Incidence of canine parvovirus type 2c in a puppy with haemorrhagic gastroenteritis in Tamil Nadu, India

Surendhar M, M Vijaya Bharathi, G Selvaraju, S Rathnapraba and RA Raj Kumar

Abstract
Canine parvovirus enteritis is caused by canine parvovirus -2 (CPV-2), which is highly contagious and often fatal disease, characterized by vomiting, fowl smelling bloody diarrhoea and myocarditis in young dogs. In the present study, a total of 150 faecal and blood samples were collected from dogs with the symptoms of haemorrhagic gastroenteritis from Madras Veterinary College Teaching Hospital (MVCTH), Chennai to study the molecular epidemiology and haematological changes in CPV-2 infected dogs respectively. Seventy one (47.33 per cent) dogs were positive for CPV-2 by PCR assay. Strain specific assay reveal CPV-2a and CPV-2c variants were recorded in 70 and 01 dogs respectively. Incidence of CPV-2c is the first report in Tamil Nadu. DNA sequencing was done for 8 PCR positive samples, out of which three were characterized as CPV-2c, indicating that this CPV type 2c is currently circulating in India.

Keywords: Canine parvovirus-2, PCR, CPV-2c, genotyping, mutants

Introduction
Canine parvovirus enteritis is caused by canine parvovirus-2 (CPV-2). CPV-2 is a highly contagious and often fatal disease, characterized by vomiting and hemorrhagic enteritis in dogs of all age (Appel et al., 1979) [2] and myocarditis leading to heart failure in pups of less than 3 month of age (Appel et al., 1978) [1]. Canine parvovirus (CPV) is a small (diameter of 25 nm), non-enveloped virus with a single-stranded DNA molecule of a approximately 5000 bases infecting vertebrates. The parvovirus virion consists of a spherical capsid, which is composed by three structural proteins namely VP1, VP2 and VP3 (Muzychka and Berns, 2001) [3] and two non-structural (NS1 and NS2) proteins.

The virus primarily originated as a host variant from feline panleukopenia virus (FLV), which was sooner adapted to the canine host via wild carnivores, such as foxes and minks (Truyen et al., 1992) [16]. After few years of spreading, the original CPV-2 virus was replaced completely replaced by CPV-2a, a newer type virus that can infect both dogs and cats (Truyen et al., 1996a). The difference between CPV-2a from CPV-2 was five amino acid changes in the VP2 coat protein. (Truyen et al., 1995) [17]. In 1984, yet another antigenic CPV variant emerged and was designated as CPV-2b, which is in cocirculation with the CPV-2a within the dog population all around the world.

The first confirmatory incidence of CPV-2 in dogs was reported at United States in 1978 (Appel et al., 1979) [2]. Ever since the emergence of CPV-2 in 1978, its strain have been continuously mutating with the emergence of CPV-2a in 1979, CPV-2b in 1984 (Parrish et al., 1985) [10] and CPV-2c in 2000 (Italy) (Buonavoglia et al., 2001) [4].

In India, the disease was first reported at Madras in 1981 by Balu and Thangaraj. The incidence of CPV-2 variants in dogs were reported from different states viz., Puducherry (Parthiban et al., 2011) [11], Kerala (Deepa and Saseedranath, 2000) [5], Haryana (Sanjukta et al., 2008) [14], Uttar Pradesh (Nandi et al., 2009) [8] and Assam (Phukan et al., 2004) [13] and West Bengal (Biswas et al., 2006). The first confirmatory of CPV-2c was reported by Nandi et al., 2010 [9] at New Delhi.
Polymerase Chain Reaction (PCR)
Stool DNA Extraction Kit (Bio Basic), 2X Red dye Master Mix (Lot No: 5200300-12L4, Ampliqon), DEPC treated nuclease free water (Bio Basic) and oligonucleotide primer (Table 2) were used in this study.

| DNA extraction from faecal samples |
| DNA was extracted from collected faecal samples by using tissue DNA extraction kit (Bio Basic) as per the protocol of manufacturer guidelines |

| PCR assay for full length VP1/VP2 gene (2.2 kb) |
| PCR for amplification of full length VP1/VP2 gene (2.2 kb) was carried out as per Nandi et al. (2009) [8] for confirmation of canine parvovirus infection in dogs presented with gastroenteritis as well as for apparently healthy dogs for checking canine parvovirus carrier status |

| PCR reaction mixture |
| The PCR was performed in an Eppendorf thermal cycler. All the reactions were carried out in volume of 25µl in 0.2 ml PCR tubes. The reaction mixture contains PCR master mix (12.5µl), forward primer (FP) (1.0 µl), reverse primer (RP) (1.0 µl), nuclease free distilled water (7.5 µl) and template DNA (3.0 µl) |

| PCR cyclic conditions |
| Details of PCR cyclic conditions |

Table 1: Reverse primers Primer Sequence

<table>
<thead>
<tr>
<th>S. No</th>
<th>Forward and Reverse primers</th>
<th>Primer Sequence 5’------3’</th>
<th>Product size</th>
<th>Reference</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>CPV2 (FP)</td>
<td>CAGGTGATGAAATTTGCTACA</td>
<td>630bp</td>
<td>Buonavoglia et al. 2001 [4].</td>
</tr>
<tr>
<td>2</td>
<td>CPV2 (RP)</td>
<td>CATTTGGAATAAATCGTGTTG</td>
<td>379bp</td>
<td>Kaur et al. 2014 [6].</td>
</tr>
<tr>
<td>3</td>
<td>CPV2a (FP)</td>
<td>AGACATATTGGGCTTACCACC</td>
<td>427bp</td>
<td>Peraira et al. 2000 [12]</td>
</tr>
<tr>
<td>4</td>
<td>CPV2a (RP)</td>
<td>ATCTTACCTGTATCTTGTTGCG</td>
<td>470bp</td>
<td>Kaur et al. 2014 [6].</td>
</tr>
</tbody>
</table>

Table 2: Initial denaturation annealing extension final extension

<table>
<thead>
<tr>
<th>CPV2</th>
<th>Initial denaturation</th>
<th>Denaturation</th>
<th>Annealing</th>
<th>Extension</th>
<th>Final extension</th>
<th>No. of cycles</th>
</tr>
</thead>
<tbody>
<tr>
<td>95°C for 5 mins</td>
<td>95°C for 1 min</td>
<td>55°C for 2 mins</td>
<td>72°C for 30 sec</td>
<td>72°C for 10 mins</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>94°C for 20 sec</td>
<td>94°C for 60 sec</td>
<td>55°C for 2 mins</td>
<td>72°C for 30 sec</td>
<td>72°C for 10 mins</td>
<td>34</td>
<td></td>
</tr>
<tr>
<td>95°C for 5 mins</td>
<td>95°C for 1 min</td>
<td>55°C for 2 mins</td>
<td>72°C for 30 sec</td>
<td>72°C for 10 mins</td>
<td>30</td>
<td></td>
</tr>
<tr>
<td>94°C for 60 sec</td>
<td>94°C for 60 sec</td>
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<td>72°C for 30 sec</td>
<td>72°C for 10 mins</td>
<td>34</td>
<td></td>
</tr>
</tbody>
</table>

Result and Discussion
In the present study, a total of 150 faecal and blood samples were collected from dogs with the symptoms of haemorrhagic gastroenteritis from Madras Veterinary College Teaching Hospital (MVCTH), Chennai to study the molecular epidemiology and haemato-biochemical changes in CPV-2 infected dogs respectively. Seventy one (47.33 per cent) dogs were positive for CPV-2 by PCR assay.

Fig 1: L1 - Positive Control 2a (Vanguard), L2 - Negative Control, L3 - Sample, L4 - Positive Control 2b (Vanguard), L5 - Sample, L6 - Positive Control (Puppy DP), L7 - Ladder, L8 - Positive Control (Puppy DP), L9 - Sample, L10 - Positive Control (Puppy DP), L11 - Sample, L12 and 13 - Positive Controls
The PCR products of five CPV-2a and one CPV-2c positive samples were purified and 25ul of this was sent for sequencing by primer walking at Eurofins Genomics India Pvt Ltd, Bangalore. The sequencing was carried out with the help of an automated DNA Sequencer.

Screening of the samples for presence of CPV-2 variants was carried out by PCR using CPV-2 strain specific primers for all the 150 samples and revealed 70 and 01 sample were positive for CPV-2a and 2c respectively.

On analysis of nucleotide sequences, T to A substitution at the third codon of 4064 position of VP2 gene and aspartic acid in CPV-2b has been replaced by glutamic acid at amino acid position of 426 in CPV-2c.

>PCR_CPV_2a_CPV_2A_F-D02.ab1
GCAAGGCGGAATTTAATCTATACTAATTATATATATATAC
TATATTGCTTTTAAATCTAGCAATTATAGTACCC
AGTTTACCAAATGATCCTTTGCAAATAATATGGGATAAGAT
TGATAGTACCTTTAAAAACCAAGACCTTCTATGAAATGC
ATATGTTTATCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_CPV_2c_CPV_2C_F-E02.ab1
TTCTTCCGAGTCAAGGATGTTTGAATCAGCACAA
ACTCGAGAGACGTCTTATGCAATAATGGCAGAA
GTTAAAATATATAAGAGGTGTTGAAATATAGTCC
ATTAATATCAGTTAAGAACACGCTTATGAAATG
ATATGGCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_CPV_2a_CPV_2A_F-D01.ab1
ATCCTGAGAATGTGTTGAAATCAGCACAA
ACTCGAGAGACGTCTTATGCAATAATGGCAGAA
GTTAAAATATATAAGAGGTGTTGAAATATAGTCC
ATTAATATCAGTTAAGAACACGCTTATGAAATG
ATATGGCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_CPV_2c_CPV_2C_F-D02.ab1
AGATAGTAATAATACTATGCCATTTACTCCAGC
GTTTATCTCCCAGCAGCTA
GTTATTTGTAAAATCTTTGTTTGGTCAGATCAAAT
TTATGAGTATATTGAAATCTATAGTGGTCTTGAATT
ATACATTTGGTTCAATATTGCCATGTTAATGGTT

>PCR_2B1_2BF_D03.ab1
AATCCCATTGGAGGTAACACAGGAATTAATGATA
AACATTTAATATCTATGCTTCTCTAATACGTGATTAA
ACATTGACTACCCACGTGTTATCTCAAAATGTTCAAT
GGCTATAAACAGGAATTTGATCAATGCTCAAAATGTTCAAT
TTCTGACTTAACTTCTGTGTTCTTTAATTGGGTGCAAT
ACTCTGACTTAACTTCTGTGTTCTTTAATTGGGTGCAAT

>PCR_2C1_2CF_E03.ab1
CATTGCAACTTACAGGGATTTTTCCTCATTACCGAAG
ACGAGGATGTTGAAATCAGCACAAATAGGAGGACA
GTTAAAATATATAAGAGGTGTTGAAATATAGTCC
ATTAATATCAGTTAAGAACACGCTTATGAAATG
ATATGGCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_2C2_2CF_E03.ab1
CATCATTAGATAGTAATAATACTATGCCATTTACTCCAGC
GTTTATCTCCCAGCAGCTA
GTTATTTGTAAAATCTTTGTTTGGTCAGATCAAAT
TTATGAGTATATTGAAATCTATAGTGGTCTTGAATT
ATACATTTGGTTCAATATTGCCATGTTAATGGTT

>PCR_2C1_2CF_F03.ab1
GTTAATTGCTTTATCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

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ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_2C1_2CF_G03.ab1
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TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_2C2_2CF_F03.ab1
GTTAATTGCTTTATCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_2C2_2CF_E03.ab1
CATCATTAGATAGTAATAATACTATGCCATTTACTCCAGC
GTTTATCTCCCAGCAGCTA
GTTATTTGTAAAATCTTTGTTTGGTCAGATCAAAT
TTATGAGTATATTGAAATCTATAGTGGTCTTGAATT
ATACATTTGGTTCAATATTGCCATGTTAATGGTT

>PCR_2C3_2CF_G03.ab1
ACGTAGAGAACACGCTTATGCAAAAATAGGAGGACA
GTTAAAATATATAAGAGGTGTTGAAATATAGTCC
ATTAATATCAGTTAAGAACACGCTTATGAAATG
ATATGGCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_2C3_2CF_F03.ab1
ACGTAGAGAACACGCTTATGCAAAAATAGGAGGACA
GTTAAAATATATAAGAGGTGTTGAAATATAGTCC
ATTAATATCAGTTAAGAACACGCTTATGAAATG
ATATGGCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

>PCR_2C3_2CF_E03.ab1
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GTTAAAATATATAAGAGGTGTTGAAATATAGTCC
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ATATGGCGCAAAATAATGTCCTGTACATAATTTAGT
TTTGTTGAAAATGGTCCCTAATTTACAAATATGGGATAAT
ACTCGTGTGCTACCTTAAATATGGGATAAAGATGTA
GACTTATGGTTAATATGGGATAAAGATGTA

From the sequence obtained, it can be concluded that T to A substitution indicates the presence of CPV-2c mutants, with a change (Asp→Glu) occurring in the strategic residue 426 (Strassheim et al., 1994).[15] However, further epidemiological surveillance and sequence analysis will help to elucidate if there is any mutation and will provide insights about the prevalence of different antigenic variants of CPV.
Sequencing of the samples was carried out, which revealed that sample no 2 shown in Table 1 were having the T to A mutation. Although limited number of samples have been screened for the presence of CPV-2c mutants, it is to be emphasized that CPV-2c mutants have been evolved to emerge as pathogens of dogs in India. This is the first report of CPV-2c in the South India and it also represents the frequency of this type mutant observed in a dog population. Its presence in India supports the assumption that CPV-2c is reaching a worldwide distribution and provides new information to understand the evolution of antigenic variants of CPV-2.

Reference