A sensitive method to detect canine parvoviral DNA in faecal samples by nested polymerase chain reaction

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Canine parvovirus 2 (CPV-2) is the causative agent of acute hemorrhagic enteritis and myocarditis in dogs. A nested polymerase chain reaction (n-PCR) was developed using published pCPV-2ab as external primer set and self designed and custom synthesized pCPV-2N as internal primer set for the detection of canine parvovirus in faecal samples of dogs. Out of 52 faecal samples, 27 and 31 were tested positive by one step PCR and n-PCR, respectively. Among the different breed of dogs, German shepherd was found to be highly susceptible (69.2%), followed by non-descript breeds (63.1%) and overall positivity was 59.6%. Ten-fold dilution of known cell culture supernatants was tested and it was observed that one step PCR had a detection limit of $10^{-5}$ dilution, whereas nested-PCR had up to $10^{-8}$ dilution. Thus, the nested-PCR seems to be a sensitive, specific and practical method for the detection of CPV in faecal samples.

Keywords: Canine parvovirus, gastroenteritis, nested-PCR.

Introduction

Canine parvovirus 2 (CPV-2) causes a highly contagious and often fatal disease in puppies. It is characterized by vomiting and hemorrhagic gastroenteritis in dogs of all age and myocarditis in pups of less than 3 months of age. CPV-2 is a member of the genus Parvovirus in the family Parvoviridae. The virus has icosahedral symmetry, 25 nm in diameter and non-enveloped with a linear single-stranded and negative-sense DNA genome of about 5.2 kb, which has two promoters resulting in the expression of two structural (VP1 and VP2) and two non-structural proteins (NS1 and NS2) through alternate splicing of the viral mRNAs. VP2 (64 kDa) is an NH2-terminally truncated form of VP1 (84 kDa) and plays an important role in virus pathogenicity and the host immune response. 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 in wild dogs. The main source of the infection seems to be the faeces of infected dogs as more than $10^9$ virus particles/g of faeces can be shed during acute phase of the enteric form and virus survives at least 4 months in the faeces in the environment. CPV-2 in the faeces can be detected by several methods, viz., virus isolation in cell culture, haemagglutination (HA) assay, electron microscopy (EM), ELISA, DNA hybridization and PCR. In Recent years, the PCR technique has been increasingly used as a tool for the diagnosis of CPV infection. Further, nested-PCR (n-PCR), real time PCR, loop-mediated isothermal amplification (LAMP), nucleic acid hybridization or dot blot, in situ hybridization, etc with varying degree of sensitivity and specificity have been employed for detection of CPV-2 genomic DNA. To increase the sensitivity and specificity of the PCR reaction, n-PCR was developed for detection of CPV genomic DNA.

In this study, n-PCR for the detection of CPV was developed to provide sensitive and accurate diagnosis with increased sensitivity and specificity. The n-PCR was designed with double primer sets, pCPV-2ab as external primer set and pCPV-2N as inner primer set. The sequences of the inner PCR primer pair were selected based on the nucleotide sequences of VP2 gene of CPV-2 using PrimerSelect software of DNAStar Inc, USA.

Materials and Methods

Sample Preparation

Faecal samples were collected from 52 dogs presented to the referral polyclinic of Indian Veterinary Research Institute (IVRI), Izatnagar, showing the symptoms of fever, diarrhoea or hemorrhagic diarrhoea and vomiting, similar to CPV
infections. The 52 clinical cases were comprised of 13 Pomeranian, 7 Doberman, 13 German shepherd and 19 non-descriptive breeds. Of this, 33 cases were from males and 19 from females. The fecal 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 (100,000 IU/L). They were centrifuged at 10,000 rpm at 4°C for 3 min in a refrigerated centrifuge and the supernatant was carefully pipetted out, followed by filtration through a disposable syringe filter (0.45 µm; Millex, Milipore).

**Genomic DNA Extraction**

The genomic DNA of CPV-2 from the stool samples was extracted by phenol-chloroform method. To remove inhibitory substances, 200 µL of sample was treated with sodium dodecyl sulphate (SDS) and proteinase K with a final concentration of 1% and 250 µg/mL, respectively and kept at 56°C for 30 min. 200 µL of Tris saturated phenol, chloroform and amyl alcohol (25:24:1) was added to 200 µL of sample in an eppendorf tube and mixed thoroughly. Then, it was centrifuged at 10,000 rpm for 5 min and the supernatant was collected in another eppendorf tube. Then 1/10th volume of 3 M sodium acetate (pH 5.5) and 1 mL of chilled ethanol were added, mixed and kept at –20°C for overnight or at least 1 h. The tubes were centrifuged at 12,000 rpm for 15 min. The supernatant was discarded and the pellet was washed with 500 µL of 70% ethanol, followed by centrifugation at 12,000 rpm for 2 min. The ethanol was discarded and pellet was dried and re-suspended in 25 µL nuclease free water. The purity of DNA was checked by Nanodrop spectrophotometer (Thermo-scientific, USA) by taking 260/280 nm ratio, which was found to be between 1.7 to 1.8 and the concentration of DNA was 20 ng/µL.

**Nested-Polymerase Chain Reaction (n-PCR)**

The n-PCR was standardized using primer set specific for pCPV-2ab as external primer, while internal primer set (pCPV-2N) was designed (Table 1).

The pCPV-2ab primer set is known to amplify part 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 681 bp. Similarly, pCPV-2N primer set is known to amplify part of VP1/VP2 gene (3188 to 3630 nucleotide position of CPV genomic DNA) to yield a product size of 442 bp.

The one-step or conventional PCR was performed in a total volume of 50 µL using pCPV-2ab as primer. The reaction mixture consisted of 5 µL template DNA, 5 µL of 10× Taq buffer (containing 15 mM MgCl$_2$), 1 µL each of forward and reverse primer (10 pmol), 1 µL of dNTPs mix (10 mM) 1 µL of Taq DNA polymerase (1 U/µL) and nuclease free water up to 50 µL. The cycling conditions for pCPV-2ab primers included one cycle of initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 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 and run along with 100 bp DNA ladder as marker in 1× TAE electrophoresis buffer.

The n-PCR was carried out following the same procedure and ingredients except primer set and amplicon of first round of PCR as template. The cycling conditions included one cycle of initial denaturation at 94°C for 5 min, followed by 30 cycles of 94°C for 30 sec, 55°C for 1 min and 72°C for 1 min, and a final extension at 72°C for 5 min. For knowing the relative sensitivity of the n-PCR over conventional PCR, 10-fold serial dilution of known titre of CPV-2 (10$^{4.5}$ TCID$_{50}$/mL) was put for conventional PCR and n-PCR as described above and the results were compared.

**Results and Discussion**

The CPV-2 was reported for the first time in India in 1982 by Ramadass and Khader. Since then, a large number of outbreaks have been reported from

<table>
<thead>
<tr>
<th>Primers</th>
<th>Sequences (5’→3’)</th>
<th>Position</th>
<th>Product size</th>
</tr>
</thead>
<tbody>
<tr>
<td>pCPV-2ab (F)</td>
<td>GAA GAG TGG TTG TAA ATA ATT (21mer)</td>
<td>3025-3045</td>
<td>681 bp</td>
</tr>
<tr>
<td>pCPV-2ab (R)</td>
<td>CCT ATA TAA CCA AAG TTA GTAC (22mer)</td>
<td>3685-3706</td>
<td></td>
</tr>
<tr>
<td>Nested primers</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>pCPV-2N (F)</td>
<td>TGA GCT GCA TTT AGT TAG TTT GGA (24mer)</td>
<td>3188-3211</td>
<td>442 bp</td>
</tr>
<tr>
<td>pCPV-2N (R)</td>
<td>TGT TTG CCA TGT ATG TGT TAG TCT (24mer)</td>
<td>3607-3630</td>
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</tbody>
</table>
different parts of the India in dogs involving different variants of CPV (2, 2a, 2b & 2c) both in vaccinated and non-vaccinated animals. A number of methods are available to diagnose the disease that includes virus isolation in the cell culture (MDCK, CRFK, A-72 cell line), HA test, electron microscopy, ELISA and agarose gel precipitation test. The virus isolation and electron microscopy are highly specific but time consuming and expensive. The HA and HAI tests are simple, inexpensive and easy to perform but are less sensitive and may give non-specific results. In recent years, the polymerase chain reaction (PCR) has become a useful and indispensable technique for detection of CPV-2 in clinical samples. PCR can detect fewer virus particles than ELISA and it is also 10 to 100-folds more sensitive than electron microscopy. N-PCR for the detection of CPV-2 was developed to increase the sensitivity and specificity of detection of the virus. Hirasawa et al. developed and compared n-PCR with one-step PCR and found that the n-PCR is more sensitive, specific and practical method for the detection of CPV in faecal samples.

In the n-PCR, pCPV-2ab primer set was used as external primer and pCPV-N primer set as internal primer. Of 52 samples tested, 27 were found positive with one-step PCR and 31 samples with n-PCR (Table 2). The pCPV-2ab primer amplified part of the VP2 gene of both CPV-2a and CPV-2b variants to yield a product size of 681 bp as expected (Fig. 1). The designed internal primer set pCPV-N amplified a product of 442 bp in length as desired (Fig. 2). However, only the 442 bp bands were observed after the optimization of the test. In some samples, a 681 bp band was also observed, probably due to the transfer of external PCR products used as a template for the second round of PCR. Of 31 dogs with confirmed parvoviral gastroenteritis, 8 were Pomeranian, 2 were Doberman, 9 were German shepherd and 12 were nondescript. The overall per cent positivity was 59.6% and it was highest in German shepherd (69.2%), followed by non-descript breeds (63.1%), Pomeranian (61.5%) and Doberman (28.5%). The 4 samples, which were negative with the conventional PCR, were also found to be positive with the n-PCR, reflecting the more sensitive assay than conventional PCR. The sample containing very few numbers of virus particles along with some inhibitory substances sometimes can not be visualized after amplification by conventional PCR. This can be easily resolved by using nested primers in n-PCR, as inhibitory substances are largely reduced and the product quantity is greatly enhanced to be visualized.

Table 2—Showing the relative performance of conventional PCR and n-PCR for detection of CPV-2 in faecal samples

<table>
<thead>
<tr>
<th>No.</th>
<th>Breed</th>
<th>Samples</th>
<th>Conventional PCR</th>
<th>Nested PCR</th>
<th>Positive cases (%)</th>
<th>Negative cases</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Pomeranian</td>
<td>13</td>
<td>8</td>
<td>9</td>
<td>61.5</td>
<td>4</td>
</tr>
<tr>
<td>2</td>
<td>German Shepherd</td>
<td>7</td>
<td>2</td>
<td>2</td>
<td>28.5</td>
<td>5</td>
</tr>
<tr>
<td>3</td>
<td>Doberman</td>
<td>19</td>
<td>10</td>
<td>12</td>
<td>63.1</td>
<td>7</td>
</tr>
<tr>
<td>4</td>
<td>Nondescript</td>
<td>52</td>
<td>27</td>
<td>31</td>
<td>59.6</td>
<td>21</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>52</td>
<td>27</td>
<td>31</td>
<td>59.6</td>
<td>21</td>
</tr>
</tbody>
</table>

Fig. 1—Agarose gel showing amplicon of 681 bp of CPV-2a or CPV-2b positive samples using primer set pCPV-2ab (F & R): Lane M: DNA marker 100 bp-5kbp; lanes 1-3: 681 bp amplicon of sample nos 1-3; lanes 4 & 5: Faecal sample nos 4 & 5, negative in PCR.

Fig. 2—Agarose gel showing the amplicon size of 442 bp confirmed in n-PCR using internal primer set pCPV-N (F & R): Lane M: DNA marker 100 bp-5kbp; Lanes 1-5: 442 bp amplicon of sample nos 1-5.)
in the agarose gel. The sensitivity of the n-PCR over conventional PCR was also confirmed by the serial dilution of known titre of CPV-2. It was found that n-PCR can detect up to $10^{-8}$ dilution, while conventional PCR can detect only up to $10^{-5}$ dilution of cell culture supernatant having known titre ($10^{3.5}$ TCID$_{50}$/mL) (Figs 3 & 4). So, n-PCR can detect genomic DNA in samples containing low concentration of virus particles. The importance of n-PCR is largely felt as the number of virus particle excreted in faecal samples is variable and depends on stress condition, immune status of host and time of collection of sample. The present study was undertaken to develop such a test that specifically detects only CPV-2 variants. The n-PCR developed previously has a drawback of giving positive result with CPV related viruses as the primers have been designed using conserved region of CPV-2 genome, while the external primers pCPV-2ab, used in the present study, were designed from variable portion of VP2 gene that detect only CPV-2 variants. The non-specific amplification in the conventional PCR is a real problem in providing accurate diagnosis and can be avoided by n-PCR, thus improving the sensitivity and specificity of the assay. The n-PCR could be 100 times or more sensitive than conventional PCR as the product which was invisible in agarose gel in conventional PCR could be easily visualized after second round of PCR. The n-PCR allows the diagnosis of CPV infection more rapidly than the traditional tests, such as, virus isolation, even when CPV gets inactivated and remains available for the diagnosis of CPV in faecal samples or when the number of virions is less than $10^6$ particles/g of faeces and can not be detected by EM examination. Therefore, n-PCR can help in early, sensitive and accurate detection of CPV-2 infection even when dogs are suffering from sub-clinical infection and, thus, can help in the prevention and control of the disease in a better manner by providing fluid, antiemetic and antibiotic therapy to the ailing animals.

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References


