

RAPID AND SENSITIVE METHOD OF DETECTION OF CANINE PARVOVIRUS INFECTIONS IN DOGS BY POLYMERASE CHAIN REACTION

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Canine parvovirus-2 (CPV-2) has been considered to be an important pathogen of domestic and wild canids and has spread worldwide since its emergence in 1978. It has been reported from Asia, Australia, New Zealand, the Americas and Europe. There are two distinct parvoviruses known to infect dogs – the pathogenic CPV-2 and the non pathogenic canine minute virus (CnMV). The disease is characterized by two prominent clinical forms, enteritis with vomiting and diarrhea in dogs of all ages and myocarditis and subsequent heart failure in pups of less than 3 months of age with high morbidity (100%) and frequent mortality up to 10% in adult dogs and 91% in pups. The disease condition has been complicated further due to emergence of a number of variants namely CPV-2a, CPV-2b, CPV-2c, New CPV-2a, New CPV- 2b and Asp 300 (2a/2b) over the years and involvement of domestic and wild canines. Vaccination is the most cost effective and ideal method to control the canine parvovirus infections in canines. A galaxy of immunoprophylactic agents is available in the market for use in dogs to control the disease. In spite of vaccination against CPV infections in dogs, the outbreaks has been reported in unvaccinated and vaccinated animals. So, the prompt and accurate diagnosis of the disease is of major importance to provide the therapeutic and supportive therapy to the affected animals. In this study, PCR has been employed to amplify the genomic DNA of CPV in the clinical samples and found to be rapid, sensitive and specific in detecting the positive cases in ailing animals.

Key words: Canine parvovirus 2(CPV-2), CPV-2a, CPV-2b, CPV-2c, New CPV-2a, new CPV-2b, Asp 300(2a/2b), DNA, Diagnosis, Polymerase chain reaction.

Introduction

Canine parvovirus – 2 (CPV-2), the causative agent of acute haemorrhagic enteritis and myocarditis in dogs, is one of the most important pathogenic viruses. It is a highly infectious and often fatal disease. CPV-2 was first recognized in 1977 and since then it has been well established as an enteric pathogen of dogs throughout the world with high morbidity (100%) and frequent mortality up to 10% in adult dogs and 91% in pups (Appel and Parrish, 1987). The disease is characterized by two prominent clinical forms (i) enteritis with vomiting and diarrhea in dogs of all ages (ii) myocarditis and subsequent heart failure in pups of less than 3 months of age. The virus was named CPV-2 in order to differentiate it from a closely related parvovirus of canine minute virus (CnMV). CPV is believed to have originated as a host range variant from feline panleucopenia. The original type (CPV-2) which emerged in the late 1970s was rapidly replaced by two antigenic variants, CPV-2a in

1979 and CPV-2b in 1984 (Parrish *et al.*, 1991; Truyen *et al.*, 1996). Further in 2000, a third type CPV-2c was first detected in Italy and found to be progressively replacing other variants in many countries of the European Union, South America, North America and Asia but Australia has been declared free of CPV-2c. Subsequently New CPV-2a, New CPV-2b and Asp-300 (2a/2b) have emerged in the canine population (Decaro and Buonavoglia, 2012). CPV affects puppies much more frequently than it affects adult dogs. The virus grows more frequently in rapidly dividing cells in intestinal lining and myocardium. The main source of infection is faeces of infected dogs and large number of virus particles (10^9 virus particles/g of faeces) is excreted in the faeces (Kumar and Nandi, 2010). The original virus (CPV-2) has been replaced by different antigenically variants viruses over the time and poses a real threat to the canine populations around the world (Decaro *et al.*, 2005a; Decaro *et al.*, 2006a; Decaro *et al.*, 2006b; Decaro *et al.*, 2007).

In India, the disease is highly prevalent and reported by many authors on the basis of various diagnostic tests namely ELISA, HA, HI, LAT, IF, IPT and PCR(Biswas *et al.*, 2005 ; Kaur *et al.*, 2006; Gunaseelan and Ramkrishna, 2003 ; Singh *et al.*, 2004 ; Phukan *et al.*, 2004). In spite of presence of a number of live attenuated and inactivated immunoprophylactic agents available in the markets, the disease is reported in pet dogs and other dog populations in India. The parvovirus vaccines available in the Indian market are as monovalent killed (Vanguard-CPV, Pfizer) or live attenuated polyvalent vaccines along with other viruses and bacterial vaccines (Megavac-6, Indian Immunologicals ; Duramune DA2 P+PV, Fort Dodge ; Novivac-Puppy DP, Intervet ; Novivac DHL, Intervet). In spite of presence of a number of vaccines in the market, disease outbreaks have been reported in unvaccinated as well as in vaccinated dogs (Dr J.P. Varshney, Personal communication). In this report, the clinical samples suspected

for CPV infections in dogs have been tested to diagnose the cases by PCR.

Materials and Methods

Faecal samples: A total of 79 faecal samples from dogs registered in Veterinary Polyclinics, Indian Veterinary Research Institute, Izatnagar, U.P., suspected for canine parvovirus infections were collected in virus transport medium (VTM) containing DMEM (with 2% FCS) supplemented with Streptomycin (0.1mg/ml) and Penicillin (500IU/ml) in sterilized container in the ratio of 1:10 over the period of 2year (January 2012 to December, 2013). The samples were brought to the laboratory maintaining the cold chain. The samples were stirred in solution and centrifuged at 10,000 rpm for 2 min at 4°C to settle down the debris at the bottom. 200 ul from each sample was collected from each sample and processed for DNA extraction. The details of the sample have been given in Table no 1.

Table 1. Breed wise, age wise and sex wise distribution of samples of dogs

Breeds	Male		Female		Total/PCR +	% +
	<1 yr (PCR+)	<1 yr (PCR+)	<1 yr (PCR+)	<1 yr (PCR+)		
GSD	05 (04)	05 (03)	03 (03)	02 (02)	15(12)	80
Doberman	02 (02)	05 (03)	03 (02)	06 (04)	16(11)	68.7
Spitz	05 (04)	09 (07)	04 (03)	07 (05)	25(19)	76
Non Descript	04 (04)	03 (03)	06 (05)	10 (08)	23(20)	86.9
Total	16 (14)	22 (16)	16 (13)	25 (19)	79 (62)	78.4

Extraction of DNA from sample: The total genomic DNA of the different rectal swabs was isolated by standard phenol-chloroform method. 200µl of sample supernatant was taken in a 1.5 ml microfuge tube to which 200 µl of Tris saturated phenol was added and centrifuged at 10,000 rpm for 3 min. Approximately 200 µl supernatant was taken out and put in a fresh microfuge tube.

100 µl of chloroform and 100 µl of Tris saturated phenol was added to it and centrifuged at 10,000 rpm for 3 min. Again 200 µl of supernatant was collected in a fresh microfuge tube and 200 µl of chloroform was added and centrifuged at 10,000 rpm for 3 min. 200 µl of supernatant was collected in a fresh microfuge tube and 20 µl of 3M sodium

acetate (pH 5.5) and 1 ml of chilled absolute ethanol were added, mixed and kept at -20°C for overnight or at -80°C for 2 h. The tubes were centrifuged at 10,000 rpm for 15 min. The supernatant was discarded and the pellet was washed with 70% ethanol at 10000 rpm for 5 min. The pellet was air dried and resuspended in 20 µl nuclease free water. The quantity and the purity of DNA were checked in Nanodrop spectrophotometer (ND-1000, Thermo-Scientific, USA).

Primer design: Nucleotide sequences of VP2 gene for CPV-2, CPV-2a, CPV-2b, CPV-2c, New CPV-2a and New CPV-2b were retrieved from GenBank

(www.ncbi.nlm.nih.gov/nucore) and assembled into multiple sequence alignment using the Clustal W program of Lasergene 6.0 (DNASTAR Inc, USA). Highly conserved region within the VP2 gene segment were identified and the primer was designed by GeneTool Lite 1.0 software (BioTools Inc., Edmonton, Canada). The primer sequence, position and the amplicon length is given in Table 2. Restriction sites of *Eco* RI and *Hind* III were incorporated in the forward and reverse primer sequence to clone the product in the expression vector for future studies. The primers were custom synthesized by Metabion GmbH, Martinsried, Germany.

Table 2 : Primer sequence and position in VP2 gene of CPV.

Primer name	Sequence of the primer	Length and position of nucleotide	Product length	Annealing temp.
CPVM-FP	5' GGCGAATTCTGATTGTAAACCATGTAGACTA 3'	31 (3590-3611)	648 bp	56°C
CPVM-RP	5' GGCAAGCTTTAAGTCAGTATCAAATTCTTTA TC3'	33 (4197-4220)		

PCR assay: The PCR for amplification of the antigenic region of the VP2 gene was performed by using 100 ng DNA, 5 µl 10X PCR buffer containing 15 mM MgCl₂, 1µl 10 mM dNTP, 10 µM of forward and reverse primers, 1.25 U of Taq DNA Polymerase (Fermentas, Lithuania) and the volume was made up to 50 µl with nuclease free water (NFW). The PCR amplification consisted of initial denaturation at 95°C for 5 min followed by 35 cycles of denaturation at 95°C for 1 min, annealing at 56°C for 1 min and extension at 72°C for 1 min, followed by a final incubation at 72°C for 10 min. The amplified products were analyzed on 1.0% agarose gel containing ethidium bromide to a final concentration of 0.5 µg/ml. 10 µl of amplified product was mixed with 2 µl of gel

loading (6X) dye and loaded into the well and run along with 100 bp to 1 Kb DNA ladder in 1X TAE electrophoresis buffer at 5 volts/cm² and the progress of mobility was monitored by migration of dye. At the end of the electrophoresis, the gel was visualized and photograph taken in gel documented system (Bio-Rad, USA).

Results and Discussion

Out of 79 faecal samples collected from different breeds of dogs particularly German Shepherds, Spitz, Doberman and nondescript suspected for CPV infection were subjected to genomic DNA extraction. 62 samples were found positive by PCR as revealed by the presence of specific DNA band of 648 bp (Fig.1). A PCR product of 648

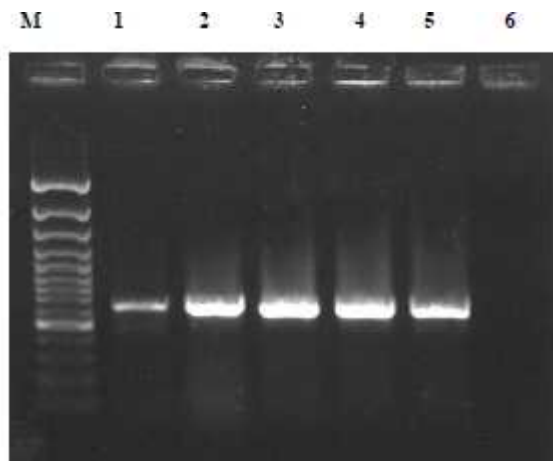


Fig. 1 : Showing amplification of specific region of VP2 gene by using primer set to yield a product of 648 bp. (M- Marker 100 bp ladder. 1- Positive control, 2- Faecal sample no 1, 3- Faecal sample no 2, 4- Faecal sample no 3, 5- Faecal sample no 4, 6- Negative control.)

The polymerase chain reaction has been applied to the detection of CPV-2 in clinical samples and shown to be rapid, sensitive and specific diagnostic method (Truen *et al.*, 1994; Uwatoko *et al.*, 1995). At the beginning FPLV vaccines were used to protect dogs against canine parvovirus infections. In pups, maternally derived antibodies (MDA) represent the main protection against CPV infections but at the same time they may interfere with CPV vaccination (Gooding and Robinson, 1982; Pollock and Carmichael, 1982; Chappuis, 1998). With the reporting of CPV-2 infections in dogs, vaccines using the homologous virus adapted in the cell culture system have become available to be used in dogs. However, vaccine manufacturing companies do not mention the strains of CPV used in the vaccine for immunization purposes. The vaccine strains might have been picked up at the time of evolution of the CPV-2. With the time, CPV-2a and CPV-2b strains have replaced the original CPV-2 around the world (Parrish *et al.*, 1985) and there is an urgent need to pick up the strain currently available in the field and responsible for causing the disease in dogs both wild and domesticated. Another reason

is that as the CPV-2 vaccines is along with other vaccines there is competitive interference /suppression of one antigen by other potent antigen to be processed by the immune system in eliciting the protective immune response. Further, it is to be noted that field veterinarians do not follow either the uniform strategy in vaccinating the pups at the particular age or prior estimating the MDA. In the present study, it has been observed that CPV infections in prevalent in different breeds of dogs and particularly rampant nondescript dogs. All the commercially available vaccines mentioned here contain the CPV as tested in our laboratory by PCR.

The maternally derived immunity strongly protects young animals from CPV infection by sequestering the virus prior to the onset of viraemia, thus preventing colonization of the intestinal epithelium (Macartney *et al.*, 1988). Although CPV-2 based vaccine is mostly used in the fields but the incorporation of new variants (CPV-2a/2b) would have made the vaccine more effective in eliciting protective immune response as dogs infections by parvovirus is caused by the CPV-2a/2b and New CPV-2a/2b in the recent years. Further, it is

prudent to use the CPV-2a/2b vaccines based on the prevalence of the strain present in the area and more efficiently based on the testing of the random samples of a herd/flock for the presence of MDA to make the vaccination programme a successful one and to control the disease to a great extent (Decaro *et al.*, 2005).

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