International Journal of Chemical Studies

P-ISSN: 2349–8528
E-ISSN: 2321–4902
IJCS 2018; 6(2): 740-742

Received: 04-01-2018
Accepted: 06-02-2018

Lancy Mammen
Ph.D scholar, Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

M Parthiban
Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

K Shoba
Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

TMA Senthilkumar
Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

S Ramesh
Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

K Vijayarani
Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

Correspondence
Lancy Mammen
Ph.D scholar, Department of Animal Biotechnology, Madras Veterinary College, TANUVAS, Chennai, Tamil Nadu, India

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

Lancy Mammen, M Parthiban, K Shoba, TMA Senthilkumar, S Ramesh and K Vijayarani

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, jejunum, 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) [3]. Canine Parvovirus pathogen emerged in early 1978 in USA with signs of myocarditis and gastroenteritis (Appel *et al*., 1979) [2]. Virus infection spread rapidly worldwide causing high rate of mortality in pups (Carmichael and Binn, 1981) [4]. 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 Immunosorbent 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, jejunum, colon and rectum was collected (Decaro *et al*., 2006) [7] 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) [18]. The final DNA pellet was dissolved in 30 µl of nuclease free water and stored at -20ºC.

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), l2.5µl 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

S. No	Primer	Sequence	Product Size (bp)	Gene Region
1.	CPV-FP	5'AGCTATGAGATCTGAGACAT-3'	1198	VP_2
	CPV-RP	5'AGTATGTTAATATAATTTTCTAGGTGC-3'		
2.	CPV-NPCR-FP	5'TGAGCTGCATTTAGTTTTGA-3'	442	VP_2
	CPV-NPCR-RP	5'TGTTTGCCATGTATGTGTTAGTCT-3'		

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

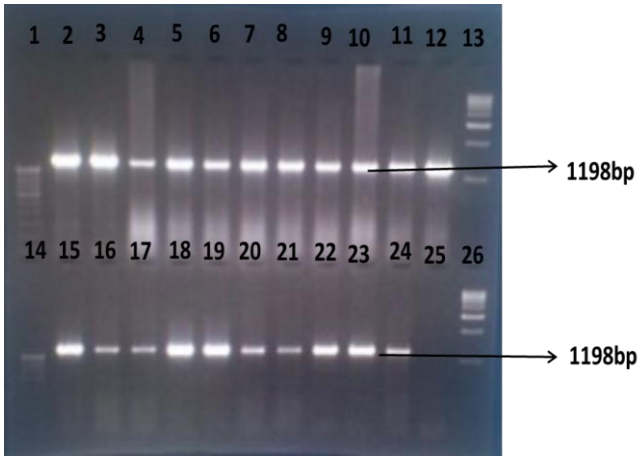

Fig 1: Row 1- Lane 1-100 bp ladder, Lane 2 - CPV positive, Lane 3to12- brain, cerebellum, cerebral bulb, tonsil, retro-pharyngeallymph node, thymus, lungs, myocardium, bone marrow, liver, Lane13-1Kb ladder.

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.

Screening of samples by nested PCR

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

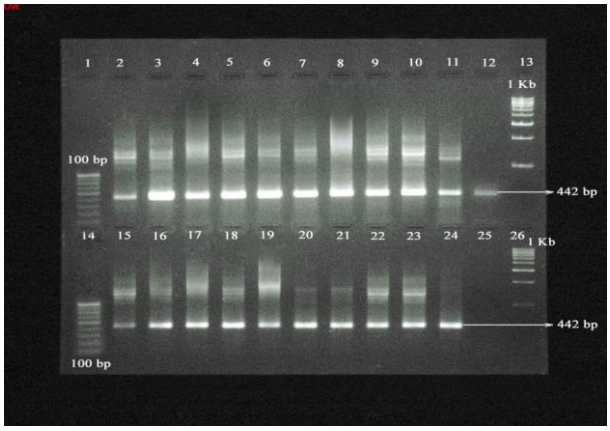

Fig 2: Row 1-Lane 1: 1-100 bp ladder, Lane 2- CPV positive, Lane 3 to12- brain, cerebellum, cerebral bulb, tonsil, retro-pharyngeal lymph node, thymus, lungs, myocardium, bone marrow, liver, Lane 13-1Kb ladder

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.

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.

Parvovirus replication in cats and dogs takes place in highly mitotically active tissues such as bone-marrow, lymphoid organ and intestinal crypts (Appel and Parrish, 1987*)* [3]. Involvment of the nervous tissues has been described in cats

(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) [6]. As per Zhao *et al*., (2013) [20], the viraemiadetected 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-intesinal tissues. The systemic infection was probably due toviral 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 PCRs, 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 lin ked 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

1. Agungpriyuono DR, Uchida K, Tabaru H, Yamaguchi R, Tateyama S. Subacute massive necrotizing myocarditis by canine parvovirus type 2 infection with diffuse leukoencephalomalaciain a puppy. Vet. Pathol. 1999; 36:77-80.
2. Appel MJG, Scott FW, Carmichael LE. Isolation and immunization studies of a canineparvo-like virus from dogs with haemorrhagic enteritis. Veterinary Record. 1979; 105:156-59.
3. Appel MJG, Parrish CR. Canine parvovirus type 2. Virus infection of carnivores, Elsevier, New york. 1987; 1:69-92.
4. Carmichael LE, Binn LN. New enteric viruses in the dog. Adv. Vet. Sci. Comp. Med. 1981; 25:1-37.
5. Csiza CK, Scott FW, DeLahunta A, Gillespie JH. Respiratory signs and central nervous system lesions in cats infected with panleukopenia virus. A case report. Cornell vet. 1972; 62:192-195.
6. Decaro N, Campolo M, Desario C, Elia G, Martella V, Tempesta M, *et al*. Maternally derived antibodies in pups and protection from canineparvo virus infection. Biologicals. 2005; 33:261-267.
7. Decaro N, Elia G, Martella V, Campolo M, Desario C, Camero M, *et al*. Characterisation of the canine parvovirus type 2 variants using minor groove binder probe technology. J Virol. Meth. 2006; 133:92-99.
8. Decaro N, Desario C, Addie DD, Martella V, Vieira MJ, Elia G, *et al*. The study of molecular epidemiology of canine parvovirus, Europe. Emerg. infect dis. 2007; 13:1222-4.
9. Elia G, Cavalli A, Cirone F, Lorusso E, Camero M, Buonavoglia C, *et al*. Anti body levels and protection to canine parvovirus type 2.J.Vet.Med. 2005; 52:320-322.
10. Holzer K, Shackelton LA, Holmes EC, Parrish CR. Within host genetic diversity of endemic and emerging parvoviruses of dogs and cats. J Virol. 2008; 82(22):11096-11105.
11. Kumar M, Chidri S., Nandi S. A sensitive method to detect canine parvoviral DNA in faecal samples b nested polymerase chain reaction. Indian J Biotechnol. 2011; 10:183-187.
12. Mizak B, Rzezutka A. Application of nested PCR for detection of canine parvovirus infaeces. Bull. Vet. Inst. Pulawy. 1999; 43(1):19-24.
13. Notomi TH. Okayama Masubuchi H. Loop mediated isothermal amplification of DNA. Nucleic Acids Research. 2000; 28:63.
14. Parthiban M, Saranya R, Magesh M. Detection of *Theileria* parasite in cattle of Tamilnadu using Nested PCR. Tamilnadu. J Veterinary & Animal Sciences. 2010l 6(4)162-165.
15. Senda M, Hirayama N, Yamamoto H, Kurata K. An improved haemagglutination test for study of canine parvovirus. Veterinary Microbiology. 1986; 12:1-6.
16. Url A, Schmidt P. Do canine parvovirus affect canine neurons? Animmunohisto chemical study. Res.Vet. Sci. 2005; 79:57-59.
17. Url A, Truyen U, Rebel-Bauder D, Weissen bock H, Schmidtv P. Evidence of parvovirus replication in cerebral neurons of cats. J Clin. Microbiol. 2003; 41:3801-3805.
18. Walter J, Tannock GW, Tilsala-Timisjarvi A, Rodtong S, Loach DM, Munro K, Alatossava T. Detection and identification of gastrointestinal Lactobacillus species by using Denaturing Gradient Gel electrophoresis and species specific PCR primers. Appl. Environ. Microbiol. 2000; 66(1):297-303.
19. Wilcox GE, Flower RL, Cook RD. Recovery of viral agents from the central nervous system of cats. Vet. Microbiol. 1984; 9:355-366.
20. Zhao Y, LinY, Zeng, Lu C, Hou J. Genotyping and pathobiologic characterization of canine parvovirus circulating in Nanjing, China. Virology Journal. 2013; 10:272.