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Molecular Cloning and Restriction Endonuclease Analysis of Canine Parvovirus DNA Amplified by Polymerase Chain Reaction

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Abstract: Canine parvovirus 2 (CPV-2) is a single stranded DNA virus of *Parvoviridae* family that causes enteritis and myocarditis in canines. DNA was isolated from an isolate of CPV-2 and 765 bp sequence of VP-2 gene was amplified by PCR using VP2 specific primer. The amplification was highly efficient as evident by single DNA band in agarose gel. The PCR product generated was ligated in pGEM-T Easy vector by TA cloning. The recombinant plasmid DNA was isolated and digested with *Bam* H I and *Sal* I restriction enzymes, which yielded the fragment of vector DNA of 3015 bp and the insert of 765 bp.The insert was further characterized by restriction enzyme (RE) mapping using *Hha* I. It was concluded that PCR along with RE analysis of amplicons can be employed successfully for detection and characterization of CPV which in turn would help in proper and effective management and control of the disease.

Key words: Canine parvovirus · Enteritis · PCR · RE mapping

INTRODUCTION

Canine parvovirus-2 (CPV-2) is the causative agent of acute hemorrhagic enteritis and myocarditis in dogs and it is one of the most common pathogenic viruses causing diarrhoea in dogs. CPV-2was first identified in USA in 1978 [1]. Canine parvovirus is antigenically and genetically related to Feline Panleukopenia virus (FPV). Around 1979, a variant of CPV-2 designated as CPV-2a became wide spread [2]. By 1981 CPV-2a was most frequently isolated from domestic dogs with clinical diseases in United States, Japan, Denmark, Australia and CPV-2 was rarely seen after that time [2-4]. In 1984, a further antigenically variant virus was detected which differed in only a single epitope, designated as CPV-2b [2]. In India, Ramadass and Khader [5] was the first to confirm that CPV-2 is the etiologic agent of disease in dogs. Subsequently several incidences of the disease have been reported from different parts of the country by several workers [6-8]. A number of methods are available to diagnose the disease namely virus isolation in the cell culture (MDCK, CRFK, A-72 cell line), agarose gel precipitation test, electron microcopy, haemagglutination (HA) test, IFT, ELISA and PCR [4]. Virus isolation is highly specific and gold standard, but it is a time consuming and expensive method. The HA and HI tests are simple, inexpensive and easy to perform, but requires continuous supply of RBCs of porcine origin and presence of non-specific agglutinin in faeces makes HA test less reliable for CPV diagnosis [4]. The polymerase chain reaction has been applied to the detection of CPV-2 in faecal samples with high sensitivity and specificity [9].

The present work was taken to amplify part of VP-2 gene of CPV-2 using specific primer followed by its characterization by RE mapping.

MATERIALS AND METHODS

Virus: A cell culture adapted CPV-2 isolate maintained at Virus Laboratory, CADRAD, IVRI, Izatnagar was cultivated in MDCK cells and grown in Dulbecco's modified Eagles medium (DMEM).

Madin Darby Canine Kidney (MDCK) cell line: MDCK cell line was obtained from National Centre for Cell Science, Pune. It was maintained at the Virology lab. by using Dulbecco's modified Eagle's medium (DMEM) (Life Technologies) with 10% fetal calf serum (FCS) as growth medium. Gentamicin was added in the medium at the rate of 50 mg/l (Life Technologies).

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Isolation of Virus and DNA Extraction: MDCK cells were grown in 25cm^2 cell culture plastic flask containing Dulbecco's MEM medium with 10 % fetal calf serum and were used to isolate the virus by adsorption method. When cell monolayer is grown to 70 per cent of cell culture flask, it was washed with medium. The infection was given with 0.5ml of processed faecal sample as inoculum and incubated for 1 h at 37°C for adsorption. After incubation, the infected cell monolayer was washed three times with DMEM and 5ml of DMEM medium with 2 % fetal calf serum (FCS) was added. The infected cells were incubated at 37°C for 3-5 days. After three passages in the MDCK cell line, specific changes including rounding of cell, granulation and aggregation of cells indicative of cytopathic effect were observed in CPV-2 infected MDCK cells. Then the virus was harvested and kept at -20°C for further use.

The genomic DNA of CPV-2 was extracted using DNAzol reagent from the CPV-2 infected MDCK cell lines [10].

PCR Assay: The primers pCPV-2bC (F and R) selected from the variable regions of the VP2 capsid gene of the CPV-2 were designed and custom synthesized to yield a product of 765 bp. The primer set used to clone the PCR product having the forward primer was having the restriction site for *Bam* H I, while the reverse primer was having the restriction site for *Sal* I in order to facilitate its sub cloning into the expression vector in *E. coli* system.

pCPV-2bC FP 5' CCG GCC GGA TCC ATG AGA CCA GCT GAG GTT GGT 3' (33mer)

pCPV-2bC RP- 5' CCG GCC GTC GAC TTA ATA TAA TTT TCT AGG TGC 3' (33mer)

The PCR was performed in 200 µl thin layered PCR tubes (Axygen) with a reaction volume of 50 µl. The pCPV-2bC primer sets (Imperial Biomedics) was used to amplify part of VP2 gene of CPV to yield a product of 765 bp. The reaction mixture included 5 µl of *Taq* DNA polymerase buffer, 3 µl of $MgCl_2$ (25 mM), 1 µl of each dNTP (200 µM), 10 pmol of each forward and reverse primer, 5 µl of extracted DNA template and 1 µl of *Taq* DNA polymerase (1U/µl) were added. Amplification was performed in a thermocycler (Applied Biosystems). The cyclic condition was denaturation at 95°C for 30 sec, primer annealing at 58°C for 1 minutes and extension at 72°C for 2 minutes. The cyclic condition was repeated for 30 times and a final extension at 72°C was given for 5 minutes. After PCR, the amplified products were analyzed on 1.0% agarose gel containing ethidium bromide [11].

The PCR products were purified from gel using QIAquick gel extraction kit (QIAGEN Inc. Valentia, USA) as per the manufacturer's protocol. After purification one µl of purified product was checked by agarose gel electrophoresis.

Cloning of PCR Product: The PCR product was cloned in pGEM-T Easy vector (Promega, Madison, USA) following the manufacturer's instruction. The ligation reaction mixture of 20 µl volume was made in a pre-chilled 0.5 ml microfuge tube and ligation reaction contains 6 µl of purified PCR product (about 25 ng DNA), 2 µl of T_4 ligase buffer (10 X), 1 µl of vector, 1 µl of T_4 DNA ligase (3U/µl) and rest nuclease free water. It was then mixed gently and incubated at 4°C temperature for overnight. The competent cell was prepared from overnight culture of *E. coli* DH5á by calcium chloride method and transformation was carried out by heat shock method. The recombinant colonies were screened by blue and white screening [10].

Plasmid DNA Isolation and Confirmation of the Recombinants: The plasmid DNA from recombinant bacterial colonies was extracted by alkaline lysis method and purified by extracting with phenol-chloroform method [10]. The recombinant plasmid was checked for the presence of desired insert by restriction endonuclease (RE) analysis. The plasmid DNA was digested with restriction enzymes *Bam* H I and *Sal* I (Life technologies) in order to release the insert for confirmation of positive clone. The reaction mixture consist of 3.0 µl of 10 X RE universal buffer, 1.0 µl of *Bam H* I, 1.0 µl of *Sal* I, 5 µl of plasmid DNA and nuclease free water to make 20 µl. The released products were purified from gel using QIAquick gel extraction kit (QIAGEN Inc. Valentia, USA) as per the manufacturer's protocol. After purification, purified product was checked by agarose gel electrophoresis. The insert of 765 bp was carried out using restriction enzymes *Hha* I (Life technologies) selected on the basis of sequence analysis by using M/S DNASTAR Inc, USA Software which have restriction site at 520 bp and gives two products of 520 bp and 245 bp.

RESULTS

Specific cytopathic changes including rounding of cell, granulation and aggregation of cells were observed in CPV 2 infected MDCK cells after 72h of post infection which increased subsequently and was widely distributed

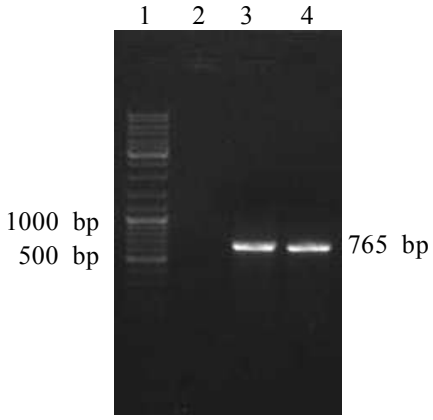

Fig. 1: Agarose gel showing amplification of specific region of VP1/VP2 gene by using pCPV-2bC primer set to yield a product of 765 bp.
M- Marker 100 bp ladder.
1- Negative control (Sample from healthy dog).
2- Cell culture adapted isolate.
3- Positive control.

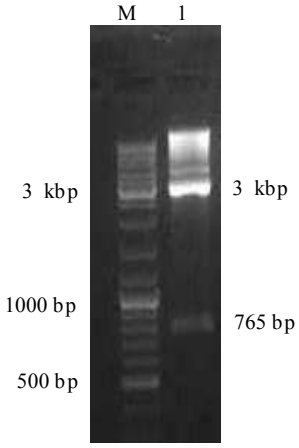

Fig. 2: Agarose gel showing release of insert (765 bp) from recombinant plasmid after double digestion with *Bam H*I and *Sal I* and vector (3 kb).

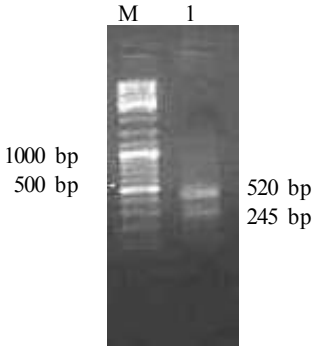

Fig. 3: Agarose gel showing RE analysis of insert of 765 bp with *Hha* I

in whole monolayer resulting in detachment of cell monolayer from the surface 4 days post infection. The viral DNA, isolated from culture fluid using DNAzol reagent method, was sufficient for PCR amplification. A specific product of 765 bp was observed in 1% agarose gel electrophoresis after amplification whereas, in the negative control, no amplification of template DNA was visualized on an agarose gel (Fig 1).

The purified product of 765 bp from the VP-2 genes was used for ligation into the pGEM-T Easy vector and transformation was carried out in *E. coli* DH5á cells. Three recombinant colonies were picked and grown in LB broth containing ampicillin and recombinant plasmid DNA was extracted. 3 µl of extracted plasmid DNA was electrophoresed on a 1% agarose gel and three bands (super coiled, open circular and linear) were observed on gel documentation.

The restriction enzymes analysis of recombinant plasmid was carried out with *Bam H* I and *Sal* I enzyme to release the 765 bp insert DNA from 3.015 kbp vector DNA as expected (Fig. 2). As the PCR have several disadvantages like non-specific amplification and carry over the contamination, so to confirm the authenticity of the PCR product RE digestion was carried out. The released insert of 765 bp was RE digested using *Hha* I enzyme. The RE enzyme *Hha* I cut the PCR product at 520 bp and produced 520 bp and 245 bp fragments as expected (Fig. 3) and further substantiated the released insert.

DISCUSSION

Parvovirus infection is an important emerging disease of dogs that mainly the intestinal tract and causes vomiting, diarrhea, fever and decreases the ability to fight against the infections [12]. After its emergence, CPV spread to most populations of domestic and wild carnivores [13]. Khadilkar *et al*. [14] reported that the disease is widely prevalent in Asia. After the isolation of CPV-2 for the first time in India by Ramadass and Khader [5], several incidences of the disease have been reported from different parts of the country [6, 8, 15]. The incidence of CPV infections of dogs in India and its diagnosis employing several diagnostic tests namely LAT, HA, AGPT, ELISA and PCR have been reported by several authors [7, 16, 17]. In spite of the presence of a number of diagnostic tests, PCR followed by RE analysis would be a valuable addition and considered to be the highly sensitive, specific and rapid to provide accurate diagnosis which in turn has enormous impact in the efficient and effective management of the disease.

In the present study, specific cytopathic changes were observed in CPV 2 infected MDCK cells after 72h of post infection. The viral DNA, isolated from culture fluid using DNAzol reagent method, was sufficient for PCR amplification.. However, presence of inhibitory substances in the faecal specimens may interfere with the PCR and produces negative results even when the virus is present in the faecal samples [18]. Elimination of inhibitory substances increases the sensitivity of PCR tests for identification of pathogens in the faecal samples [19]. Schunk *et al.* [9] reported that both phenol/chloroform extraction of DNA after SDS and proteinase-K treatment and boiling protocol can be used effectively for sensitive detection of CPV in stool samples. Uwatoko *et al.* [18] reported that gel filtration and boiling of the samples can remove or inactivate inhibitory substances in the faecal substances. However, DNA extraction with DNAzol can successfully eliminate the inhibitory substances and amplification of desired product. The amplified DNA was purified from gel and could be successfully ligated into pGEM-T Easy vector and used to transform *E. coli* DH5á cells.

The recombinant plasmid developed in this study may be used to characterize the CPV-2 genomic fragment and to carry out further molecular studies like sequencing and to develop probe by labeling either with radioisotopic or non-isotopic labels. This kind of labelled DNA probe can be used to detect CPV-2 in clinical sample or cell culture infected with CPV-2. The released insert may be further subcloned in expression vector like pQE-30 in *E. coli* M15 cells for expression of recombinant protein as this part of VP-2 gene encodes some immunodominant antigenic sites [20]. Such recombinant protein may be used to develop indirect ELISA for screening large number of serum samples for sero-serveillance studies and to measure the antibody level following vaccination against CPV infection in dogs.

In conclusion, CPV has been isolated successfully in MDCK cell line and self designed primer set has been used for precise amplification of CPV genomic DNA. The result has been further confirmed by digestion of amplicons with *Hha* I enzyme. The PCR followed by RE analysis based diagnosis of CPV suspected samples would be a valuable addition as diagnostic for prompt and more accurate diagnosis of the disease. Further, the amplicons can be used as cold or hot probe in hybridization experiment to detect the CPV genomic DNA or can be expressed as recombinant protein in *E. coli* based expression system to be used in indirect ELISA for serosurveillance of the disease.

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