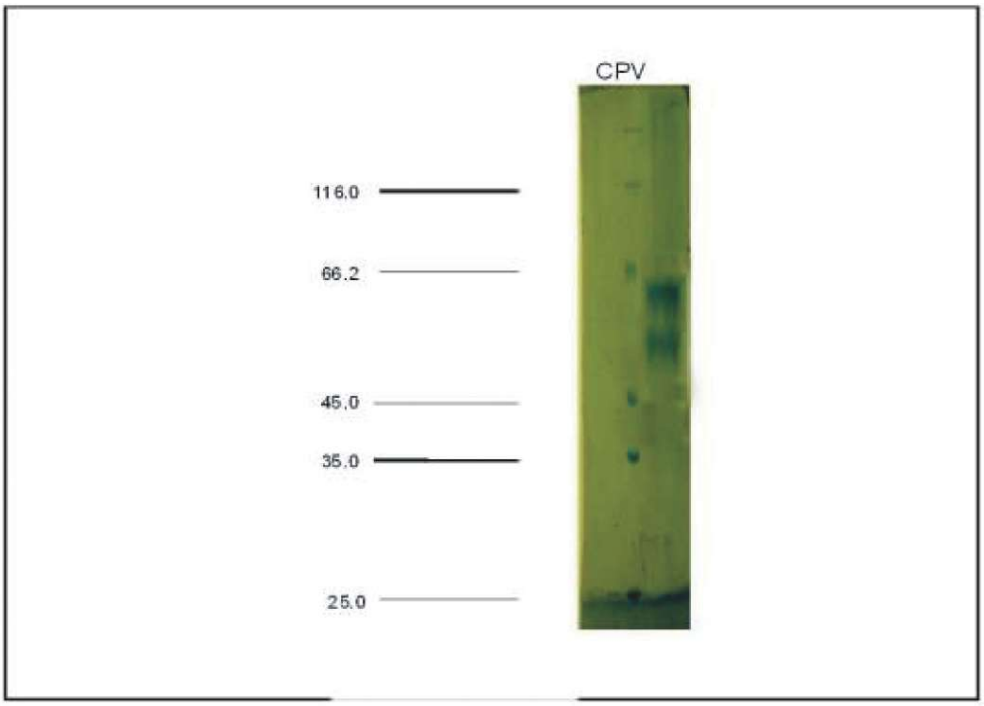
The positive facal samples were filtered through 0.22µm membrane filters and attempts were made to isolate the parvovirus from the ten fecal samples in CRFK and MDCK cell lines. The CRFK cell lines were best suited for the growth and isolation of the virus with the production of good minimal cytopathic effect [22]. The characteristic CPE was observed after 72h of post infection only after the 10$^{th}$ passage as focal rounding and clear aggregation.At each passage level the presence of virus was confirmed by PCR. The virus growth in MDCK cell lines was not significant with any detectable CPE. The CPV growth in MDCK cell lines may be varied depending on the strain of the virus [14]. Moreover the virus can be best studied in CRFK cell lines which are from felines than MDCK cell lines which are from canine origin [2].

The virus isolated from fecal samples in CRFK cell lines was grown in large quantities and concentrated by sucrose density gradient. The band formed at 20% sucrose level was subjected for polypeptide analysis by SDS-PAGE. The viral capsid contains three major proteins VP1 (82Kda), VP2 (65 KDa), VP3 (63 KDa) and VP2 is responsible for attachment, assembly of viral capsid and neutralization. The SDS-PAGE analysis revealed only 2 protein bands at 65 KDa and 62 KDa (Figure 3) The VP1 protein was not significantly observed, which might be due to the low concentration of the protein in the purified virus or the relative concentrations of the polypeptides may varied from batch to batch as reported by *Turiso* [24].

**Table 3:** Comparison of HA, PCR and Rapigen kit for Diagnosis

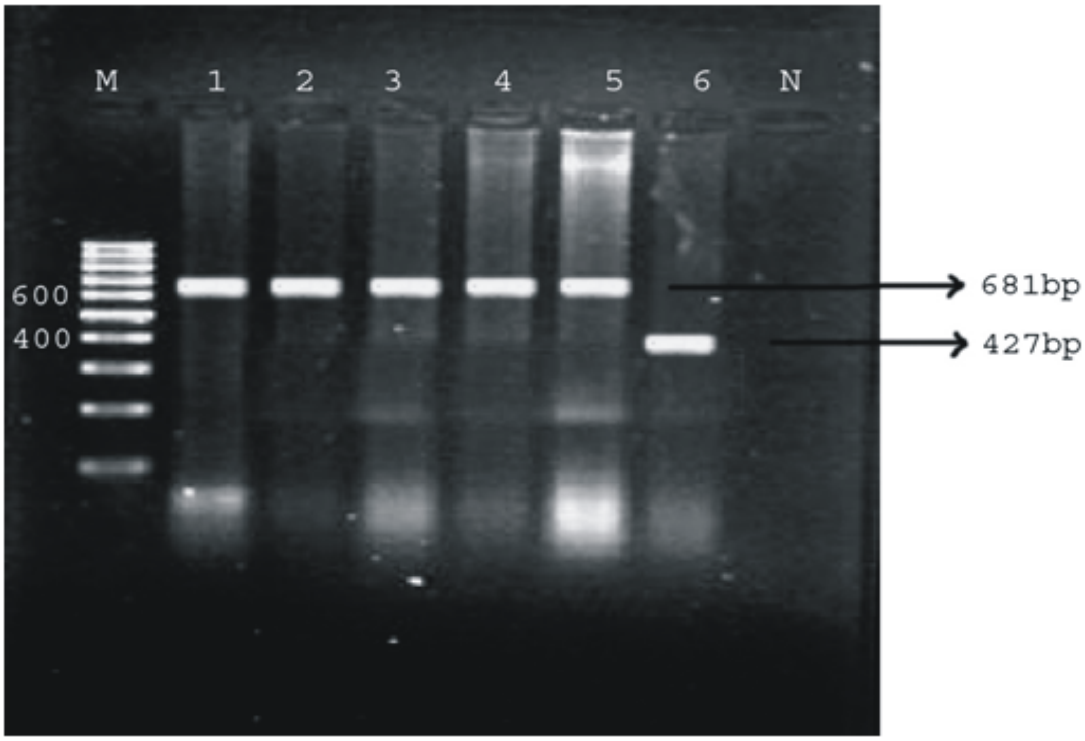
| Sample No. | Results | | |
|---|---|---|---|
| | Haemagglutination test | PCR assay | Rapigen Kit Test |
| 1 | 32 | + | - |
| 2 | 32 | + | - |
| 3 | 512 | + | + |
| 4 | 64 | + | - |
| 5 | 1024 | + | + |
| 6 | 32 | + | – |
| 7 | 64 | + | – |
| 8 | 512 | + | + |
| 9 | 128 | + | - |
| 10 | 512 | + | + |

**Fig. 3:** Polypeptide profiles of CPV in SDS-PAGE gel
Two bands of CPV isolate visible with molecular weights 65KDa and 62 KDa



The CRFK infected cell culture fluid was directly used for amplification of Canine parvoviral DNA. The avoidance of DNA extraction step from the infected cell lines was more economical and time saving. Two sets of primers, one specific for VP2 gene of both CPV2 a and b strains (681bp product size) and another set for VP2 gene of CPV2b strain (427 bp product size) were used in PCRfor detection of the strain variation parvovirus [Panda].All the ten samples were amplified with CPV2ab primers with a VP2 gene product size of 681 bp. But out of ten samples, one sample was successfully amplified with the CPV 2b set of primers with a product size of 427bp. The control sample was amplified only with CPV 2ab set of primers with amplicon size of 681bp. The use of two different sets of primers for detection of strain variations in CPV infections was most succeeded in the present study. It detects the presence of CPV 2b strain in one sample out of the ten (figure 4).The past reviews emphasizing that the most prevalent strain of CPV in India was CPV2a. The incidence of high CPV2a strain infections in canines, out of ten, 9 samples were detected as CPV2a, strongly correlates with the previous reports. [7, 17].

**Fig. 4:** Polymerase Chain Reaction




M - Marker (100 bp)

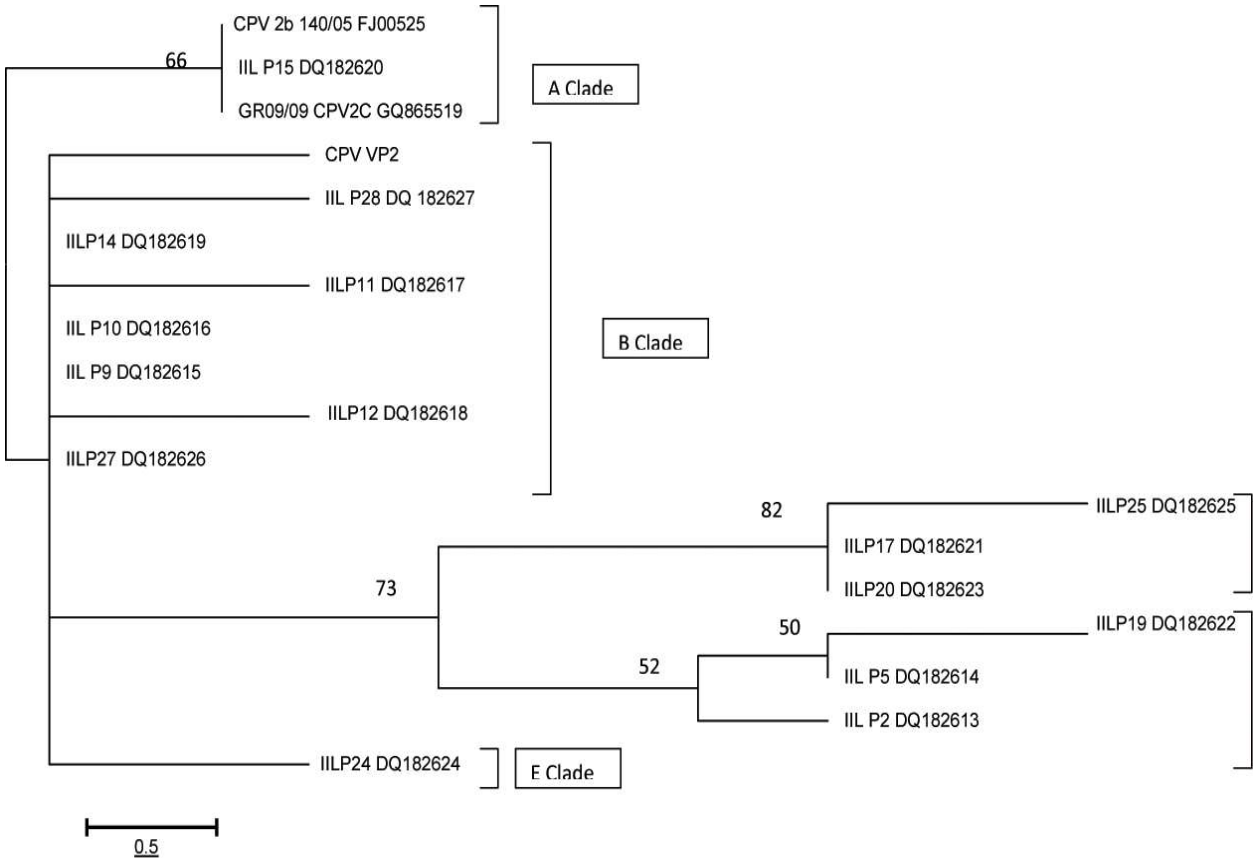
1 to 5- Samples positive for CPV 2a strain with CPV- 2ab primer (681bp)

6 - Sample positive for CPV 2b strain with CPV-2b primer (427bp)

N - Negative control

One of the isolated CPV2astrain's VP2 gene was partially amplified by specific primers with a PCR product size of 681 bp and was sequenced for further analysis.The Phylogenetic analysis was carried out and a phylogenetic tree was constructed using MEGA 4.0 software with the available CPV VP2 gene sequences of Indian isolates and two European isolates.A total of five different clades were formed (figure 5) and the tree emphasizing that the isolated CPV2a strain was fallen under clade B, along with other Indian isolates [7,12,16]. The European isolates were fall under a separate clade – A. However clade A contained both Indian and European isolates probably because phylogenetic analysis was based on partial VP2 sequence and not on complete sequence.

**Fig. 5:** The Partial nucleotide sequence of VP2 gene of CPV isolate




*The Partial nucleotide sequence of VP2 gene of CPV isolate*

5′ACAAATTGTAACACCTTGGTCATTGGTT GATGCAAATGCTTGGGGAGTTT GGTTTAATCCAGGAGATT GGCAACTAATTGTTAATACTAT GAGTGA GTTGCATTTAATTAGTTTTGAACAA GAAATTTTTAATGTTGTTTTAAAGAC TGTTTCAGAATCTGCTACTCAGCCA CCAACTAAAGTTTATAATAA TGATTTAACTGCATCATTGATGGTTGCATT AGATAGTAATAATACTATGCCATTTAC TCCAGCAGCTATGAGATCTGA GACATTGGGCTTTTATCCATGGAAACCAACCA TACCAACTCCATGGAGATATTATTTTCAATG GGATAGAACATTAATACCATCTCA TACTGGAACTAGTGGCAC ACCAACAAATATATACCA TGGTACAGATCCAGATGA TGTTCAATTTTATACTATTGA AAATTCTGTGCCAGT ACACTTACTAAGAAC AGGTGATGAATTTGC TACAGGAACATTTTTTTTT GATTGTAAAC CATGTAGACTAACACATA CATGGCAAACA AATAGAGCAT3′

## Summary

The Clinical diagnosis of CPV infection in dogs was based on symptoms like fever, gastritis, haemorrhagic enteritis is quite suggestive to collect right samples for isolation or for molecular diagnosis. HA with swine RBC is an economical test for diagnosis of CPV infection and Rapigen kit test is less sensitive when compared to HA or PCR for the diagnosis of CPV infection. CPV can be isolated from fecal samples more preferentially in cells of feline origin than in cells of canine origin. By using two sets of primers the strain variation was diagnosed and CPV2a strain was detected as the most predominant strain in India. On phylogenetic analysis, it detected five distinct clades of which the isolated canine parvovirus was more genetically

related with that of the other seven CPV isolates of Indian origin.

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