DETECTION OF CANINE PARVOVIRUS (CPV) CIRCULATING STRAINS IN ANDHRA PRADESH BY EMPLOYING MULTIPLEX PCR AND RESTRICTION FRAGMENT LENGTH POLYMORPHISM–PCR

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ABSTRACT

Canine parvovirus (CPV) is considered as one of the important cause of viral gastroenteritis in dogs. In recent years, studies on CPV indicated that it underwent mutational changes and led to the formation of new antigenic variants which differed from the strains earlier existed. With an attempt, to find out the present circulating variants in Andhra Pradesh, the study was carried out with a total of 342 clinically suspected fecal samples for canine parvovirus representing the various districts of Andhra Pradesh, South India from dogs of different age groups exhibiting signs of gastroenteritis. Initially, fecal samples were subjected for HA test and PCR with CPV-2ab primers. Out of 342, 71 samples had a titre of 1:32 and above and 233 samples were positive by PCR. Genotyping of CPV was done by employing multiplex PCR using CPV-2ab and CPV-2b primer pairs. Out of 233 positive samples, 216 (92.70%) samples produced an amplicon size of 681 bp characteristic of CPV-2a and 17 samples (7.29%) yielded two specific amplicons of 681 bp and 427 bp and thus categorized as CPV-2b. The fecal samples not reacted with CPV-2ab and CPV-2b primers were further analyzed by PCR-RFLP using restriction enzyme *Mbo*II for the detection of CPV-2c strain. Only one sample reacted with CPV-555 primers and produced an amplicon size of 583 bp but remain undigested with restriction enzyme. From the above studies, it can be inferred that the CPV-2a was the predominant strain and no CPV-2c was detected in Andhra Pradesh.

Keywords: Antigenic variants, Canine parvovirus, Multiplex PCR, PCR-RFLP

Canine parvovirus (CPV) was discovered in the feces of normal dogs in 1967 and was called as Minute virus of canines (MVC) or Canine parvovirus (CPV-1). A second CPV emerged in 1978 (Appel et al., 1979) and was termed as Canine parvovirus-2. Canine parvovirus-1 was less pathogenic and not serologically related to CPV-2 (Carmichael and Binn, 1981). In 1980s, the original CPV-2 underwent antigenic changes and was completely replaced by new antigenic variants designated as CPV-2a, CPV-2b. Changes in the position of amino acids have been evolving leading to a new antigenic variant of CPV and had been reported in Italy (Buonavoglia et al., 2001) and named it as CPV-2c. The variant had an amino acid substitution, Asp- $426 \rightarrow$ Glu, which occurs in a residue of the capsid protein that is considered important for the antigenic properties of CPV-2. The CPV-2c was co-circulating in Italy with types CPV-2a and CPV-2b (Martella et al., 2004). Studies on circulation of CPV strains in different geographical regions of the world during 2009-2015 reported that CPV-2a was more predominant strain when compared to CPV -2b/2c. In Asia, CPV-2a is the predominant strain whereas CPV-2c was the predominant variant in Europe and Latin America (Zhou et al., 2017). In India, CPV variants were earlier reported by many authors (Parthiban et al., 2010, Deepika et al., 2015, Sharma et al., 2016). The prevalence

of CPV variants circulating in Andhra Pradesh was not documented, so an attempt was made to investigate the prevailing strains (CPV-2a, CPV-2b and CPV-2c) responsible for the CPV infections in dogs.

MATERIALS AND METHODS

Three hundred and forty two fecal samples suspected of CPV were collected from 11 districts of Andhra Pradesh during the months of June (2017) to December (2018). The fecal material was collected with the help of sterilized swabs from dogs at Super Speciality Veterinary Hospital, Vijayawada; Teaching Veterinary Clinical Complex, NTR College of Veterinary Science, Gannavaram; College of Veterinary Science, Tirupati and from various Veterinary Polyclinics across different districts of Andhra Pradesh. The fecal samples were initially screened for the viral pathogen by haemagglutination test and later confirmed by conventional PCR. Antigenic variants were detected by employing multiplex PCR and Restriction Fragment Length Polymorphism (RFLP)-PCR. Commercially available vaccine-CanigenTM (Virbac India) was used as a positive control for CPV.

Preparation of fecal sample: The swabs were properly rinsed in PBS and taken out. The fecal contents in PBS were centrifuged at 6,000 rpm for 15 min at 4 °C. The supernatant was collected and stored at -80 °C until further use.

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Screening of samples by HA test: Packed pig erythrocytes of 0.8 ml was suspended in 100 ml of 0.2 M Sorenson's phosphate buffered saline of pH 7.0, after three washings with the same solution. The haemagglutination test was done as per the method described by Carmichael and Binn (1981). The fecal samples/rectal swabs obtained from the suspected dogs were emulsified in 1 ml of 0.2 M PBS of pH 7.4 and centrifuged at 6000 rpm for 15 min at 4 °C. Ninety microlitre of processed fecal sample supernatant was treated with 10 μ l of chloroform and mixed well. The mixture was kept at 4 °C for 10 min and centrifuged at 10000 rpm at 4 °C for 10 min. The supernatant was collected and used for haemagglutination test.

Detection of CPV by Conventional Polymerase Chain Reaction: The DNA was isolated from fecal samples as per Vieira et al. (2008). Processed fecal sample of 100 µl was used for template DNA preparation. The samples were boiled at 96 °C for 10 min and immediately chilled in crushed ice (Schunck et al., 1995; Uwatoko et al., 1995 and Decaro et al., 2005). Then, the samples were centrifuged at $12,000 \times g$ for 10 min at 4 °C. The supernatants were diluted 1:5 in distilled water to reduce residual inhibitors of DNA polymerase activity (Decaro et al., 2006) and used as template for PCR. The concentration of DNA was measured with Nanodrop 200C at 260/280 Å. Conventional PCR was carried out targeting the VP2 gene using CPV-2ab published primers, Forward primer - GAAGAGTGGTT GTAAATAATT and reverse primer CCTATATAACCA AAGTTAGTAC. The PCR amplification was optimized in 20 µl PCR reaction with the following conditons: Initial denaturation at 95 °C for 5 min, followed by 30 cycles denaturation at 95 °C for 30 sec, annealing 55 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min.

The samples positive for PCR targeting the VP2 gene with CPV-2ab primers were subsequently employed for multiplex PCR for the simultaneous detection of CPV-2a and CPV-2b types in the fecal samples using CPV set of CPV-2ab and 2b published primers (Senda *et al.*, 1995): CPV-2ab(F)–GAAGAGTGGTTGTAAATAATT and CPV- 2ab(R) CCTATATAACCAAAGTTAGTAC, CPV-2b(F) CTTTAACCTTCCTGTAACAG and CPV- 2a(R) CATAGTTAAATTGGTTATCTAC.

Polymerase chain reaction– Restriction fragment length polymorphism: The samples which were negative with CPV-2ab primers subjected to amplification with a primer pair 555 F/555 R which amplified a 583 bp fragment of the gene encoding for capsid protein. PCR products generated with the primer pair were digested with enzyme MboII, which selectively recognize the restriction site "GAAGA" unique to CPV-2c. The PCR amplification was optimized in 20µl PCR reaction mixture amplification of VP2 gene using 555 primers (Buonavoglia *et al.*, 2001) 555 F– AGGAAGAT ATCCAGAAGGA and 555 R– GGTGCTAGTTGATAT GTAATAAACA. The sequence of cycles followed were an initial denaturation at 94 °C for 5 min, followed by 40 cycles denaturation at 94 °C for 30 sec, annealing 55 °C for 2 min, extension at 72 °C for 2 min and final extension at 72 °C for 10 min.

A 10µl reaction was prepared for the PCR-RFLP with the amplified product along with the following reagents. The whole mixture was placed at 37 °C for 4 hrs in a thermal cycler and finally inactivated the enzyme at 65°C for 5 min. After completion of the cycles, the PCR product obtained was subjected to 2% agarose gel electrophoresis.

Reagent	Quantity (µl)
Buffer	2.0µl
Mbo II (Moraxella bovis enzyme)	1.0µ1(10 units)
PCR product run with CPV-555 primer	7.0µl
Final Volume	10.0 µl

RESULTS AND DISCUSSION

As a preliminary test, HA detected 71 (20.76%) samples positive for CPV with HA titres ranging from 1 in 32 to 1 in 512. Fecal samples with < 1 in 32 was considered to be negative. Out of 71 HA positive, 23 samples were highly positive (HA titre 1:64 and above) and 48 samples were weakly positive (HA titre 1:32). Eighty one fecal samples had a titre ranging from 1 in 2 to 1 in 16 and remaining 190 did not haemagglutinate the swine RBC.

The processed 342 samples were screened by primer pair CPV-2ab and negative samples further by CPV-555 primers for the presence of VP2 gene encoding the capsid protein. Two hundred and thirty four (68.42%) samples were positive for PCR with 233 producing an amplicon product size of 681 bp with CPV-2ab primer (Fig.1) and 1 sample with CPV-555 primers produced a product size of 583 bp. The vaccine strain also reacted with the primers specific to VP2 gene. The size of the PCR products specific to partial VP2 gene by 1.5% electrophoresis was 681 bp. The fecal sample from a healthy dog as negative



Fig. 1. Polymerase chain reaction assay amplification product of 2ab primers pair for CPV. Lane M: 100 bp ladder; Lane N: Fecal sample from a healthy dog as negative control; Lane C: Vaccine strain as positive control

control was unresponsive to primer pair.

Polymerase chain reaction technique is known for its increased usage as a diagnostic tool for the detection and had been a rapid, sensitive and accurate test for the confirmation of CPV infection (Nandi and Kumar, 2010). The present study recorded 68.42% molecular prevalence of the canine parvoviral disease in Andhra Pradesh. Raj *et al.* (2011) recorded a prevalence rate of 66.23 % in and around pondicherry, whereas, Agnihotri *et al.* (2017) reported 36% of CPV in Haryana from the dogs suspected of viral gastroenteritis using CPV-2ab primers.

All the 233 samples found positive by PCR using CPV-2ab primer were further screened for the detection of CPV types, CPV-2a and CPV-2b by multiplex PCR using CPV-2ab and CPV-2b primer pairs (Senda et al., 1995). Out of 233 samples, 17 (7.29%) yielded two specific amplicon sizes of 681bp and 427 bp responding to both CPV-2ab and CPV-2b primers, respectively and therefore characterized as CPV-2b types. 216 (92.70%) samples reacted with CPV-2ab primer but not to CPV-2b primer, producing only a single band at 681 bp and was characterised as CPV-2a. The vaccine strain CPV-2 also reacted only with CPV 2ab primer pair and produced an amplicon size of 681 bp. Incidence of 7.29% of CPV - 2b and a variant of CPV-2a was reported only in border districts like Kurnool, Tirupati and Visakapatnam which share boundary with states of Telangana, Karnataka, Tamil Nadu and Odisha, suggesting the probable reason may be the transborder movement of animals across the borders. The results were in agreement with those reported by Parthiban et al. (2010) and Sharma et al. (2016). From the results it was confirmed that the predominant strain



Fig. 2. Multiplex polymerase chain reaction of Canine parvovirus with CPV-2ab and CPV-2b specific primers. M: 100 bp ladder; Lane 1-13: Positive samples from suspected clinical fecal samples; N: Fecal sample from a healthy dog as negative control

prevailing in Andhra Pradesh was CPV-2a. Similar reports of high prevalence of CPV-2a in Southern India was also reported by Srinivas *et al.* (2013) and Deepika *et al.* (2015).

All 109 CPV samples negative by multiplex PCR were subjected to PCR with CPV-555 primer pair and the product was digested with enzyme MboII that selectively recognises the restriction site "GAAGA" unique to CPV-2c and form two fragments of 500 and 83 bp, respectively (Desario *et al.*, 2005).

Out of 109 negative samples tested with multiplex primers, only one sample could produce an amplicon product size of 583 bp, indicating all others were negative and when further digested with MboII restriction enzyme, it remained undigested indicating that it was not CPV-2c strain but variant of CPV-2a or CPV-2b. 'D' means it was digested with the enzyme and later run on agarose gel but could get the bands that were relevant to CPV-2c. Those bands appearing may be non-specific bands or the DNA



Fig. 3. Restriction fragment length polymorphism analysis of CPV-555 amplicons. Lane M: 100 bp ladder; Lane U: undigested amplicon; Lane D: digested amplicon with MboII.

might have chances of impurities. Similar results were reported by Parthiban *et al.* (2010) where sixty samples negative by multiplex PCR were subjected to PCR using CPV-555 primer pair of which only three samples produced 583 bp product and on further digestion with MboII restriction enzyme remained undigested confirming that they were not CPV-2c strain but may be a variant of CPV-2a or CPV-2b. Sharma *et al.* (2016) also used the MboII restriction enzyme for detection of CPV-2c in the negative samples but all the 50 samples were not amplified with CPV-555 primers indicating that they were negative for CPV infection. Dastmalchi *et al.* (2017) were successful in detecting one CPV-2c out of 10 samples by CPV-555 primers that were further analyzed by MboII digestion of PCR products.

From the above studies, the predominant strain circulating in Andhra Pradesh is CPV-2a when compared to CPV-2b and prevalence of CPV-2c was not observed. Molecular detection by PCR was more efficient than the conventional haemagglutination assay in the preliminary screening of CPV suspected samples.

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