Strain differentiation and characterization of canine parvovirus by PCR and RE mapping

S Nandi*, R Anbuzhagan and Manoj Kumar
Centre for Animal Disease Research and Diagnosis (CADRAD)
Indian Veterinary Research Institute (IVRI), Izatnagar 243 122, India

Received 20 January 2009; revised 1 June 2009; accepted 6 August 2009

Canine parvovirus 2 (CPV-2) is a highly contagious and fatal disease of dogs, causing acute hemorrhagic enteritis and myocarditis. In this study, different mutant of CPV2 prevalent in India have been isolated in Madin-Darby canine kidney (MDCK) cells and characterized by polymerase chain reaction (PCR) and restriction endonuclease (RE) mapping. The faecal samples from gastroenteritic dog suspected for CPV2 infection were collected in a suitable medium, processed and inoculated in MDCK cells. The viral DNA from faecal samples was extracted using phenol-chloroform method. PCR were carried out with two different primer pairs, pCPV-2ab and pCPV-2b, to distinguish the strain prevalent in field condition. The primer pCPV-2ab recognized both the variant CPV-2a and CPV-2b, whereas the primer pCPV-2b recognized only the variant CPV-2b, enabling the differentiation of CPV-2a variant from CPV-2b in field isolates. The different PCR products were further analyzed by using RE mapping.

Keywords: Canine parvovirus, haemorrhagic enteritis, MDCK, PCR, RE mapping

Introduction

Canine parvovirus 2 (CPV-2) causes a highly contagious and often fatal disease, characterized by vomition and hemorrhagic gastroenteritis in dogs of all age, and myocarditis and subsequent heart failure in pups of less than 3 month of age. CPV-2 emerged in 1978 as the cause of new disease in dogs throughout the world, when it rapidly spread in domestic dog populations as well as wild dogs with high morbidity (100%) and frequent mortality (up to 10%). The main source of infection is faeces of infected dogs for large numbers of virus particles (10^9 virus particles/g of faeces) are excreted in the faeces. Between 1979 and 1981 the original (1978) strain of the virus (CPV-2) had been replaced by a genetically and antigenically variant strain termed as CPV-2a. The two viruses differed in 5-6 amino acids, which constitute two different neutralizing antigenic sites on the surface of the capsid. In 1984, a further antigenically variant virus was detected which differed in only a single epitope, designated as CPV-2b. The CPV-2, a non-enveloped virus with an approximate diameter of 20 nm, is a member of the genus Parvovirus of the family Parvoviridae.

In the present study, CPV-2 present in India has been isolated in Madin-Darby canine kidney (MDCK) cells, followed by characterization and strain differentiation by polymerase chain reaction (PCR) and restriction enzyme (RE) mapping. PCR were carried out using two different primer pairs pCPV-2ab and pCPV-2b to distinguish the different strain of CPV-2 prevalent in India.

Materials and Methods

Collection of Faecal Sample

The faecal samples were collected from 12 dogs came to Veterinary Polyclinics, Indian Veterinary Research Institute, Izatnagar and showing the symptoms of fever, diarrhoea or hemorrhagic diarrhoea and vomition, clinically suspected for CPV infections. The faecal samples were collected in the form of rectal swab in Hank’s balanced salt solution (HBBS) in the ratio of 1:9, containing Streptomycin (100 mg/L) and Penicillin (1 lakh IU/L).

Faecal Sample Preparation

The faecal samples collected were filtered through a disposable syringe filter (0.45 µm; Millex, Milipore) and then centrifuged at 10,000 rpm at 4°C for 3 min in a refrigerated centrifuge. The supernatant was carefully pipette out and used for PCR amplification of VP2 gene of CPV2. A commercially available
inactivated vaccine was used as positive control of CPV. As a negative control stool sample from the healthy dog processed similarly was used.

**Virus Isolation**

MDCK cells were grown in 25 cm² cell culture plastic flask containing Eagle’s minimal essential medium (EMEM) with 10% fetal calf serum (FCS). The infection was given with 0.5 mL of processed faecal sample as inoculum and incubated for 1 h at 37°C for adsorption when cell monolayer was 70% complete. After incubation, the infected cell monolayer was washed three times and 5 mL of EMEM medium with 2% FCS was added. The infected cells were incubated at 37°C for 3-5 d or till cytopathic effect was observed. The genomic DNA of CPV2 was extracted by DNAzol reagent method from the CPV2 infected MDCK cell lines.

**PCR Assay**

The PCR protocol had been standardized for the primer set pCPV-2ab and pCPV-2b, as reported by Pereira et al with slight modifications. The details of primers are given in Table 1. The primer set pCPV (F & R) used to amplify the entire genome of the CPV was custom designed and synthesized to yield a PCR product of 2.2 kbp. The PCR protocol had been standardized employing the varying concentration of MgCl₂ and annealing temperature as mentioned and there by pCPV-2ab and pCPV-2b, as reported by Pereira et al.

The PCR reaction mixture contained 200 µM dNTPs, 10 pmol of each primer, 1× PCR reaction mixture containing 15 mM MgCl₂ and 5 µL of processed sample as source of template DNA. Amplification was performed in a thermocycler (Applied Biosystems). 1 µL of DNA polymerase (1 IU/µL) was added to above reaction mixture after initial denaturation was done at 95°C for 5 min in the thermocycler. The cyclic condition was denaturation at 95°C for 1 min, primer annealing at 55°C for 2 min and extension at 72°C for 30 sec. The cyclic condition was repeated for 30 times and a final extension at 72°C was given for 10 min. After PCR, 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 bromophenol dye (6×) and loaded into the well and run along with 100 bp to 1 Kbp DNA ladder in 1× 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 under the UV transilluminator.

The PCR products were purified from gel using QIAquick gel extraction kit (QUIAGEN Inc. Valenta, USA) as per the manufacturer’s protocol. After purification 1 µL of purified product was checked by agarose gel electrophoresis.

**Characterization of PCR product by RE Mapping**

RE mapping of PCR product of VP1/VP2 gene of CPV2 was carried out using REs, PstI, BglII and PvuII (Life Technologies), selected on the basis of sequence analysis by using M/S DNASTAR Inc, USA Software.

To 4 µL of PCR product taken in 3 separate Eppendorf tubes, 1U of RE, either PstI, BglII or PvuII (10 U/µL), was added with 4.5 µL of 10× respective RE buffer and the volume was made to 30 µL with nuclease free water and incubated at 37°C for 4-6 h. The enzyme activity was stopped by freezing at −20°C. The RE digests, thus, obtained was

<table>
<thead>
<tr>
<th>No.</th>
<th>Forward and reverse primers</th>
<th>Primer sequence</th>
<th>Position of the genome</th>
<th>Annealing temperature and product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>pCPV-2ab (F)</td>
<td>5’-GAA GAG TGG TTG TAA ATA ATT-3’ (21 mer)</td>
<td>3025-3045 to 3685-3706</td>
<td>55°C 61 bp</td>
</tr>
<tr>
<td></td>
<td>pCPV-2ab (R)</td>
<td>5’-CCT ATA TAA CCA AAG TTA GTA C-3’ (22 mer)</td>
<td>4043-4062 to 4449-4470</td>
<td>55°C 427 bp</td>
</tr>
<tr>
<td>2</td>
<td>pCPV-2b (F)</td>
<td>5’-CTT TAA CCT TCC TGT AAC AG-3’ (20 mer)</td>
<td>2285-2305 to 4517-4540</td>
<td>57°C 52 bp</td>
</tr>
<tr>
<td></td>
<td>pCPV-2b (R)</td>
<td>5’-CAT AGT TAA ATT GGT TAT CTA C-3’ (22 mer)</td>
<td></td>
<td>55°C 427 bp</td>
</tr>
<tr>
<td>3</td>
<td>pCPV-(F)</td>
<td>5’ – GGG GAA TTC ATG GCA CCT CCG GCA AAG AGA – 3’ (30 mer)</td>
<td></td>
<td>2.2 kb</td>
</tr>
<tr>
<td></td>
<td>pCPV-(R)</td>
<td>5’ – GGC TCT AGA TTA ATA TAA TTT TCT AGG TGC TAG – 3’ (33 mer)</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>
electrophoresed in 1.2% agarose gel at 80 volts for 1 h. The gel was visualized in UV transilluminator/Gel documentation system.

**Results and Discussion**

In the present study, PCR was carried out taking 12 stool samples collected from CPV suspected dogs and used as template to amplify the VP1/VP2 structural gene of CPV genome. The PCR assays have been found to be the highly sensitive as only 5 µL of processed samples revealed the amplified product. Of 12 stool samples from the suspected cases of CPV infections, 9 were found to be positive by pCPV-2b primer set, whereas all of them are amplified by pCPV-2ab primer set\(^5,6\). The pCPV-2ab primer set amplified portion of VP1/VP2 gene of both CPV-2a and CPV-2b variants (3025 to 3706 nucleotide position of CPV genomic DNA) to yield a product size of 681bp (Fig. 1). The pCPV-2b primer pair amplified specific portion VP1/VP2 gene of only CPV-2b (4043 to 4470 nucleotide position of CPV genomic DNA) to yield a product size of 427 bp and thereby differentiate between CPV-2a and CPV-2b\(^8\) (Fig. 2). So the results showed that out of 12 samples 9 were of CPV-2b variant, while 3 were CPV-2a strain.

The 2.2 Kbp amplicons denoting the entire length of the structural gene coding for the structural protein of the CPV-2 was amplified using the primer set pCPV (Fig. 3). RE mapping of all the PCR product of VP1/VP2 gene of 2.2 kbp obtained from processed faecal sample were carried out using PstI, BglII or PvuII as explained. The Pvu II had unique cleavage site at 1501/1502 bp that yielded two fragments of...
1501 bp and 755 bp in length (Fig. 4). RE mapping was also carried out with REs BglII and PstI, which had unique cleavage site at 1072/1073 and 774/775, respectively that yielded two DNA fragments of 1072 bp and 1184 bp, and 774 bp and 1482 bp in length, respectively (Fig. 5). This further substantiates the result of PCR.

Canine parvovirus infections have been emerged as a most important killer disease of pups in recent time as it causes vomition, myocarditis and hemorrhagic gastroenteritis. Although adult dogs show less severe symptoms of gastroenteritis, the dogs serve as source of infection. Further due to its immunosuppressive nature, CPV decreases the animal’s ability to fight against infections. After emergence of CPV-2, two more mutants, namely CPV-2a and CPV-2b, have been reported and completely replaced the original strain (CPV2) around the world. Decaro et al identified different variants of CPV circulating in dog population in Spain. Truyen et al studied that CPV-2a and CPV-2b have almost completely replaced the original CPV2 in canine population in Germany. Pereira et al reported that the predominant strain found in Brazil during 1980 was CPV-2a and CPV-2b during 1990-1995. Wang et al reported both antigenic types CPV-2a and CPV-2b prevailing in Taiwan. Battilani et al showed that both antigenic types 2a and 2b co-exist in canines in Italy. Ramadass and Khader have isolated the CPV2 for the first time in India. Although several workers have reported the incidence of CPV infection in India, very little work has been carried out about the prevalence of novel mutants of CPV in dogs.

Thus, PCR technique can be adopted to diagnose rapidly, reproducibly and accurately the CPV infections. Further, different antigenic variants of CPV can also be differentiated by employing PCR with different combination of primer sets. RE digestion further substantiates the specificity of the PCR. From the present study, it is inferred that CPV-2b is more prevalent in dog population in India as revealed in PCR based diagnosis. So, necessary measures should be taken to control the disease in dogs by incorporating the indigenous strain of CPV in the preparation of vaccine.

**Acknowledgement**

Authors thank the Director, Indian Veterinary Research Institute, Izatnagar for providing facilities to carry out the work.

**References**

4. Parrish C R, Aquadro C F & Carmichael L E, Canine host range and a specific epitope map along with variant sequences in the capsid protein gene of canine parvovirus


