RESEARCH ARTICLE
Open Access

Journal of Veterinary and Animal Sciences
ISSN (Print): 0971-0701, (Online): 2582-0605
https://doi.org/10.51966/jvas.2024.55.1.178-183

Molecular analysis of partial VP-2 gene amplified from rectal swab samples of diarrheic dogs in Kerala confirms the circulation of canine parvovirus genetic variant CPV-2c#

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Citation: Krishna, D.L., Bipin, K.C., Deepa, P.M., Rathish, R.L., Sebastian, R. and Asaf, M. 2024. Molecular analysis of partial VP-2 gene amplified from rectal swab samples of diarrheic dogs in Kerala confirms the circulation of canine parvovirus genetic variant CPV-2c.
J. Vet. Anim. Sci. **55**(1):178-183
DOI: https://doi.org/10.51966/jvas.2024.55.1.178-183

Received:13.10.2023 *Accepted: 29.12.2023* *Published: 31.03.2024*

Abstract

Canine parvovirus enteritis is caused by canine parvovirus -2 (CPV-2), which is a highly contagious disease, characterized by vomiting, foul-smelling bloody diarrhoea and myocarditis in young dogs. In the present study, a total of 50 faecal samples were collected from dogs with the symptoms of haemorrhagic gastroenteritis from hospitals under Kerala Veterinary and Animal Sciences University to study the molecular epidemiology of the CPV-2. The samples were examined for parvovirus by using VP2 gene-specific PCR for parvovirus. Forty-one (82 per cent) dogs were positive for CPV-2 by PCR assay. DNA sequencing was done for five PCR-positive samples, out of which four were characterized as CPV-2c, indicating that CPV type 2c is currently circulating in India. The partial VP2 analysis showed that these CPVs are closely related to Asian-CPV-2c, with unique amino acids at positions 297A, 324I, 370R and 426E. The remaining one was characterised as the new CPV-2a. Incidence of CPV-2c is the first report in Kerala.

Keywords: *Canine parvovirus (CPV), CPV-2c, new CPV-2a strain, sequence*

 # Part of M.V.Sc thesis submitted to Kerala Veterinary and Animal Sciences University, Pookode, Wayanad, Kerala

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Canine parvoviral infection is a highly contagious and life-threatening disease characterized by haemorrhagic gastroenteritis and myocarditis. *Canine parvovirus type-2* (CPV-2) has been identified as a major pathogen in domestic dog populations, causing significant morbidity and mortality, especially in young unvaccinated pups (Appel *et al*., 1979).

In the late 1970s, *canine parvovirus type 2* (CPV-2) emerged and quickly spread worldwide. The most probable hypothesis behind the emergence of CPV is that it emerged as a host range variant from feline parvovirus (FPV). Canine parvovirus enteritis (PVE) is caused due to three mutations in canine parvovirus type 2 (Shruti and Ajay, 2023). CPV-2 constantly changes its genetic and antigenic character through regular mutations in the VP2 gene. Between 1979 and 1985, the original CPV-2 was replaced by two new types, *viz*., CPV-2a and CPV-2b. Another antigenic variant, CPV-2c, with an amino acid substitution at Asp 426 to Glu, was first reported in Italy and has since been identified in various countries, including India (Decaro *et al*., 2006; Perez *et al*., 2007; Streck *et al*., 2009 and Nandi *et al*., 2009). Additionally, both CPV-2a and CPV-2b exhibited an amino acid change at position 297 (Ser to Ala), and were classified as "new CPV-2a" and "new CPV-2b." (Martella *et al*., 2005). It has been reported that CPV-2a is the major antigenic variant of CPV-2 present in India, followed by CPV-2b (Thomas *et al*., 2017).

Materials and methods

Rectal swab samples were collected from dogs with haemorrhagic or watery diarrhoea, vomiting, fever and dehydration, which were brought to Veterinary Hospitals at Pookode, Mannuthy, Kakkavayal and Kokkalai, under Kerala Veterinary and Animal Sciences University. Sterile rectal swabs were used for the collection of samples and after collection, the swabs were immersed in sterile phosphate-buffered saline (PBS, pH 7.2). The faecal samples immersed in PBS were clarified by centrifuging at 3000 x g for 10 min in a cooling centrifuge. The supernatant of samples was collected and stored at -20° till further use.

For parvovirus identification, viral DNA was extracted using the conventional Phenol – Chloroform method. The viral DNA was stored at –20°C until use. PCR assay for parvovirus identification was conducted as previously described (Buonavoglia *et al*., 2001). Specific primer pair for VP2 gene H for: 5′-CAGGTGATGAATTGCTACA-3′ and Hrev: 5′- CATTTGGATAAACTGGTG GT-3′, located at positions 3556– 3575 and 4166–4185 of CPV-2, respectively, were used to amplify 630 bp fragment of the capsid protein gene (VP2) (Buonavoglia*et al*., 2001). The PCR condition was set as initial denaturation step at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 30 sec, annealing at 56°C for 30 sec and extension at 72°C for 1min and final extension at 72°C for 10 min. The PCR was performed in BIORAD® thermal cycler. All the reactions were carried out in a volume of 25µL in 0.2 ml PCR tubes. The reaction mixture contains PCR master mix (12.5µL), forward primer (FP) (1.0 µL), reverse primer (RP) (1.0 µL), nuclease-free distilled water (7.5 µL) and template DNA (3.0 µL). A single-step polymerase chain reaction (PCR) was performed to amplify viral DNA from clinical specimens. The PCR amplified products were run on 1.0 per cent agarose gel containing ethidium bromide in Tris-acetate EDTA (TAE) buffer and then visualized under UV transilluminator.

Representative amplicons obtained were further subjected to purification and DNA sequencing. DNA nucleotide sequence determination was carried out by the sequencing service, Genespec, Kochi with the same primers that were used for amplification. Automated Sanger dideoxy nucleotide sequencing method was used for bidirectional sequencing. The nucleotide sequences were edited using BioEdit application and EditSeq (DNA Star) The nucleotide sequences thus obtained were further aligned with prototype CPV strains using CLUSTALW2 multiple sequence alignment program in order to identify the nucleotide variations of the VP2 gene and then translated into amino acid sequences for further alignment**.** Finally, the amino acid mutations at the key sites were predicted. The global sequence comparisons were performed using basic alignment search tool (BLAST)

hosted by the National Centre for Biotechnology Information (NCBI).

Results and discussion

Molecular characterisation of CPV-2 variants

During the study period, a total of 50 faecal samples were collected from dogs with gastroenteritis. Forty-one (82%) out of the 50 samples were confirmed positive for CPV with amplification of a fragment of 630 bp size (Fig.1). A total of five samples positive for CPV by PCR were selected for genetic characterisation and partial VP2 gene (Fig.2).

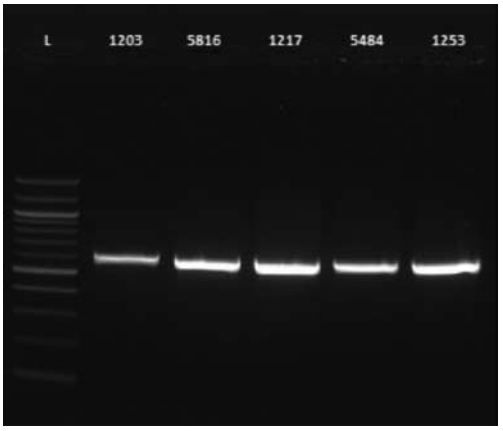

Fig. 1. Agarose gel electrophoresis of PCR products targeting *VP2* gene using CPV-2H primer

Lane 1: 1000 bp Ladder; Lane 2-5: Positive samples for CPV 2 PCR product showing 630 bp size

Antigenic types of CPV-2 as CPV-2c and CPV new 2a were identified in the present study. For CPV typing, the differences in the amino acid 426 were analysed (Martella *et al.,* 2006). A total of four, (two properly vaccinated and two unvaccinated) samples were sequenced, which revealed the presence of a glutamic acid at position 426 of the VP2 protein, which was identified as CPV-2c. The remaining samples revealed an amino acid asparagine at position 426 and hence classified as CPV-2a. The CPV-2a variant detected in this study had an Alanine at amino acid position 297 instead of serine indicating that they were "new CPV-2a" types. No CPV-2 and CPV-2b variants were detected among the sequenced CPV samples.

Additional substitutions were detected in the sequenced type 2c CPVs at amino acid position 370(Q370R) and 324(Y324I). In addition, the new 2a strain harboured amino acid Isoleucine at position 324 instead of Tyrosine (Y324I) and amino acid Alanine at position 440 instead of Threonine (T440A).

Sequence similarity searches of the CPV sequences obtained in the study using BLAST analysis revealed that the sequences were highly specific to CPV, as indicated by the maximum identity (99-100 per cent) obtained with VP2 gene sequences of other CPV strains available in the GenBank.

The amino acid change Tyr324Ile was noticed in all the sequences analysed in this study. Similar codon changes at amino acid positions 324 were also reported in the CPVs isolated in different countries (Zhang *et al.* 2010; Dogonyaro *et al.* 2013; Mittal *et al.* 2014; Mukhopadhyay *et al.* 2017; Csagola *et al.* 2014 and Nookala *et al.* 2016). Horiuchi *et al.* (1994) revealed that residue 324 was prone to strong positive selection in all carnivorous CPV isolates. The residue 324 was adjacent to residue 323, responsible for TfR binding, and together with residue 93, determined the canine host range. Therefore, mutation at amino acid residue 324 is likely to have an impact on the parvovirus host range (Hueffer and Parrish, 2003).

Additional substitutions were detected in the sequenced type 2c CPVs at amino acid position 370(Q370R). Thus, the present CPVs identified are closely related to Asian-CPV-2c with unique amino acids at position 297A, 370R and 426E of VP2, suggesting predominant Asian-CPV-2c in the country. Unique amino acid substitutions at positions Y324I and Q370R were only observed in Asian strains of CPV-2c (Charoenkul *et al.*, 2019). Mutation Q370R first appeared in CPV-2a isolated from giant pandas in Sichuan, China and subsequently became the dominant mutation site of CPV-2c. This mutation may affect the host range and interaction between the host DNA and VP2 protein (Guo *et al.* 2013).

Another important amino acid change Thr440Ala was observed in CPV-2a type. The

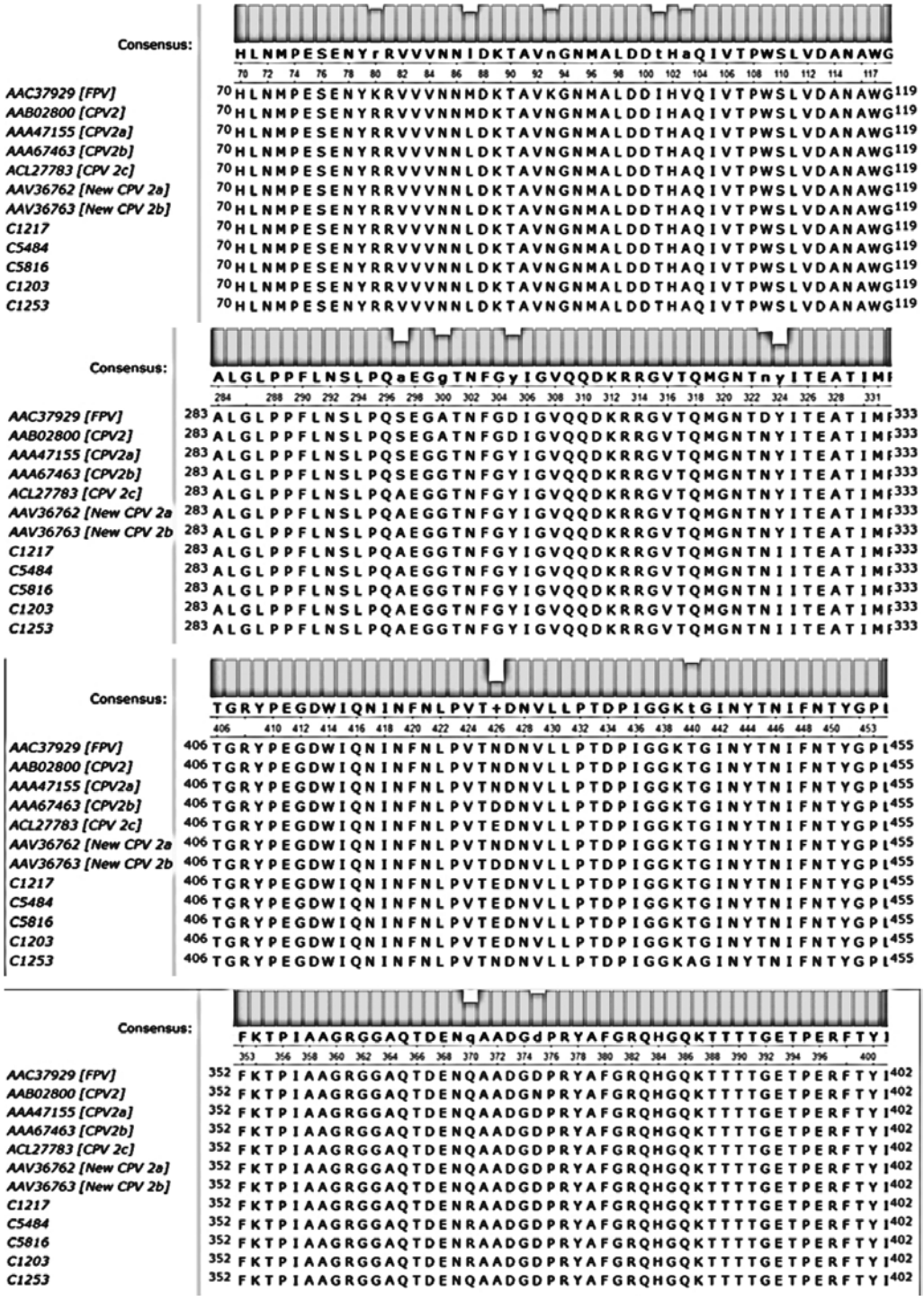

Fig.2. CLUSTALW 2.1 Multiple Sequence Alignment of CPV Sequences under Study with the Prototype CPV Strains in the Database

amino acid residue 440 is important because it is located at the top of the 3-fold spike (GH loop) of the VP2 protein on the surface of the capsid, the main antigenic site of the virus (Battilani *et al.* 2002; Tsao *et al.* 2008). This residue has undergone positive selection for the past few years and has evolved independently in different populations (Decaro *et al.* 2007). Similar codon

changes at amino acid position 440 were also reported in CPVs isolated in different countries (Castro *et al.* 2010; Mukhopadhyay *et al.* 2017; Mittal *et al.* 2014; Nookala *et al.* 2016).

Conclusion

Although the sample size in the present study is smaller, it is important to note that CPV-2c mutants have evolved to emerge as pathogens of dogs in India. The occurrence of CPV-2c antigenic variant has been reported from northern and southern parts of India (Nandi *et al*., 2010; Surendhar *et al*., 2019). Larger epidemiological studies are needed to establish the current epidemiological profile and prevalence of antigenic variants in Kerala.

Acknowledgement

The authors wish to place on record their sincere gratitude to Kerala Veterinary and Animal Sciences University for the facilities provided and funds granted for the research.

Conflict of interest

The authors declare that there is no conflict of interest in publishing this paper.

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