Tissue distribution of the antigenic variants of canine parvovirus in various organs using nested PCR

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Abstract
Canine parvovirus-2(CPV-2) is a highly contagious and fatal disease of dogs causing acute haemorrhagic enteritis and myocarditis. In this study, tissue samples such as brain, cerebellum, cerebral bulb, tonsil, retropharyngeal lymph node, thymus, lungs, myocardium, bone-marrow, liver, spleen, pancreas, kidney, bladder, mesenteric lymph node, stomach, duodenum, jejenum, colon and rectum collected from 3-9 months old dead dogs reported with severe clinical symptoms of CPV were subjected to conventional and nested PCR assay, CPV-DNA was detected in all tissues including brain tissue.

Keywords: dogs, Parvovirus, nested PCR, tissue distribution

Introduction
Canine parvovirus belongs to the family Parvoviridae and genus Parvovirus. CPV-2 has icosahedral symmetry, 25nm in diameter and is non-enveloped with a linear, single stranded negative sense DNA genome of 5.2 kb in length encoding structural proteins VP1, VP2, VP3 and non-structural proteins NS1, NS2 (Notomi et al., 2000). Canine Parvovirus pathogen emerged in early 1978 in USA with signs of myocarditis and gastroenteritis (Appel et al., 1979). Virus infection spread rapidly worldwide causing high rate of mortality in pups (Carmichael and Binn, 1981). The dogs are infected through oro-nasal route and after 3-10 days they develop an acute gastroenteritis characterised by loss of appetite, vomition, fever, diarrhoea (from mucoid to haemorrhagic) and leukopenia. The replication of the virus takes place in the epithelial villi of the small intestine that are rapidly dividing and the virus is shed in large quantity in the faeces particularly 4-7 days post-infection. There are a number of methods that are used to diagnose CPV such as, virus isolation using cell culture, Haemagglutination (HA), Haemagglutination Inhibition (HI), Electron Microscopy (EM), Indirect Fluorescent Antibody Test (IFT) and Enzyme Linked Immunosorbert Assay (ELISA). Nowadays, Polymerase Chain Reaction (PCR) and Nested PCR (NPCR) could be used for detection of Canine Parvovirus in clinical samples because of its high sensitivity and specificity. Hence, in this study using conventional and Nested PCR, the distribution pattern of the CPV in different tissues of dogs which had died as a consequence of parvoviral infection was investigated.

Materials and Methods
The carcasses of five (n=5) 3-9 months old mixed-bred dogs, that had died of CPV infection after the onset of the clinical signs from the Department of Pathology, Madras Veterinary College, Chennai, Tamil Nadu, India during the year 2016-2017 were utilised for the study. Tissue samples such as brain, cerebellum, cerebral bulb, tonsil, retro-pharyngeal lymphnode, thymus, lungs, myocardium, bone-marrow, liver, spleen, pancreas, kidney, urinary bladder, mesenteric lymph node, stomach, duodenum, jejenum, colon and rectum was collected (Decaro et al., 2006) in phosphate buffered saline (PBS, pH-7.2) and the homogenates were frozen and thawed twice, subsequently clarified by centrifuging at 10000 rpm for 15 minutes. 200μl of the supernatant was treated with 1ml of proteinase K buffer and 4μl of proteinase K (10mg/ml). The DNA was extracted by Phenol: Chloroform: Iso-amyl alcohol method (Walter et al., 2000). The final DNA pellet was dissolved in 30 μl of nuclease free water and stored at -20°C.
The extracted DNA from different tissue samples were subjected to conventional PCR using the already published primers CPV F1 and CPV R1 (Mizak and Rzezutka, 1999) [12]. The PCR reaction mixture consisted of 2μl of the template DNA, 1.0μl each of forward and reverse primer (25pmol/μl), 12.5μl Mastermix (2X) containing TaqDNA polymerase to make the final reaction of 25μl using nuclease free water, and kept in a thermocycler with 35 cycles of denaturation at 94°C for 60s, annealing at 55°C for 60s, elongation at 72°C for 150s and a final elongation at 72°C for 10 minutes (Mizak and Rzezutka., 1999) [12]. PCR product was electrophoresed in 1.0% agarose gel with ethidium bromide at 5 volts/cm along with 100bp and 1Kb Gene Ruler ladder as molecular weight marker (Biogene) and it was visualized and photographed using gel documentation system (Mega Bio-print, France). For Nested PCR assay, the already published primers (Kumar et al., 2011) [11] were used in this study. Nested PCR reaction was set up by adding 2μl of the PCR product, 12.5μl of Mastermix (2X) containing Taq DNA polymerase 1.0μl each of forward and reverse primer (25pmol/μl), and the final volume was made up to 25μl by adding nuclease free water. The reaction was placed in a ther mocycler with 30 cycles of denaturation at 94°C for 30s, annealing at 50°C for 60s, elongation at 72°C for 60s and final elongation at 72°C for 5 minutes. PCR product was electrophoresed in 1.5% agarose gel with ethidium bromide at 5 volts/cm along with 100bp and 1Kb gene ruler ladder (Biogene) and was visualized and photographed using gel documentation system (Mega Bio-print, France). In both the PCR and Nested PCR, rectal swab from a healthy dog was used as a negative control and a DNA from a vaccine was used as a positive control. The primer sequence for both conventional and nested PCR are given in the following table.

Table 1

<table>
<thead>
<tr>
<th>S. No</th>
<th>Primer</th>
<th>Sequence</th>
<th>Product Size (bp)</th>
<th>Gene Region</th>
</tr>
</thead>
<tbody>
<tr>
<td>1.</td>
<td>CPV-FP</td>
<td>5’AGCTATGAGATCGTACAT-3’</td>
<td>1198</td>
<td>VP2</td>
</tr>
<tr>
<td></td>
<td>CPV-RP</td>
<td>5’AGTATGTTAATATAATTTCTTAGTC-3’</td>
<td></td>
<td></td>
</tr>
<tr>
<td>2.</td>
<td>CPV-NPCR-FP</td>
<td>5’TGAGTCTGATTAGTTTTGA-3’</td>
<td>442</td>
<td>VP2</td>
</tr>
<tr>
<td></td>
<td>CPV-NPCR-RP</td>
<td>5’TGGTTGCATGTATGTAGTCT-3’</td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Results and Discussion

The presence of CPV viral nucleic acid were observed in tissue samples such as brain, cerebellum, cerebral bulb, tonsil, retro-pharyngeal lymph node, thymus, lungs, myocardium, bone-marrow, liver, spleen, pancreas, kidney, urinary bladder, mesenteric lymph node, stomach, duodenum, jejunum, colon and rectum by amplifying 1198 bp product in conventional PCR and 442bp in Nested PCR.

Screening of samples by conventional PCR primers

Row 1-Lane 1: 1-100 bp ladder, Lane 2- CPV positive, Lane 3 to12- brain, cerebellum, cerebral bulb, tonsil, retro-pharyngeal lymph node, thymus, lungs, myocardium, bone marrow, liver, Lane13-1Kb ladder.

Row 2- Lane14- 100bp ladder, Lane 15to24- spleen, pancreas, kidney, bladder, mesenteric lymph node, stomach, duodenum, jejunum, colon, rectum, Lane 25- negative control, Lane 26-1Kb ladder.

CPV infects dogs through oro-nasal route and reaches the intestinal mucosa after an initial spread to lymphoid tissues (Appel and Parrish, 1987) [3]. The replication of the virus takes place in the epithelial villi of the small intestine that are rapidly dividing. Viraemia may reach very high titres of viral DNA, persists for several weeks, even after the virus has disappeared from the intestinal content and the infected faeces serves as a source of infection (Hoelzer et al., 2008). In this study, all tissues analysed were shown to contain CPV DNA, probably as a consequence of viral spread in the organs through blood-stream. Pups of mixed-bred approximately 3-9 months of age died after a similar duration of disease (4-5 days) as a consequence of single CPV infection.

Screening of samples by nested PCR

1% agarose gel electrophoresis for demonstration of conventional and 1.5% agarose gel electrophoresis for demonstration of Nested PCR product in various tissues.

Row 2-Lane 14-100bp ladder, Lane 15 to 24-spleen, pancreas, kidney, bladder, mesenteric lymph node, stomach, duodenum, jejunum, colon, rectum, Lane25-negative control, Lane 26-1Kb ladder.

Fig 1: Row 1- Lane 1-100 bp ladder, Lane 2 - CPV positive, Lane 3 to12- brain, cerebellum, cerebral bulb, tonsil, retro-pharyngeal lymph node, thymus, lungs, myocardium, bone marrow, liver, Lane13-1Kb ladder.

Fig 2: Row 1-Lane 1: 1-100 bp ladder, Lane 2- CPV positive, Lane 3 to12- brain, cerebellum, cerebral bulb, tonsil, retro-pharyngeal lymph node, thymus, lungs, myocardium, bone marrow, liver, Lane 13-1Kb ladder.
(Csiza et al., 1972; Wilcox et al., 1984; Url et al., 2003) [5, 19, 17], whereas in dogs CPV antigen has never been detected in neurons, inspite of the presence of neurodegeneration (Agungpriyono et al., 1999; Url and Schmidt, 2005) [16]. In this study we have demonstrated the presence of CPV nucleic acids in all tissues including brain, cerebellum and cerebral bulb (Zhao et al., 2013) [20].

CPV-DNA detected in the faeces were lower than that observed in lymphoid organ (Decaro et al., 2007) [8]. It has been shown previously that shedding of CPV DNA in the faeces reaches maximal load in the first 4 days after infection (with a peak at 7-8 days post infection) with a rapid decrease in 10-11 days post-infection (Decaro et al., 2005; Elia et al., 2005) [6]. Detection of CPV using haemagglutination and virus isolation gives false negative results (Decaro et al., 2005) [8]. As per Zhao et al., (2013) [20], the viraemidetected from 4 days post-infection suggested that the disease may lead to a generalised infection and cause viral replication which infects other tissues also, otherthan the gastro-intestinal tissues. The systemic infection was probably due viral spread to the various internal organs through blood circulation. Hence, molecular methods such as conventional PCR and Nested PCR should be carried out on internal organs rather than confining with the faeces or intestinal contents in post-mortem samples (Zhao et al., 2013) [20].

The appearance of a mere band of the expected size does not always correlate with the presence of the virus genome. The conventional PCR method works poorly because of sub-optimal conditions in unpurified samples which can be salvaged by the use of nested PCR. Among the two PCR's, the sensitivity of Nested PCR is extremely high which will amplify the specific target region of the viral genome. It confirms the PCR positivity by further re-amplification with the second set of internal primers which serves to verify the specificity of the first round product. This is brought about with the transfer of reaction products from the first reaction which effectively serves to dilute out inhibitors that might be present in the sample initially (Parthiban et al., 2010) [14]. Therefore, in this study Nested PCR was used to confirm the specific sequence of CPV viral genome present in different organs.

Summary
The traditional methods that are used to diagnose CPV are virus isolation using cell culture system, Haemagglutination (HA), Haemagglutination inhibition (HI), Electron microscopy (EM), Indirect fluorescent antibody test (IFT) and Enzyme linked immunosorbent assay (ELISA). However, sensitivity and specificity of these traditional diagnostic methods has proven to be inferior to molecular assays. Hence in this study, a highly sensitive and specific nested PCR has been used to detect distribution of CPV in various tissues including the nervous system in order to help in early and accurate diagnosis of CPV especially in post mortem samples of dogs.

References