RESEARCH ARTICLE Indian Journal of Animal Research, Volume 59 Issue 1: 120-125 (January 2025)

Molecular Characterization and Phylogenetic Analysis of CPV-2 from Diarrheic Dogs in and Around Nagpur

V.A. Dhas[1], S.R. Warke[1], S.A. Ingle[1], Mehak Tikoo[1]

10.18805/IJAR.B-5305

ABSTRACT

Background: *Canine Parvovirus* (CPV) is a major viral infection that results in acute hemorrhagic gastroenteritis and myocarditis in puppies aged 3-4 months. Despite successful vaccination, it produces significant morbidity and mortality. Inadequate immunity and current kennel practices such as early weaning have predisposed the young canines to CPV. The present study aimed to characterize different antigenic types of CPV-2 in Nagpur region.

Methods: In the current investigation, 50 fecal samples were obtained from dogs suspected of having CPV-2 from 2020-2023 in Nagpur, India and subjected to various detection tests such as haemagglutination test, polymerase chain reaction (PCR), Nested PCR (NPCR) followed by VP2 gene sequencing and phylogenetic analysis.

Result: A total of 9 (18%) of the 50 samples tested positive for the haemagglutination test, while 19 (38%) and 29 (58%) tested positive for conventional PCR and NPCR. NPCR was found to be the most sensitive detection tool of all of them. Sequence analysis found 99.85%-100% similarity with CPV-2b and 2c antigenic type sequences from China and 99.79% identity with sequences from Mizoram state of India. CPV-2b and CPV-2c were the major antigenic types circulating in Nagpur, India. This study will help us to understand the circulating antigenic types of CPV-2 in Nagpur region and promote the formulation of novel diagnostics and vaccines.

Key words: Canine Parvovirus, Haemagglutination test, NPCR, Phylogeny, Polymerase chain reaction (PCR), Sequencing.

INTRODUCTION

Canine Parvoviral gastroenteritis, caused by *Canine parvovirus* -2 (CPV-2) is a highly contagious viral disease that primarily causes hemorrhagic gastroenteritis and myocarditis in puppies under the age of six months (Kaur *et al.,* 2014; Sheikh *et al.,* 2017). This disease is caused by the smallest known viruses belonging to the genus *Protoparvovirus* of the family *Parvoviridae* (Cotmore and Tattersall, 1990). CPV is a non-enveloped icosahedral virion with a linear, non-segmented, negative sense, single-stranded DNA genome of 5323 bases. It is comprised of two Open Reading Frames (ORFs) that encode two structural proteins, VP1 and VP2, as well as two non-structural proteins, NS1 and NS2. The VP2 is regarded as a key capsid protein involved in the regulation of antigenicity and host range. CPV was first discovered in mid-1978 as an emerging pathogen, it was referred to as CPV-2 to distinguish it from CPV-1 (Appel, 1978; Appel *et al.,* 1979; Parrish and Carmichael, 1983). In the Indian scenario, Balu and Thangaraj originally reported the presence of CPV-2 in Madras, India and Ramadass and Khader were the first ones responsible for its isolation (Balu and Thangaraj, 1981; Ramadass and Khader, 1982). *Canine parvovirus* accounts for 27% of all diarrheic infections in dogs (Raj *et al.,* 2010).

Substitution of some amino acids has contributed to mutations in CPV which is the major cause of the emergence of different antigenic variants of CPV (Phromnoi *et al.,* 2010; Kaur *et al.,* 2016). So far, four CPV antigenic variants have been documented around the world including CPV-2, CPV-2a, CPV-2 b and CPV-2c. In some recent studies, CPV-2c antigenic variant has been noted as the cause of *Canine Parvovirus* illness in pups (Magar *et al.,* 2020). As the knowledge concerning the various antigenic variants of CPV causing gastroenteritis in India is still evolving, the extensive research conducted to analyze the prevalence and sequencing of the VP2 gene for ascertaining variable mutations may help in the identification of possible new strains of CPV in a specific selected region. Thus, the present study was designed in a manner to detect the prevailing antigenic types of CPV in the Nagpur region of Maharashtra.

[1]Department of Veterinary Microbiology and Animal Biotechnology T and R cell, Maharashtra Animal and Fishery Sciences University, Nagpur-440 006, Maharashtra, India.

Corresponding Author: S.R. Warke, Department of Veterinary Microbiology and Animal Biotechnology T and R cell, Maharashtra Animal and Fishery Sciences University, Seminary Hills, Nagpur-440 006, Maharashtra, India.
Email: shubhangi_warke@rediffmail.com

How to cite this article: Dhas, V.A., Warke, S.R., Ingle, S.A. and Tikoo, M. (2025). Molecular Characterization and Phylogenetic Analysis of CPV-2 from Diarrheic Dogs in and Around Nagpur. Indian Journal of Animal Research. 59(1): 120-125. doi: 10.18805/IJAR.B-5305.

Submitted: 17-01-2024 **Accepted:** 24-05-2024 **Online:** 27-06-2024

MATERIALS AND METHODS

Sample collection, haemagglutination test and DNA extraction

The study was conducted during the time-period of September 2020-March 2023. A total of 50 canine fecal

samples were collected from clinics throughout Nagpur, with clinical indicators such as vomiting, high temperature, hemorrhagic gastroenteritis and anorexia. The fecal samples were emulsified in 1 mL of 0.1 M phosphate-buffered saline (pH 7.2) and the solution was centrifuged at 10,000 × g for 10 minutes to remove cellular debris. The supernatant was collected and kept at -20°C until further investigation. The previously collected samples were then screened using a haemagglutination test in accordance with the OIE rules. The DNA was isolated from fecal samples according to the manufacturer's instructions using a DNA extraction kit (HiMedia DNA Extraction Kit). DNA was also extracted from a rectal swab sample of a healthy dog and used as a negative control whereas DNA extracted from a commercial Vanguard® Plus5 vaccine procured from a local drug store, in Nagpur, Maharashtra was used as the positive control.

Conventional PCR

The polymerase chain reaction was performed using the method and primers described by Sheikh *et al.* (2017). The VP2 gene was amplified by PCR using the primers forward 5′-GAAGAGTGGTTGTAAATAATT-3′ and reverse 5′CCTATATAA CCAAAGTTAGTAC-3′. A 25 µl reaction mixture containing 12.5 µl master mix, 2 µl each forward and reverse primer (20 Mm), 8.5 µl nuclease-free water and 2 µl template was used for PCR amplification. Initial denaturation at 94°C for 5 minutes was followed by 30 cycles of denaturation at 94°C for 1 minute, annealing at 50°C for 2 minutes, extension at 72°C for 2 minutes and a final extension step of 10 minutes at 72°C. All PCR products were run on a 1% agarose gel and visualized under a UV transilluminator (Syngene G box, UK) in a gel documentation system.

Nested-PCR

The primers used for Nested polymerase chain reaction (NPCR) were as follows: forward 5′- ATACAGGAAG ATATCCAGAAG-3′ and reverse 5′-AGTATGTTAATATAAT TTTCTAGGTGC-3′ (Mizak and Rzezutka, 1999). NPCR reaction was set up by adding 5 µl of the PCR product (from the previous reaction), 2.5 µl of 10X PCR buffer (with 15 mM $MgCl_2$), 1.0 µl each of forward and reverse primer (25 pm/l), 1.0 µl of dNTPs (10 Mm each), 0.5 µl $MgCl_2$ (50 Mm), 1U Taq DNA polymerase and the final volume was made up to 25 µl by adding nuclease-free water (Kaur *et al.,* 2014). All PCR products were run on a 1% agarose gel and visualized under a UV transilluminator (Syngene G box, UK) in a gel documentation system.

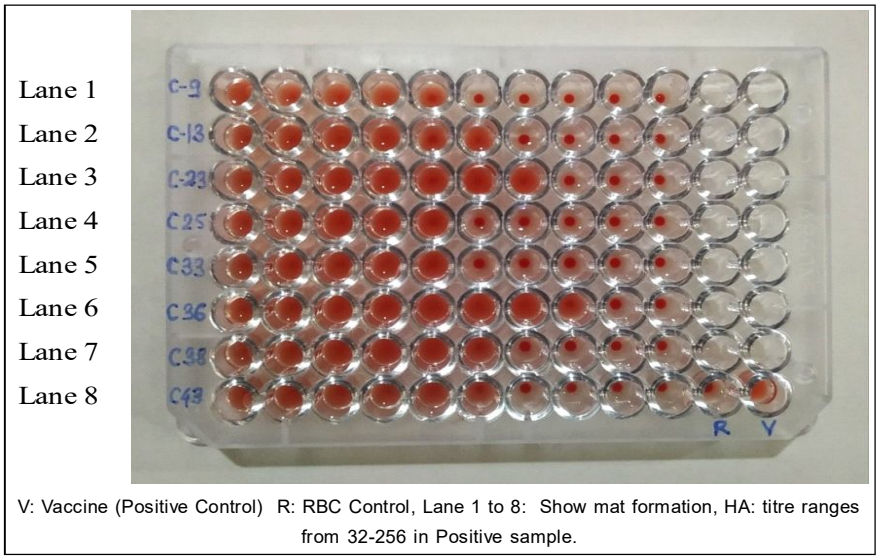
Sequencing and phylogenetic analysis

A total of 2 PCR products with amplicon size (681 bp) and (548 bp) each was subjected to purification using the QIAquick PCR Purification Kit (Mfg. Qiagen Inc, MD, USA) according to the manufacturer's instructions and submitted for sequencing (Eurofins Genomic (India) Pvt. Ltd. Bangalore). The sequences retrieved were then analyzed and compared with the CPV sequences available from GenBank using NCBI BLAST (http:// blast.ncbi.nlm.nih.gov/ blast.cgi) and ClustalW (https://www.ebi.ac.uk/Tools/msa/ clustalw2/). Systematic phylogenetic research was conducted, with a focus on CPV-2 sequences from India and nearby countries (Srinivas *et al.,* 2013). Phylogenetic analysis of both sequences under study was done using MEGA X software (Kumar *et al.,* 2018).

RESULTS AND DISCUSSION

Haemagglutination test

Out of 50 fecal samples screened by HA test, 09 (18%) samples agglutinated porcine RBCs with a titer ranging from 32-256 (Fig 1). Our findings agreed with Nair, (2022) who had screened a total of 50 samples by HA test, of them 05 (10%) samples agglutinated porcine RBCs with a titer ranging from 32-256. In a similar study by (Kumari *et al.,* 2020).



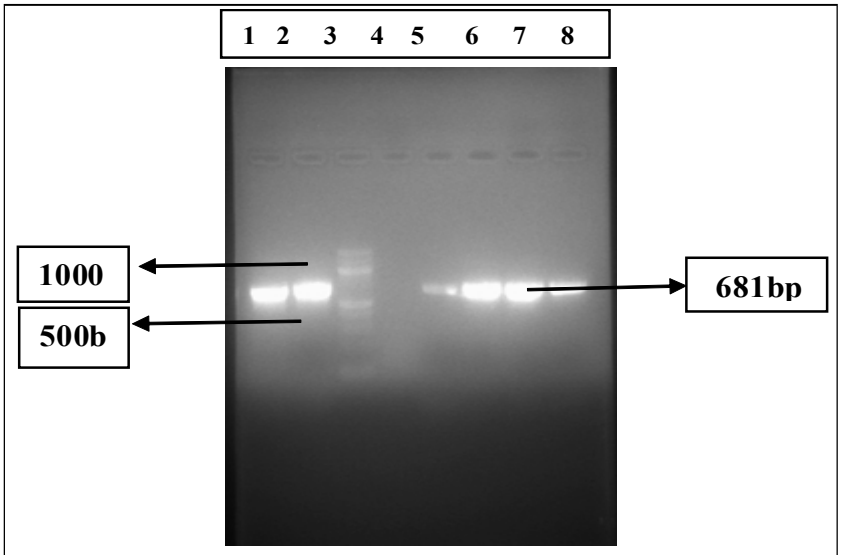
V: Vaccine (Positive Control) R: RBC Control, Lane 1 to 8: Show mat formation, HA: titre ranges from 32-256 in Positive sample.

Fig 1: Haemagglutination test of CPV with pig RBCs.

HA test was conducted on 342 samples and found that 71 (20.76%) of the samples agglutinated pig RBC with HA titers ranging from 1 in 32 to 1 in 512.

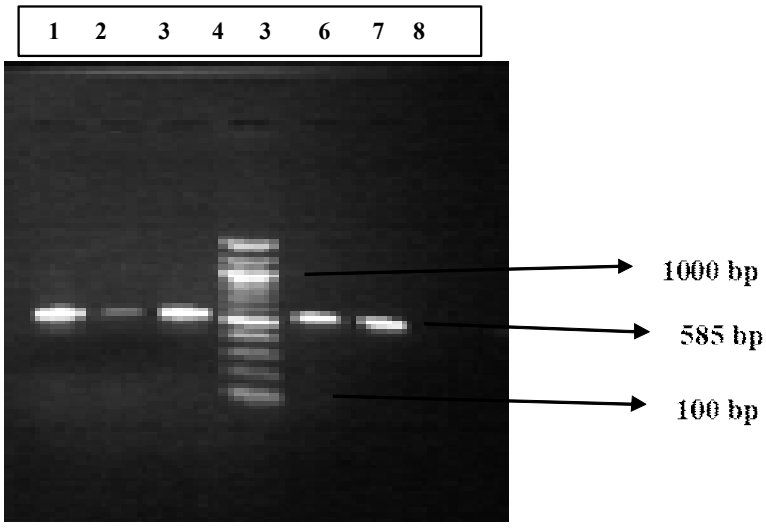
PCR and NPCR

Out of the total 50 samples subjected to PCR and NPCR, 19 (38%) samples were found positive by PCR and produced an amplicon size of 681 bp (Fig 2), whereas 29 (58%) were found positive by NPCR amplifying a product size of 548 bp (Fig 3). Similar results were obtained in a study conducted by (Khadse *et al.* 2023) which revealed the prevalence of CPV to be (18/50, 36%) by conventional PCR assay. Consequently, a study by (Navarro *et al.,* 2017) screened 104 diarrheic fecal samples collected from puppies and adult dogs, with or without hemorrhagic gastroenteritis, in which, a total of (25/104, 24%) of dogs were found positive for CPV by PCR.



Lane 1: Sample no. (CPV-9), Lane 2: Sample no. (CPV-13), Lane 3: 100 bp DNA ladderLane 4: Negative Control, Lane 5: Sample no. (CPV-23), Lane 6: Positive ControlLane 7: Sample no. (CPV-25), Lane 8: Sample no. (CPV-36).

Fig 2: Amplification of VP2 gene of CPV by Conventional PCR.



Lane 1: Sample no. (CPV -13) Lane 2: Sample no. (CPV-15) Lane 3: Positive Control Lane 4: 100 bp DNA ladder Lane 5: Sample no. (CPV- 23) Lane 6: Sample no (CPV -32) Lane 7: Negative Control Lane 8: Sample no. (CPV-20) (Negative).

Fig 3: Amplification of VP2 gene of CPV by Nested PCR.

The present study revealed that NPCR has a substantially higher sensitivity for CPV detection than conventional PCR. These results were similar to the earlier findings of (Mochizuki *et al*. 1993), who stated that nested PCR is more sensitive than conventional PCR. Similarly, the results ascertained by (Khadse *et al.,* 2023) revealed the prevalence of CPV in dogs to be (30/50, 60%). A study by (Kushwaha *et al.,* 2018) using PCR and NPCR for the detection of CPV-2 reported (18%) and (63%) positive reactions, respectively. These above-stated findings indicated that nested PCR assay was a more sensitive molecular technique than conventional PCR for the detection of CPV-2.

Sequencing analysis

Two representative PCR products each from conventional and Nested PCR (Sample no. C13 from Ayodhya Nagar, Nagpur; OQ594804.1) and (Sample no. C23 from Bhavani mandir, Pardi, Nagpur; OQ594805.1) showed 99.5% to 100% sequence identity with the sequences obtained from China, Australia, Egypt, Myanmar, Italy, Ethiopia, *etc*. BLAST analysis of OQ594804.1 showed 99.85% identity with two *Canine parvovirus* strains *viz* CPV 2b and CPV 2c obtained from different regions of the world whereas the sequences of strain 2b obtained from Australia showed 99.85% identity with the sequence under study (Fig 4). Phylogenetic analysis of sample C-13 (accession no. OQ594804.1) further revealed that it was closely related to *Canine parvovirus* VP2 gene sequence obtained from China in 2021 (Acc. No. MZ836323, strain 2c) with 99.85% homology. Our sequence also showed 99.85% homology with that obtained from Pangolin from China (Acc. No. OP208805), though it was distinctly located and placed in a separate node in the phylogenetic tree. Moreover, many other sequences of strain 2c obtained from China, Myanmar, *etc*. also showed 99.85% identity with our sequence. This pattern of homology suggests that sample C13 might possess *Canine parvovirus* belonging to both strains 2b and 2c. Earlier research by various workers involving the application of multiplex PCR for the detection of CPV strains supported this hypothesis. A study by (Deng *et al.,* 2018) established multiplex PCR to target *Canine parvovirus*-2 and revealed that a single sample suspected for CPV showed the presence of all 3 antigenic strains such as, CPV-2a, CPV-2b and CPV-2c. Another study by Agnihotri, (2017) revealed that the Hisar CPV isolates showed 99.6% identity with KU244254 (*Canine parvovirus* 2c capsid protein VP2),99.7% identity with KR869657 (*Canine parvovirus* 2a strain CPV /BJ24/VP and KR002793 *Canine parvovirus* 2b strain CPV /CN/HB1/2013).

BLAST analysis of OQ594805.1 showed (99.79-100%) homology with only one strain of *Canine parvovirus viz*. strain 2c obtained from different regions. This suggested that the CPV of sample 23 belonged to strain 2c. Similar results were obtained by Perez *et al.* (2012) which revealed that CPV-2c was the only strain detected showing high homogeneity in both nucleotide and amino acid sequences.

Phylogenetic analysis of sample C-23 (accession no. OQ594805.1) revealed that it was closely related to and placed in the same node with *Canine parvovirus* VP2 gene sequence obtained from China in 2021 (Acc. No. ON322829, strain 2c) with 100% homology (Fig 5). Our sequence

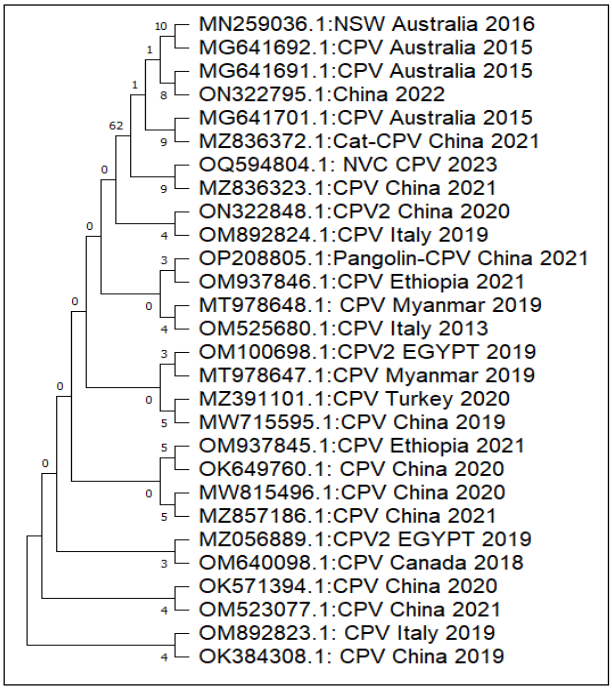


Fig 4: Phylogenetic analysis of partial gene of CPV sample-13 (OQ594804) with world isolates using MEGA X

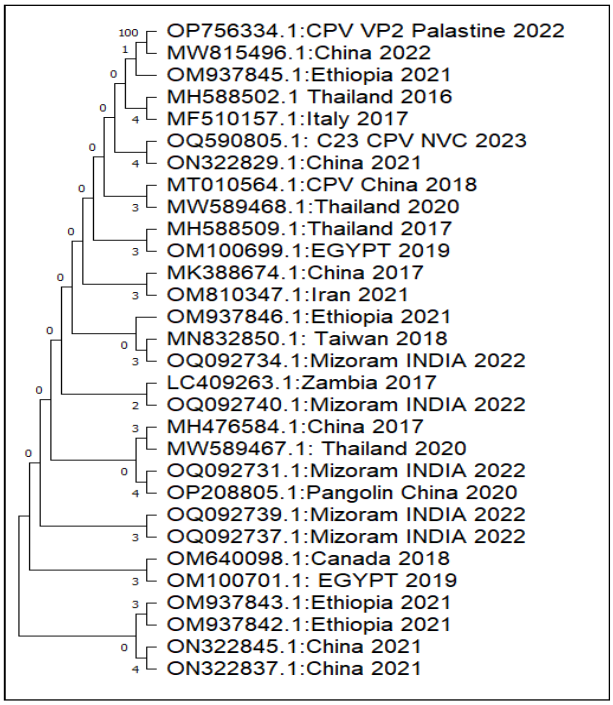


Fig 5: Phylogenetic analysis of partial gene of CPV samples-23 (OQ594805) with world isolates using MEGA X.

showed 99.79% identity with other sequences obtained from the Mizoram state of India and was distantly related to all sequences from India. Similar findings by Nair, (2022) previously revealed that the sequences of CPV isolates obtained from dog samples in Nagpur, Maharashtra showed homology to sequences obtained from China and Mizoram (India) (strain 2c).

CONCLUSION

The present study concluded that conventional PCR and Nested PCR are sensitive tools for the detection of CPV-2 from clinical fecal samples of infected dogs. The overall prevalence of CPV-2 in Nagpur city was found to be significantly high (38%) and (58%) by PCR and NPCR respectively. PCR assays were found to be more sensitive in comparison to the haemagglutination test with Nested PCR being the most sensitive of all three assays. Furthermore, partial length VP2 gene sequence analysis revealed that the sample CPV-13 belonged to both CPV-2b and CPV-2c variants and is currently the circulating antigenic type prevalent in Nagpur region of India. For a better understanding and the development of novel vaccine strains, ongoing research is required to keep track of emerging mutations of CPV-2 antigenic types and their prevalence in various geographic locations.

ACKNOWLEDGEMENT

The authors would like to thank Associate Dean, Nagpur Veterinary College, Nagpur, India for provision of research facility required to conduct this research.

Conflict of interest

All the authors declare that there are no conflicts of interest.

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