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Molecular characterization of canine parvovirus in Namakkal region of Tamil Nadu

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Abstract
The aim of this study was to know the type of canine parvovirus strain predominantly circulate in the Namakkal region of Tamil Nadu. Totally, 120 faecal samples were collected from clinically affected domestic dogs and viral DNA was extracted. The template DNA was used to detect and molecularly characterization of canine parvo virus by using polymerase chain reaction and DNA sequencing technique. $H_{For/rev}$ primers were used in PCR and DNA sequencing was done with all positive PCR products. Out of a total 120 collected faecal samples, 58 were found to be positive for CPV-2a and 14 were CPV-2b by PCR and sequencing technique. CPV-2a is the predominant strain circulates in and around Namakkal region but there is no variation in the amino acid sequence among the positive cases in this study.

Keywords: CPV-2a, H primers, PCR, DNA sequencing

Introduction
Canine parvoviral infection is a highly contagious and fatal viral disease of dogs caused by canine parvovirus-2, which is characterised by vomition, diarrhoea with dark or bloody faeces, dehydration, fever and lowered WBC counts in affected dogs of all ages and myocarditis in puppies of less than three months of age (Buonavoglia *et al*., 2001) [1]. Canine parvovirus type-2 was shown to be a variant of long recognized feline panleukopenia virus (Chinchkar *et al*., 2006) [2] and emerged in 1978 as a highly contagious and very serious disease in dogs. However, CPV-2 underwent evolutionary changes after its emergence in the late 1970s and within few years CPV-2 has been globally replaced by the new antigenic variants (Filipov *et al*., 2011) [4]. In 1979, a CPV variant (CPV-2a) emerged and contained five substitution in the capsid sequence compared to CPV-2, including changes of VP2 residues 87 from methionine to leucine, 300 from alanine to glycine, and 305 from aspartic acid to tyrosine (Hoelzer *et al*., 2010) [5]. Canine parvovirus-2a isolates were antigenically different from CPV-2 and caused disease in cats (Kapil *et al*., 2007) [6] An antigenic variant of CPV-2a (CPV-2b) was recognised in 1984, and it differed in an antigenic epitope as a result of the substitution of VP2 at residue 426 from asparagine to aspartic acid and at residue 555 from isoleucine to valine (Mohanraj *et al*., 2010) [7]. In 2000, another mutant called CPV-2c was reported in dogs from Italy and it differs from CPV-2b by one amino acid at 426 positions from asparagine to glutamine (Mohapatra *et al*., 2012) [8]. Additional amino acid difference was observed in both CPV-2a and CPV-2b at position 297 (serine to alanine). Due to mutation appeared first in 1993 in German CPV isolates, which were designated as 'New CPV-2a/2b' (Nandi *et al*., 2010) [9].
The prevalence of CPV-2a (Chinchkar *et al*., 2006) [2] and new CPV-2a (Mohanraj *et al*., 2010) [7] has been documented in South India. However, recently new CPV-2a has replaced CPV-2b as the major circulating variant in North India (Mohapatra *et al*., 2012) [8]. Occurrence of CPV-2c was first reported in India based on the sequence analysis of CPV-2b positive samples. Its presence in India supports the assumption that CPV-2c is going to reach a worldwide distribution and provides new information to understand the evolution of antigenic variants of CPV-2 (Nandi *et al*., 2013) [10]. New CPV-2a was found to be the predominant strain prevalent in different cities across five South Indian states/ union territories and there was also enough indication of circulation of New CPV-2b strain from different states of South India (Srinivas *et al*., 2013) [15]. PCR technique has been widely applied for laboratory diagnosis of the disease due to high sensitivity and specificity and also requiring only two to four hours of time for detection of viral nucleic acid.

The PCR product as it is or cloned in the suitable cloning vector can be sequenced using the suitable primer with the help of automated DNA sequencing machine for typing of CPV strains. The sequence is analyzed using the appropriate software. This is an important technique to know with certainty the particular variant of the CPV present in the field sample. Both the nucleotide and amino acid sequence data can also be used to know the percent homology and for phylogenetic analysis of CPV-2 isolates from different geographical regions (Nandi *et al*., 2009) [11]. Based on sequence analysis CPV-2a and CPV-2b type could be differentiated from original CPV-2 (Chinchkar *et al*., 2006) [2]. Canine parvovirus type 2c variants have been reported from various countries based on the nucleotide sequence analysis (Kapil *et al*., 2007) [6]. The present study was undertaken with an objective to know the type and molecular characterization of CPV strain circulate in and around Namakkal region by polymerase chain reaction (PCR) followed by DNA sequencing.

Material and methods

Sample collection: In this study, ethical clearance was not required because faecal samples were collected from clinical cases. A total of 120 faecal samples were collected from clinically affected dogs showing typical clinical signs such as foul smelling, hemorrhagic diarrhoea and vomiting presented at Teaching Veterinary Clinical Complex, Veterinary College and Research Institute, Namakkal, Tamil Nadu. The collected samples were emulsified in 1 ml of 0.1M PBS with a pH of 7.4 and centrifuged at 6000rpm for 15 min at 4°C. The supernatant was collected and used for DNA extraction (Mohanraj *et al.,* 2010) [7].

Extraction of DNA from the faecal sample: The DNA was extracted from all 120 faecal samples by using QIAamp DNA Mini Kit (Qiagen, Germany) as per the protocol.

Primers: A pair of primers H_{for} (5'-CAGGTGATGAATTTG CTACA-3') and H_{rev} (5'-CATTTGGATAAACTGGTGGT-3') was used for screening all the collected faecal samples. 'H' primer pairs amplify 630 bp fragment of the gene encoding capsid protein. The primer pairs H_{for}/H_{rev} were utilized them for sequencing studies of canine parvovirus in which the H primer pairs amplifies a portion of VP2 gene containing the critical amino acids position of 297, 300, 305, 375 and 426, which helps in characterization of canine parvovirus (Buonavoglia *et al*., 2001) [1].

Polymerase chain reaction: Polymerase chain reaction was performed using template DNA extracted from faecal samples with positive control as vaccine DNA and negative control as without any template DNA. PCR reaction mixture was set up in a standard volume of 50 µl in a PCR tubes (5 µl – template DNA, 25µl- Red dye master mix, 1µl- forward primer, 1µl- reverse primer, 18 µl- Nuclease free water). The PCR tubes with reaction mixture were transferred to automated thermal cycler, (Eppendorf Master Cycler, Germany) for amplification of template DNA with required thermal cyclic condition (Table -1).

Table 1: Steps and conditions of thermal profile involved for PCR

Serial No	Steps	Temperature	Time
Step 1	Initial denaturation	95°C	5 min
Step 2	Denaturation	95°C	1 min
Step 3	Annealing	55°C	2 min
Step 4	Extension	72°C	30 sec
Step 2 to step 4 repeated for 30 cycles			
Step 5	Final extension	72°C	10 min
Step 6	Holding temperature	4°C	

DNA sequencing of PCR products: All the positive amplified PCR products were sequenced by Sanger's Dideoxy chain termination method (Sanger *et al.* 1977) [14] at M/s. Chromous Biotech, Bangalore. Results of gene sequence data was analyzed and aligned with sequences of prototype CPV strains (M38245- CPV-2; M24003 – CPV – 2a; M74849- CPV- 2b; AY742953- New CPV – 2a; AY742955- New CPV- 2b and FJ222821- CPV-2C) by the software Clustal W and analyzed for the nucleotide variation of VP2 gene positions at 3675, 3685, 3699, 3909 and 4064 with the corresponding amino acid residues at 297, 300, 305, 375, and 426, respectively. The specificity of the sequence obtained, the nucleotide variation and amino acid variation with respect to the VP2 gene sequence of canine parvovirus were determined using BLAST (Basic Local Alignment Search Tool).

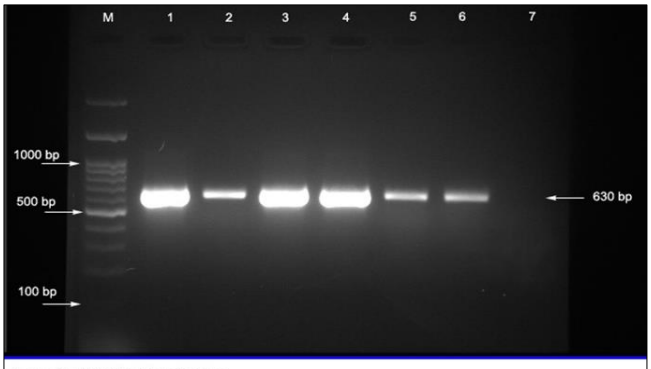

Fig 1: PCR amplified product of VP2 gene of canine parvovirus in 2.0 per cent agarose gel showing bands at 630 bp (H primers)

Results

Detection of CPV by polymerase chain reaction using 'H' primer: A total of 120 faecal samples were subjected to PCR by using 'H' primers. Only 72 faecal samples were revealed the product size of 630 bp (Figure-1).

Sequencing analysis of CPV PCR products: Sequencing of 72 positive PCR products obtained by using 'H' primers was done for strain identity and characterization of CPV. Out of 72 faecal samples, 58 and 14 samples were revealed the types CPV-2a and CPV-2b respectively.

Out of 58 CPV-2a samples, the sequence of 44, 6, 5, 2 and 1 samples had 98-100% homology that of existing isolates sequence published in gene bank by BLAST analysis from Pondicherry-India, Chennai-India, China, Thailand and Italy respectively.

All the fourteen CPV-2b isolates were 100% homology with the isolates sequence published in gene bank from China.

Discussion

Canine parvovirus (CPV) is a well known enteric pathogen of dogs throughout the world causing hemorrhagic enteritis or gastroenteritis characterised by vomition, diarrhoea with foul smelling. The virus is transmitted mainly via faecal-oral route and large number of virus particles (>10^9 virus particles/gm) are excreted in the faeces of the affected animals. The virus multiplies in the rapidly dividing cells of the intestinal crypts thus exhibiting its pathogenecity. Despite widespread vaccination, CPV has remained as a widespread disease of dogs, because the virus forms new genetic and antigenic variants and thus leads to vaccination failure. The clinical diagnosis of CPV infection should be confirmed with laboratory tests and PCR is generally employed for the diagnosis of CPV due to its higher sensitivity, specificity and rapidity and its ability to detect even fewer organisms.

In this study, 120 faecal samples were subjected to PCR using 'H' primer pairs, but only 72 samples yielded a 630 bp product. Similar results were reported by Srinivas *et al.* (2013) [15] by using H_{for}/H_{rev} primers pairs in South India. Sequencing studies revealed that CPV-2a is predominant strain circulate in and around Namakkal region of Tamil Nadu. This is in agreement with Narayanan *et al*. (2001) [12], Chinchikar *et al*. (2006) [2] and Panneer *et al.* (2008) [13] who reported that prevalence of CPV 2a is more when compared to other mutants in South India.

Published sequencing of CPV-2a in Gene Bank indicated that most of the isolates were identical to Indian isolates followed by China, Thailand and Italy. All CPV-2b isolates were identical to China. Sequencing of PCR product and BLAST are the useful tool in aiding the epidemiological diagnosis.

Conclusion

In that study, CPV- 2a is predominant strain circulated in Namakkal region of Tamil Nadu and also low prevalence of CPV-2b. As far as sequencing study concerned that there is no notable nucleotide sequence variation in the key position of canine parvovirus strain of Namakkal region of Tamil Nadu in India.

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