Molecular Typing of *Canine parvovirus* Occurring in Pondicherry by Multiplex PCR and PCR–RFLP

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Abstract The present study was aimed at molecular typing of *Canine parvovirus* (CPV) occurring in Pondicherry using PCR based assays. CPV-2a and CPV-2b types were detected by PCR in 68 (53.12%) out of 128 faecal samples/rectal swabs. All the 68 samples found positive by PCR assay were subjected to multiplex PCR assay with CPV-2ab and CPV-2b primer pairs. Sixty-seven (98.52%) samples were characterized as CPV-2b type and one sample (1.47%) was categorized as CPV-2a type. Sixty clinical samples found negative by CPV-2ab primers were subjected to another PCR assay with CPV-555 primer pair for detecting CPV-2c. Though three samples (5%) responded to this PCR, subsequent RFLP of these PCR products with MboII did not show any cleavage indicating the absence of CPV-2c in Pondicherry. It was inferred that CPV-2b was the most prevalent CPV type in Pondicherry. It was further concluded that the CPV-2 variants (CPV-2a, CPV-2b and CPV-2c) currently circulating in the field worldwide could be diagnosed by employing multiplex PCR and PCR–RFLP assays.

Keywords PCR · Multiplex PCR · PCR–RFLP · CPV-2a · CPV-2b · CPV-2c

Canine parvovirus (CPV) is responsible for a severe, highly contagious gastroenteric disease in pups. The *canine parvovirus* 2 (CPV-2) belongs to the family *Parvoviridae*. The genome consists of a single molecule of linear single stranded DNA, 5.2 Kb in size. It is a non-enveloped, icosahedral symmetry virus whose single stranded DNA encodes two capsid proteins (VP1 and VP2) and two non-structural proteins (NS1 and NS2). VP1 and VP2 are translated from alternative splicing of the same RNA [16]. VP2 mainly comprises the icosahedral capsid of CPV, and only a few amino acid substitutions in its sequence can alter relevant biological characteristics to the virus [13]. CPV-1 is known as minute viruses of canines and is usually not associated with the clinical disease. CPV-2 was derived from Feline panleucopaenia virus (FPLV) or from FPLV like viruses in wild animals by natural genetic mutation. Genetic analysis of parvovirus DNA obtained from number of wild carnivore isolates supports this hypothesis [21]. CPV-2 was first identified in the late 1970s when outbreaks of fatal myocarditis and haemorrhagic gastroenteritis were observed in young puppies worldwide [9]. Few years after its successful spreading, the original form of CPV-2 was completely replaced by CPV-2a; a new type with the ability to efficiently infect and cause disease in both dogs and cats [22]. CPV-2a mainly differs from the original type 2 in five amino acid changes in VP2 protein. In 1984, another antigenic variant emerged as a new CPV type, designated as CPV-2b, which is currently co-circulating with the CPV-2a within dog populations around the world. The antigenic variants CPV-2a and CPV-2b differ by two amino acids in the VP2 protein Asn-426 to Asp and Ile-555 to Val [12]. Presently, the original CPV-2 no longer circulates in dog population, whereas the CPV-2a and CPV-2b are distributed worldwide [4, 20]. A novel CPV mutant (Glu-426) produced by a glutamate substitution in the

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426th position was detected in year 2000 in Italy [2, 4] and named CPV-2c. Later, CPV-2c had also been reported from United Kingdom [5], United States [8] and very recently in Portugal [23].

Polymerase chain reaction (PCR) is considered as the most reliable diagnostic technique having high degree of sensitivity and specificity in detecting CPV from faecal samples [4]. PCR based molecular typing of CPV also helps to gain new insights into pathogenesis of CPV-2 types (CPV-2a, CPV-2b and CPV-2c) and is extremely useful to understand antigenic differences between CPV types 2a, 2b or 2c. Canine parvovirus was isolated in India for the first time in 1982 [15]. Occurrence of canine parvovirus infections in India were reported later by Meerarani et al. [11] and Latha and Ramadass [10]. The prevalence of CPV-2a and 2b were documented in India by [3]. CPV-2b was found to be the most prevalent type compared to CPV-2a in India [1, 17]. The prevalence of CPV-2c had not been documented so far in India and therefore the present study was undertaken to find out the prevalence of different variants of CPV-2 (CPV-2a, CPV-2b and CPV-2c) associated with the field cases of canine parvovirus infections in Pondicherry.

Faecal samples/rectal swabs from CPV suspected dogs were collected from Teaching Veterinary Hospital, Rajiv Gandhi College of Veterinary and Animal Sciences, Pondicherry, Veterinary dispensaries and Pet Clinics situated in and around Pondicherry. The faecal samples/rectal swabs obtained from the suspected dogs were emulsified in 1 ml of 0.1 M PBS of pH 7.4 and centrifuged at 6000 rpm for 15 min at 4°C. The supernatant was collected and stored at -40°C until further use.

The processed samples were screened by primer pair CPV-2ab (F) 5'-gaagagtgggtgtaataatt-3' and 2ab (R) 5'-cctatataaccaagtagtac-3' that amplified a 681 bp fragment of the gene encoding capsid protein VP2 of both CPV-2a and CPV-2b types [18]. Hundred microlitres of processed clinical samples were used for the preparation of template DNA by boiling at 96°C for 10 min and chilling immediately on crushed ice for few min. It was then centrifuged at 10,000 rpm for 10 min in a refrigerated centrifuge. The supernatant was diluted (1:5) in distilled water to reduce residual inhibitors of DNA polymerase activity [4]. The reaction mixture (50 µl) consisted of 5 µl of 10× Taq PCR buffer (containing 15 mM magnesium chloride), 20 pmol each of CPV-2ab (F) and CPV-2ab (R) primers, 10 mM dNTPs each, 1 unit of Taq DNA polymerase (Bangalore Genei) and nuclease free water to make up the volume. Template DNA amounting to 0.1 µg was added to the reaction mixture. The conditions for the PCR assay were initial denaturation at 94°C for 5 min, followed by 30 cycles of denaturation at 94°C for 30 s, annealing at 55°C for 2 min and synthesis at 72°C for 2 min, with the final

extension at 72°C for 5 min. The PCR amplification was carried out in an automated thermal cycler (Eppendorf Master Cycler, Germany) and the products were analyzed by electrophoresis in 1.5% agarose gel.

Subsequently, a multiplex PCR for simultaneous detection of CPV-2a and CPV-2b types utilizing primer pairs, CPV-2ab (F)/2ab (R) and CPV-2b (F)/CPV-2b (R) developed by Senda et al. [18] and Pereira et al. [14], respectively was attempted. The sequence of the primer used was CPV-2b (F) 5'-ctttaacctctctgtaacag-3' and CPV-2b (R) 5'-catagtttaattggttatctac-3' with the expected product size of 427 bp. The samples were subjected to multiplex PCR following the similar protocol as described above for primer pair CPV-2ab.

The screened samples, which were found negative by PCR with CPV-2ab primers (which amplified only CPV-2a and CPV-2b types but not CPV-2c), were then subjected to another PCR using primer pair CPV555 (F) 5'-agaagatatccagaagga-3' and CPV555 (R) 5'-ggtgctagtgtatgtaataaaca-3' [2] that amplified a 583 bp fragment of the gene encoding capsid protein VP2 of CPV-2a, CPV-2b and also CPV-2c types. The conditions for the CPV555 PCR assay were initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 s, annealing at 50°C for 1 min and synthesis at 72°C for 1 min, with the final extension at 72°C for 10 min. The PCR products generated with primer pair CPV555 (F)/CPV555 (R) were then digested with enzyme MboII (New England Biolabs) that selectively recognizes the restriction site "GAAGA" (nucleotide 4062-4066 of the VP2 encoding gene) unique to CPV-2c only. After digestion at 37°C for 2 h and enzyme inactivation at 65°C for 5 min, the digested products were analysed in 2.5% agarose gel. The PCR products obtained from CPV-2c would be cut by MboII, generating two fragments of 500 and 83 bp, respectively [2, 6].

One hundred and twenty-eight (128) samples were screened by PCR assay using CPV-2ab primer pair. Sixty-eight (53.12%) samples yielded a single DNA amplicon of 681 bp (Fig. 1). Similar results were also obtained by Meerarani et al. [11] and Desario et al. [6]. All the 68 samples found positive by PCR assay using CPV-2ab primer pairs were subjected to multiplex PCR assay. Out of 68 samples, 67 (98.52%) samples yielded two specific amplicons of 681 and 427 bp responding to both CPV-2ab and CPV-2b primers, respectively, and therefore characterized as CPV-2b types. One sample (1.47%) generated only one amplicon of 681 bp responding to only CPV-2ab primer and not responding to CPV-2b primer and was categorized as CPV-2a (Fig. 2). The results were in agreement with those reported by Hirasawa et al. [7] in Japan, Steniel et al. [19] in South Africa, Pereira et al. [14] in Brazil, Wang et al. [24] in Taiwan and Hong et al. [8] in

Fig. 1 Agarose gel electrophoresis pattern of CPV-2ab specific PCR product of 681 bp of clinical samples assay using CPV-2ab primers. 1 to 9 Clinical samples, 10 Negative control, 11 Positive control, 12 100 bp DNA ladder

Fig. 2 Agarose gel electrophoresis pattern of CPV-2ab and CPV-2b specific multiplex PCR products of 681 bp, 421 bp, respectively of clinical samples. 1 2220 (Sample positive for CPV-2a), 2 1061 (Sample positive for CPV-2b), 3 1414 (Sample positive for CPV-2b), 4 1379 (Sample positive for CPV-2b), 5 1533 (Sample positive for CPV-2b), 6 1496 (Sample positive for CPV-2b), 7 1544 (Sample positive for CPV-2b), 8 Positive control, 9 100 bp DNA ladder, 10 Negative control

US. In a recent report Sanjukta et al. [17] also reported CPV-2b as the prevalent strain in certain North Indian states.

Sixty clinical samples found negative by CPV-2ab primers were subjected to another PCR assay with CPV-555 primer pair. The PCR products obtained with primer pair CPV-555 upon digestion with enzyme MboII would generate two fragments of 500 and 83 bp by selectively recognizing the restriction site GAAGA (nucleotide 4062-4066 of the VP2 encoding gene) unique to the CPV-2c type alone [2]. CPV types 2a and 2b remain undigested upon MboII digestion. In these study, only three samples (5.0%) out of 60 samples (negative by CPV-2ab primers) yielded specific DNA amplicon of 583 bp with CPV-555 primer pair. But all the three products amplified by CPV-555 primer pair remained undigested after MboII digestion indicating that they are not of CPV-2c type and may be either CPV-2a or CPV-2b types [2, 6]. This finding was in support of other studies conducted in India [3, 17], which showed that the CPV-2b was the prevalent strain in India. Molecular typing of clinical samples by PCR and PCR-

RFLP based assays also did not reveal any involvement of CPV-2c type in the field cases of canine parvovirus infections in Pondicherry.

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References

1. Anbazhagan R. Molecular characterization of VP1/VP2 gene of a vaccine strain of canine parvovirus. Dissertation, Deemed University IVRI, Izatnagar; 2004.
2. Buonavoglia C, Martella A, Pratella M, Tempesta A, Cavalli D, Buonavoglia G, Bozzo G, Decaro N, Carmichael LE. Evidence for evolution of canine parvovirus type-2 in Italy. *J Gen Virol*. 2001;82:3021–5.
3. Chinchkar SR, Mohana Subramaniam B, Hanumantha Rao N, Rangarajan PN, Thiagarajan D, Srinivasan VA. Analysis of VP2 gene of canine parvovirus in India. *Arch Virol*. 2006;151:1881–7.
4. Decaro N, Elia G, Campolo M, Desario C, Lucente MS, Bellacicco AL, Buonavoglia C. New approaches for the molecular characterization of canine parvovirus type 2 strains. *J Vet Med*. 2005;52:316–9.
5. Decaro N, Martella V, Gabriella E, Desario C, Campolo M, Lorusso E, Colaianni ML, Lorusso A, Buonavoglia C. Tissue distribution of the antigenic variants of canine parvovirus type 2 in dogs. *Vet Microbiol*. 2007;121:39–44.
6. Desario C, Decaro N, Campolo M, Cavalli A, Cirone F, Gabriella E, Martella V, Lorusso E, Camero M, Buonavoglia C. Canine parvovirus infection: which diagnostic test for the virus? *J Virol Methods*. 2005;126:179–85.
7. Hirasawa T, Kaneshige T, Mikazuki K. Sensitive detection of canine parvo virus DNA by the nested polymerase chain reaction. *Vet Microbiol*. 1994;41:135–45.
8. Hong C, Decaro N, Desario C, Tanner P, Pardo MC, Sanchez S, Buonavoglia C, Saliki. Occurrence of canine parvovirus type 2c in the United States. *J Vet Diagn Investig*. 2007;19:535–9.
9. Kelly WR. An enteric disease of dogs resembling feline panleucopaenia. *Aust Vet J*. 1978;54:593.
10. Latha D, Ramadass P. Improved PCR assay for the detection of canine parvovirus from faecal samples. *Indian J Vet Med*. 2002;26:335–8.
11. Meerarani S, Ramadass P, Subashini CR, Nachimuthu K. Polymerase chain reaction assay for early detection of canine parvovirus. *Indian Vet J*. 1996;73:1013–6.
12. Parrish CR. Mapping specific functions in the capsid structure of canine parvovirus and feline panleucopaenia virus using infectious plasmid clones. *J Virol*. 1991;183:195–205.
13. Parrish CR, Carmichael LE. Antigenic structure and variation of canine parvovirus type-2, feline panleukopenia virus, and mink enteritis virus. *J Virol*. 1983;129:401–14.
14. Pereira CA, Monezi TA, Mehnert DU, D'Angelo M, Durigon EL. Molecular characterization of canine parvo virus in Brazil by polymerase chain reaction assay. *Vet Microbiol*. 2000;75:127–33.
15. Ramadass P, Khader TGA. Diagnosis of CPV infection by agar gel precipitation test and fluorescent antibody technique. *Cheiron*. 1982;11:323–5.
16. Reed AP, Jones EV, Miller TJ. Nucleotide sequence and genome organization of canine parvovirus. *J Virol*. 1988;62:266–76.
17. Sanjukta R, Maheshkumar L, Nagappa B, Joken B, Karuna I, Mandakini S. Molecular epidemiological study of canine parvovirus in some parts of India. In: International conference on

- emerging infectious diseases of animals and biotechnological applications, 28th and 29th July 2008, TANUVAS, Compendium and Souvenir; 2008 p. 65.
18. Senda M, Parrish CR, Harasawa R, Gamoh K, Muramatsu M, Hirayama N, Itoh O. Detection by PCR of wild type canine parvovirus which contaminates dog vaccines. *J Clin Microbiol.* 1995;18:110–3.
 19. Steniel A, Venter EH, Van Vuuren M, Truyen U. Antigenic and genetic analysis of canine parvo virus in Southern Africa. *Onderstepoort J Vet Res.* 1998;65:239–42.
 20. Truyen U, Steinell, Bruckner L, Lutz H, Mostl K. Distribution of antigenic types of canine parvovirus in Switzerland. *Arch Tierheilkd.* 2000;142:115–119. Cited in <http://www.ncbi.nlm.nih.gov>.
 21. Truyen U, Gruenberg A, Obermaier B, Veijalainen P, Parrish CR. Evolution of feline subgroup parvoviruses and the control of canine host range in vivo. *J Virol.* 1995;69:115–9.
 22. Truyen U, Platzer G, Parrish CR, Evermann JF, Viller E, Parrish CR. Evolution of canine parvovirus involved loss and gain of feline host range. *J Virol.* 1996;215:186–9.
 23. Vieira JM, Silva E, Oliveira J, Vieira LA, Decaro N, Desario C, Muller A, Carvalheira J, Buonavoglia C, Thompson G. Canine parvovirus 2c infection in central Portugal. *J Vet Diagn Investig.* 2008;20:488–91.
 24. Wang HC, Chen WD, Lin SL, Chan JPN, Wang ML. Phylogenetic analysis of canine parvovirus VP2 gene in Taiwan. *Virus Genes.* 2005;31:171–4.