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Detection and Molecular Characterization of Indian Isolates of Canine Parvovirus in Fecal Samples

Prasad Minakshi*, Koushlesh Ranjan, Supriya*, Basanti Brar*, Gaya Prasad*****

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Author Affiliation
*Department of Animal Biotechnology, LLR University of Veterinary and Animal Sciences, Hisar, Haryana, India, 125004.
**Department of Veterinary Physiology and Biochemistry, Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India, 250110. ***Sardar Vallabhbhai Patel University of Agriculture and Technology, Meerut, Uttar Pradesh, India, 250110.

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Minakshi Prasad, Professor, Department of Animal Biotechnology, LLR University of Veterinary and Animal Sciences, Hisar, Haryana, India, 125004.
E-mail: minakshi.abt@gmail.com

Abstract

The canine parvovirus which causes myocarditis and enteritis is considered to be a very serious pathogen causing mortality in pups. The original strain CPV2 is found to be continuously evolving and presently three antigenic strains CPV-2a, CPV-2b and CPV-2c found to be circulating worldwide. In the present study 52 fecal samples were collected from 1-2 month old pups showing clinical signs of CPV from Hisar (India) and screened using vp2 gene specific semi-nested polymerase chain reaction assay. Three fecal samples were found positive for CPV infection. The PCR products were allowed for nucleic acid sequencing and nucleotide sequences were deposited to GenBank with accession numbers KC012923, KC012928 and KC012932. The sequence analysis using various bioinformatics tools revealed that all the three isolates belong to CPV-2a genotype and are much closer to other CPV-2a genotype from India and China. The present study showed that the predominant genotype running in India is CPV2a. Therefore, CPV2a strain should be incorporated in vaccine formulation.

Keywords: CPV; CPV-2a; PCR; Genotype.

Introduction

Canine parvovirus (CPV) was first identified in USA in 1978 and was designated as CPV type 2 (CPV-2) (Appel et al., 1979). The CPV-2 was genetically distinct from previously recognized parvovirus of dogs i.e., minute virus of canines (Binn et al., 1970). After its emergence, CPV-2 became globally endemic in domestic and wild canid population (Parrish et al., 1988). It is assumed that CPV-2 was originated from Feline panleukopaenia virus (FPV) as host variant in late 1970 (Truyen et al., 1998). However, CPV-2 is still continuously emerging with nucleotide changes where as some sort of genetic stability is maintained by FPV (Decaro et al., 2008). CPV-2 is a serious pathogen which causes diarrhea and myocarditis mainly in young dogs (Perez et al., 2007). However, virus is also detected in cats (Decaro et al., 2010) and wolves (Mech et al., 2012). CPV-2 belongs to family *parvoviridae*, subfamily *parvovirinae* and genus parvovirus (Nandi et al., 2011). It has single stranded DNA genome having negative polarity with a whole genome size of 5.2kb. It has got two open reading frames which encodes for non-structural (NS) as well as structural virus protein (VP). VP proteins consist of VP1, VP2 and VP3. VP2 is major protein (80% of capsid protein), translated from an in frame ATG codon which is present within VP1 open reading frame (Rai et al., 2006). All the epitope neutralizing antibodies are found within VP2 region. However, a T cell epitope is found

within VP1 region (Rai et al., 2004). Thus, any changes in amino acid residue of VP2 protein may seriously alter the biological characteristics of virus (Parrish and Carmichael, 1986).

After a few years of its emergence CPV-2 has completely replaced by CPV-2a which may cause disease in both cats and dogs (Truyen, 1996). CPV-2a virus differ from CPV-2 by amino acid changes in the capsid protein (VP2) at positions 87 (Met to Leu), 300 (Gly to Ala), 305 (Tyr to Asp) and 555 (Val to Ile) (Castro et al., 2010). Later on, a new antigenic variant of CPV i.e. CPV-2b was emerged which is now circulating along with CPV-2a among dog population around the world. The CPV-2b differs from CPV-2a in two amino acid changes at 426 (Asn to Asp) and at 555 (Ile to Val) (Decaro et al., 2006). A third variant i.e. CPV-2c was also discovered in Italy in 2000 which has a mutation at position 426 (Asp to Glu) in main neutralizing epitope of capsid (Buonavoglia et al., 2001). The CPV-2c variant is widely distributed in Italy and is co-circulating along with CPV-2a and CPV-2b variant (Martella et al., 2004). The molecular epidemiological survey suggest that CPV-2a is predominantly found in Italy (Martella et al., 2006), India (Chinchkar et al., 2006) and Korea (Kang et al., 2008). Similarly, CPV-2b is predominantly circulating in USA, Japan, Brazil, Switzerland, Taiwan and South Africa (Shoorijeh et al., 2011). Both CPV2a and CPV2b are equally circulating in UK, Germany, Spain and Australia (Shoorijeh et al., 2011). CPV-2c is found to be circulating in Italy, (Martella et al., 2004), Vietnam, Spain, Germany, United Kingdom (Perez et al., 2007) and India (Nandi et al., 2010a).

Although, Canine parvovirus is a major infectious disease of pups, very few researches has been carried out in India with regard to various genotype circulating in different part of India. In present study we have carried out molecular genotyping of CPV-2 circulating in India so that better information can be generated for future development of vaccine against canine parvovirus.

Materials and Methods

Viral Samples

A total of 52 faecal samples from 1-2 month old pups showing various symptoms such as gastroenteritis, off smelling diarrhea with blood were collected from University Veterinary clinic of college of veterinary sciences, Hisar from July 2011 to May 2012. The fecal samples were collected in screw capped sterile cotton swab containing 0.5ml of 0.1M phosphate buffer saline (PBS).

DNA Extraction

DNA was extracted from the faecal sample by using DNAzol kit (Invitrogen, USA) according to the manufacturer's instructions. The extracted DNA was stored at -20°C for further molecular biology study.

Polymerase Chain Reaction

The samples were screened using semi nested PCR assay with published primers (Sakulwira et al., 2001). The semi nested polymerase chain reactions were performed by standardized protocol with some modifications (Sakulwira et al., 2001). In short 2µl of extracted DNA was added to a reaction mixture containing 1.25U of Taq DNA polymerase, 200µM dNTP mix, 1mM $MgCl_2$, 5 Pmol of both forward and reverse primers. The total reaction volume was made to 12.5µl by adding 9.3µ1 of nuclease free water. Primer pair: P1 (5′-TCCAGCAGCTATGAGATC-3′; nt no. 3342-3360) and P2 (5′-GATCTGTGGTAGCAATAC-3′; nt no. 4570-4588) were used for the first round amplification. For second amplification round P1 and P3 (5′-GATCTGTTGGTAGCAATAC-3′; nt no. 4070-4088) were used. The first amplification round consists of an initial denaturation step of 95^0C for 5 minute followed by denaturation at 95^0C for 30seconds, annealing at 52^0C for 50 seconds and elongation at 72^0C for 1 minute. The whole cycle repeated for 30 times and a final elongation is done at 72^0C for 10 minutes. For the second amplification step 2µl of the first PCR product were added to the reaction mixture and amplification was performed in a manner identical to that of the first step. The PCR amplicon of 747bp were visualized under UV trans-illuminator after 1% agarose gel electrophoresis.

Sequencing PCR Product

The PCR product obtained was purified with QIAquick PCR Purification Kit (Quiagen, USA) as per manufacturer's instruction. The purified PCR products were subjected to nucleotide sequencing using Genetic Analyser 3130XL (ABI, USA) machine in Department of Animal Biotechnology, LUVAS, Hisar.

Bioinformatics Analysis

The nucleotide sequence data obtained was

allowed for GenBank database search using online BlastN 2.5.0+ (Zhang et al., 2000) for similarity search with other nucleotide sequences available in GenBank. The nucleotide sequences of both ends (forward and reverse) were assembled and contig is prepared using Bioedit v7.2.5 programme (Hall, 1999). The Bioedit v7.2.5 programme was also used for multiple sequence alignment and percent nucleotide identity calculation of our CVP isolates with other isolates from Gen Bank. The phylogenetic analysis of our CPV sequences, along with other sequences from GenBank were done using Mega 5 programme (Tamura et al., 2011). The multiple sequence alignment of deduced amino acid sequences of our CPV isolates along with other CPV from genebank were done using Bioedit v7.2.5 programme (Hall, 1999).

Results and Discussion

The CPV2 was emerged in late 1970 as a host variant of Feline Panleukopenia Virus (FPV). Later on it was evolved into different variants due to continuous mutation in the nucleotide sequences which codes for capsid genes. This has raised a worldwide concern regarding the health of cats and dogs. CPV can be detected in feces by electron microscopy but only few institutes have got this facility in India. The virus can also be isolated in canine and feline kidney cells and can be confirmed by the characteristic cytopathic effect produced and also by polymerase chain reaction. The viral antigen can also be detected in clinical specimens by ELISA (Phukan et al., 2005), haemagglutination-haeminhibition tests (HA-HI), (Senda et al., 1986), nucleic acid hybridization assay (Waldvogel et al., 1992). The polymerase chain reaction were found to be 10 fold higher sensitive than others in detecting CPV in clinical samples (Truyen, 2000). The present study used PCR and found to be very effective in detecting the virus in fecal sample.

In our study a total of 52 fecal samples from 1-2 month old pups were collected. The samples were allowed for further processing and viral DNA was extracted using a commercially available DNAzol kit (Invitrogen, USA). The DNA from fecal samples was allowed for semi nested PCR using previously published primers (Sakulwira et al., 2001). The agarose gel electrophoresis revealed that three isolates from fecal samples (P4/10/HSR, P53/11/HSR and P58/11/HSR) showed specific amplification of 747 bp (Figure 1). However, remaining samples did not show any amplification. The PCR products of P4/10/HSR, P53/11/HSR and P58/11/HSR samples were purified and allowed for nucleic acid sequencing. The BlastN 2.5.0+ search revealed that these isolates showed maximum identity of >99% with nucleotide sequences of CPV-2a isolates from different regions of the world. Thus, these isolates were characterized as CPV-2a. The nucleotide sequences of P4/10/HSR, P53/11/HSR and P58/11/HSR isolates were deposited to GenBank database and Accession numbers KC012923.1, KC012928.1 and KC012932.1 respectively were assigned. These isolates showed nucleotide (nt) and deduced amino acid (aa) identity of 98.8-99.8/ 98.3-100% (nt/aa) among themselves which suggests that these isolates are distinct from each other. The multiple sequence alignment using Bioedit v7.2.5 programme showed that P4/10/HSR, P53/11/HSR and P58/11/HSR isolates have >99/ 98% (nt/aa) identity with several isolates of CPV-2a from different regions of the world.

The phylogenetic analysis of nucleotide sequences of P4/10/HSR, P53/11/HSR and P58/ 11/HSR isolates along with several other isolates from different regions of the world showed that P4/ 10/HSR and P53/11/HSR isolates form a separate close cluster with CPV-2a isolates from China (CPV/CN/JL1/2013 and CPV/CN/SD6/2014) (Wang et al., 2016) and India (Faizabad) (Doley et al., 2014) (Figure 2). However, these isolates were slightly distantly related with a CPV-2b isolate from France (04S23). Similarly, P58/11/HSR isolate was formed a separate close cluster with several CPV-2a isolates from India (WBD1 and NAG3) and China (CPV/CN/SD10/2014, CPV/CN/SD19/2014 and CPV/CN/SD18/2014) (Wang et al., 2016).

Several variants of CPV2 such as CPV2a, CPV2b and CPV2c were detected in India by different researcher (Nandi et al., 2010a, b; Mohan Raj et al., 2010). The nucleotide sequence based study revealed that CPV-2a is predominant genotype in India. With regard to the pathogenicity of the canine parvovirus the studies are showing conflicting results. Some studies suggest that CPV2b is milder pathogen when compared to other variants; however both CPV2a and CPV2b have been detected in animals with severe diarrhea (Castro et al., 2010). The CPV2c has been associated with severe hemorrhagic enteritis and mortality (Buonovoglia et al., 2001).

Since the virus has been continuously evolved to new variants like CPV-2a, CPV-2b and CPV-2c, the newer strains/isolates should be incorporated in vaccines. Since the present study has shown that predominant genotype running in India and

especially in Hisar region is CPV-2a, vaccines should be prepared keeping in view of the genotypes running regionally (Perez et al., 2007). Although, genotype specific vaccine is better than other vaccines for genotype specific vaccines against CPV, some study showed that CPV2 can give cross protection to the newly evolved CPV variants (Spibey et al., 2008). However, more detailed studies are required in this aspect. Also detailed epidemiological studies has to be performed regionally every year in order to determine which genotypes are running currently, emergence of new genotypes etc.

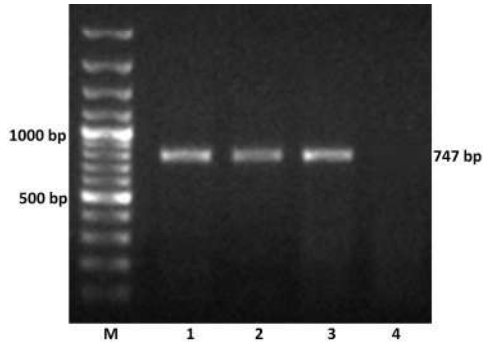

Fig. 1: Agarose gel electrophoresis of semi-nested PCR of vp2 gene of Canine Parvovirus samples showing 747 bp PCR amplicon. Lane M: Marker 1000bp: Lane 1: P4/10/HSR; Lane 2: P53/11/HSR; Lane 3: P58/11/HSR; Lane 4: Nuclease free water negative control

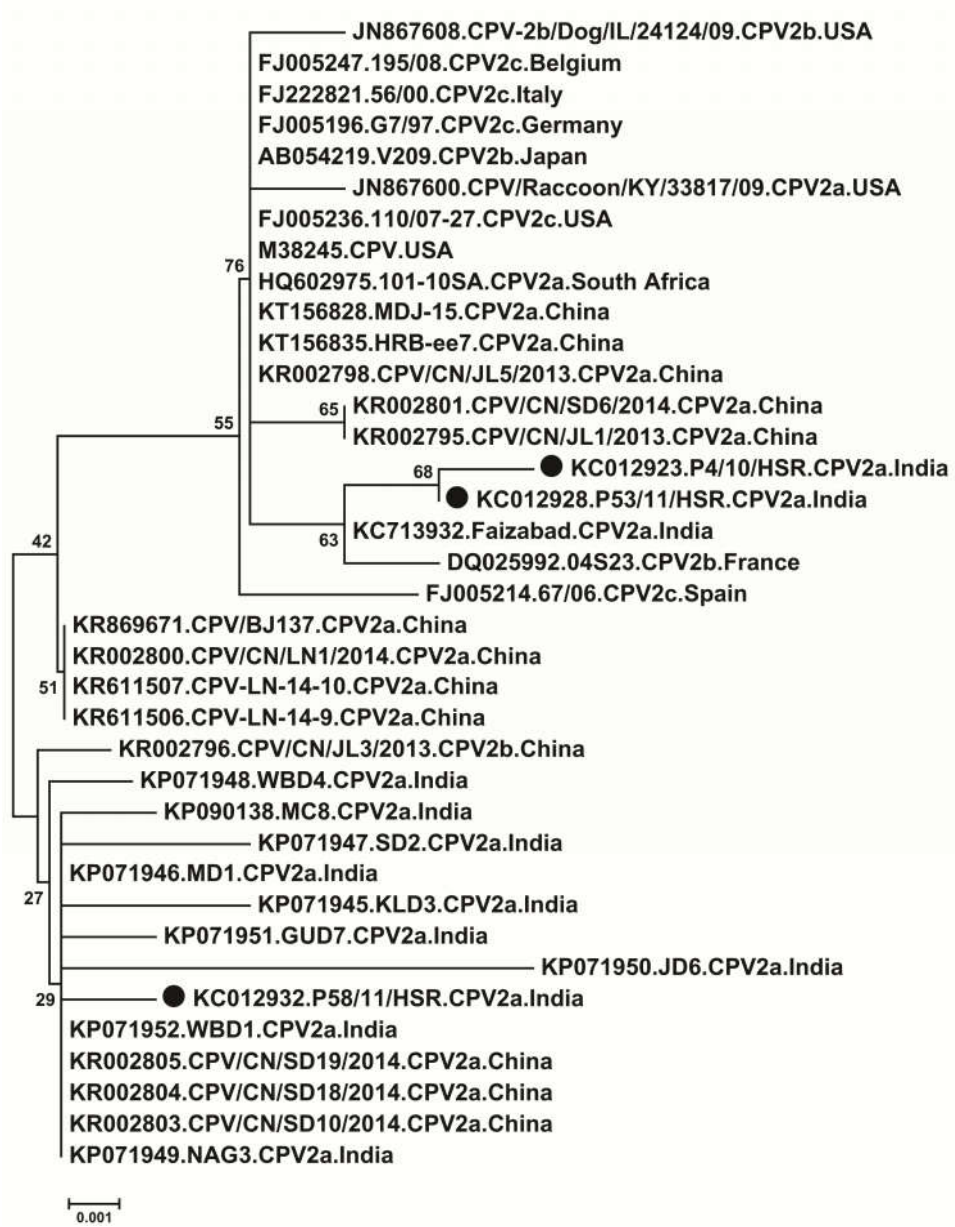

Fig. 2: Vp2 gene nucleotide sequence based phylogenetic analysis of P4/10/HSR, P53/11/HSR and P58/11/HSR isolates along with other global isolates of Canine Parvovirus 2. Phylogenetic tree was constructed using p-distance determinant of neighbor joining algorithm of Mega 5 programme with 1000 bootstrap values. ● = Isolates used in present study

Conclusions

The PCR followed by nucleic acid sequencing is a sensitive technique for molecular detection of CPV from fecal samples. The nucleic acid sequence analysis of fecal samples revealed that these isolates belong to CPV-2a genotype. The sequence analysis further revealed that P4/10/HSR, P53/11/HSR and P58/11/HSR isolates are very similar with CPV-2a isolates from India and China. The present study recommends that new vaccine should be made available in the market incorporating at least CPV-2a genotype since it is predominant genotype circulating in Hisar. Also proper awareness programs should be conducted among the dog owners for preventing the spread of the disease. Proper surveillance of CPV should be done in a regular manner to identify whether new strains are circulating or not since the sequence analysis has shown that genetic mutations are continuously occurring in virus.

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