

Real time PCR typing of canine parvovirus types in various regions of northern India

KARMAN KOUR¹, GURPREET KAUR^{2✉}, MUDIT CHANDRA³ and P N DWIVEDI⁴

Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab 141 001 India

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ABSTRACT

Canine parvovirus (CPV) is a ssDNA virus which has high morbidity in unvaccinated and some vaccinated dogs as well. It is seen to be evolving and has various antigenic types. The present study was aimed to study the prevalence of the antigenic type in northern India by real-time PCR. This study was conducted in 2019 wherein 178 samples (collected from Delhi, Punjab, Jammu, Chandigarh and Assam) available in the Department of Veterinary Microbiology, College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana were subjected to real time PCR for CPV 2, CPV 2a and CPV 2b. Out of 178 samples, 133 were found to be positive and amongst the three CPV types, CPV 2a was most prevalent. It was also observed that a few samples were positive for more than one antigenic type simultaneously. CPV 2a is most prevalent in the areas under study and a dog can be affected by more than one antigenic type simultaneously. Also, real-time PCR is a sensitive method for the diagnosis of canine parvovirus.

Keywords: Antigenic typing, Canine parvovirus, Prevalence, Real-time PCR

Canine parvovirus, the smallest DNA virus, was first isolated in 1978. Belonging to genus *Parvovirus* and family Parvoviridae, it has an icosahedral symmetry, is 25 nm in diameter and non-enveloped with a linear, single stranded DNA genome having size of 5.2 Kb in length. It has two promoters resulting in the expression of three structural (VP1, VP2 and VP3) and two non-structural proteins (NS1 and NS2) with VP2 (64 kDa) being the major component of the capsid. Main clinical signs are haemorrhagic diarrhoea and vomiting. Myocarditis may also occur in puppies infected during the first few days after birth. Puppies aged between 6 weeks and 4 months old are most commonly and severely affected by CPV, and the crossbreds are less susceptible in comparison to pure breeds (Houston *et al.* 1996). VP2 plays a major role in virus pathogenicity and host immune response (Mochizuki *et al.* 1996). In the current world, the original type CPV 2 has been completely replaced by its antigenic variants (Greenwood *et al.* 1996, Truyen *et al.* 1996, 2000; Steinel *et al.* 1998, Pereira *et al.* 2000).

The vaccines are administered at 6 weeks or 8 weeks followed by 12th week. One of the most important causes of immunization failures are maternally derived antibodies which also play an important role in the protection of the neonate. Fecal samples from parvovirus suspected dogs are routinely analyzed by conventional PCR and real-time PCR. Real-time PCR being more sensitive than conventional

PCR detects low viral titres of CPV-2 in infected dogs. By real-time PCR, a wide range of parvovirus particles has been found in the samples from 1.45×10^6 to 9.45×10^8 copies/0.01g of feces (Sin *et al.* 2005). Thus real time PCR was employed in the given study for antigenic typing of CPV from the samples collected from suspected dogs from various regions of Northern India.

MATERIALS AND METHODS

Real time PCR typing of CPV: A real time PCR was conducted for three typing strains of CPV, viz. CPV 2, CPV 2a and CPV 2b on 178 samples (rectal swabs) collected from different regions of India i.e. Punjab, Assam, Jammu and Kashmir, Delhi and Chandigarh. The DNA was extracted from the samples using Phenol-Chloroform extraction method (Sambrook and Russell 2001). The purity of the DNA was checked using Nanodrop (ThermoFisher, USA) at the absorbance ratio of 260/280.

Extraction of DNA from clinical samples: The DNA extraction was done on all the samples collected and vaccine (Nobivac, DHPPi) which was used as positive control. Two microlitre of sample in PBS (pH 7.2) was centrifuged at 3,000 rpm for 5 min at 4°C. To 600 µl of supernatant, 20 µl of proteinase K (20 mg/ml) and 20 µl of 10% SDS (Sodium Dodecyl Sulphate) were added. The tubes were vortexed and incubated at 56°C for 2-3 h in water bath. Then 600 µl of phenol-chloroform-isoamyl alcohol (PCI) mixture (25:24:1) was added and vortexed for 15 sec and centrifuged at 13,000 rpm for 15 min at 4°C. The aqueous layer of supernatant was collected and 600 µl

Present address: ¹College of Veterinary Science, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana, Punjab.

✉Corresponding author email: gurpreet7502@rediffmail.com

Table 1. Real time PCR assay for antigenic typing of three CPV types

Antigenic type	Taqman assay	Sequence	References	Position in genome
CPV 2	F	5'-AAACAGGAATTAATACTATACTAATATAT' TTA-3'	Decaro <i>et al.</i> 2005	-
	R	5'-AAATTTGACCATTGGATAAACT-3'		-
	Probe	5'-/6- FAM/TGGTCCTTT/ZEN/AACTGCATTAAAT AATGTACC/IowaBlack/3'		-
CPV 2a	F	5'-TGACCAAGGAGAACCAACTAAC-3'	Decaro <i>et al.</i> 2006	847-866
	R	5'-TGATCTGCTGGCGAGAAATATAA-3'		1013-993
	Probe	5'/6-FAM/ACGCTGCTT/ZEN/ATCTTC GCTCTGGT/IowaBlack/-3'		867-896
CPV 2b	F	5'-ACAGGAAGATATCCAGAAGGAGA-3'	Kaur <i>et al.</i> 2016	1216-1238
	R	5'-TGACCATTTGGATAAACTGGTGG-3'		1403-1381
	Probe	5'- /HEX/TATTAACCT/ZEN/TAACCTTCCTGTA ACAGATGA-/IowaBlack/-3'		1251-1280

of chloroform and isoamyl alcohol mixture (24:1) was added. It was mixed gently and centrifuged at 13,000 rpm for 15 min at 4°C. One-tenth volume of sodium acetate (3 M, pH 5.2) and 600 µl of isopropanol were added to the supernatant and mixed gently. The supernatant was kept at -20°C overnight and centrifuged at 12,000 rpm for 10 min at 4°C. The pellet was washed twice with 70% ethanol, dried in the incubator at 37°C and suspended in 50 µl NFW. It was finally stored at -20°C till further use.

Preparation of Real time PCR mix: Real Time PCR reaction mixture (20 µl) was prepared by adding 10 µl of 2× Taqman® Universal Master Mix II with UNG (Applied Biosystems), 1 µl of the 20× Taqman® assay (for the individual antigenic type) (Table 1), 2 µl of the DNA template and 7 µl of nuclease free water. The PCR reaction was carried out in CFX™ 96 Real-Time System (BioRad, USA) with the thermal condition of UNG incubation at 50°C for 2 min, polymerase activation at 95°C and 40 cycles of denaturation at 95°C for 15 sec and annealing at variable temperatures and time depending on the antigenic type detected. For detection of CPV-2, the annealing was done at 52°C for 30 sec; for CPV 2a at 61°C for 45 sec and for CPV-2b at 57°C for 45 sec. A rectal swab from a healthy dog was used as a negative control and vaccine (Nobivac DHPPi) DNA was used as a positive control.

RESULTS AND DISCUSSION

Total 178 samples (rectal swabs) collected from the various regions of northern India, viz. Punjab (n=119), Assam (n=36), Delhi (n=11), Jammu (n=6) and Chandigarh (n=6) were analysed. Out of 178, 133 samples were positive for canine parvovirus with a percent positivity of 74.71%. Out of 133 positive samples, 27 cases were positive for CPV 2, 86 for CPV 2a and 20 for CPV 2b. The number of dogs positive for CPV 2 alone were 3, 61 for CPV 2a and 7 for CPV 2b. Number of positive cases for both CPV 2 and 2a were 12, 1 for 2a and 2b, no case was positive for CPV 2 and 2b simultaneously while 12 cases were positive for CPV 2, 2a and 2b altogether.

When the distribution of three CPV types (CPV 2,

CPV 2a and CPV 2b) in various regions under study was analysed, it was observed that in Assam, 10 samples were positive for CPV 2a type and 4 were positive for CPV 2b; in Punjab, 17 samples were positive for CPV 2, 59 for CPV 2a and 9 for CPV 2b. Similarly, in Delhi, 6 samples were positive for CPV 2, 9 for CPV 2a and 5 for CPV 2b. One sample collected from Chandigarh was positive for CPV 2 type and 3 for CPV 2a. Three samples collected from Jammu were positive for CPV 2, 5 for CPV 2a and 2 were positive for CPV 2b.

The final result of RT-PCR was detected by the graph displayed at the end of procedure in CFX Manager Version 3.1. Depending on the fluorescence emitted of a particular wavelength by the respective fluorophore, the curve of the graph is also determined giving the RFU value and cut off value. The graph of the CPV 2, 2a and 2b positive samples is given in Figs 1a, 1b and 1c respectively.

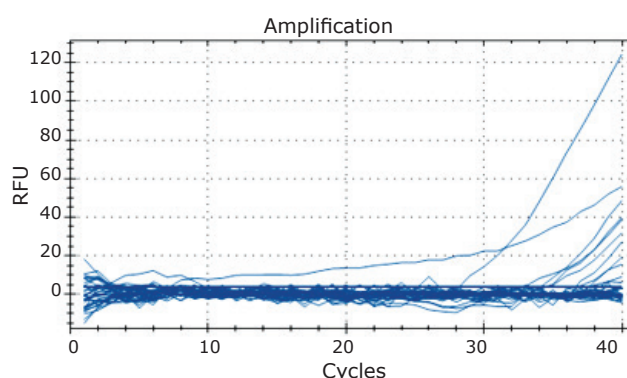


Fig. 1a. Real-time PCR graph for sample positive for CPV 2.

CPV 2a antigenic type of canine parvovirus is most prevalent followed by CPV 2 and CPV 2b. However, when the results were thoroughly observed, some cases were found to be positive for more than a single subtype simultaneously. The region wise distribution of CPV types indicated that CPV 2a type of canine parvovirus was most prevalent followed by CPV 2 and CPV 2b type.

Scientists throughout the world have used real time PCR for the studies on canine parvovirus. While the viral shedding in the feces had been detected up to 12 days by

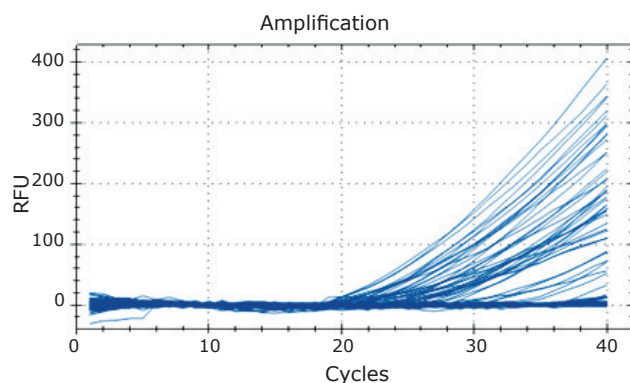


Fig. 1b. Real-time PCR graph for sample positive for CPV 2a.

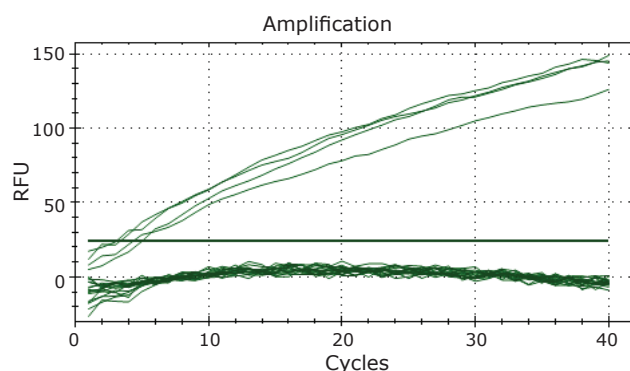


Fig. 1c. Real-time PCR graph for sample positive for CPV 2b.

virus isolation in cell culture and for up to 7 days by HA (Carmichael *et al.* 1980), it was studied that the higher sensitivity of real-time PCR allowed the detection of viral nucleic acid for a much longer period, even when titres dropped to about 104 DNA copies/mg of feces toward the end. Fecal samples from dogs suspected of CPV 2 infection were analyzed by real-time PCR, haemagglutination (HA) assay and by a conventional PCR, and the results revealed that real-time PCR is more sensitive than HA and conventional PCR (Kumar and Nandi 2010). Characterization of CPV-2c strain by means of real-time PCR assays using minor groove binder probes was also done (Decaro *et al.* 2010). Identification as well as antigenic typing of CPV using a *de novo* multiplex real time PCR has also been done (Kaur *et al.* 2016). Multiplex real time PCR assay has also been used for rapid detection of CPV as well as typing of its 3 antigenic types (CPV 2, CPV 2a and CPV 2b). The prevalence of CPV-2b by various authors in several countries namely Brazil (Pereira *et al.* 2000), USA (Parrish *et al.* 1988), Japan (Hirasawa *et al.* 1996), Switzerland (Truyen *et al.* 2000) and South Africa (Steinel *et al.* 1998) had been reported. Contrastingly, CPV-2a was found to be the prevalent antigenic type in France, Taiwan and Italy (Martella *et al.* 2004). However, both CPV-2a and CPV-2b have been found to be distributed in equal proportion in Spain and UK (Greenwood *et al.* 1996). CPV-2c has been found in Vietnam (Nakamura *et al.* 2004), Spain (Decaro *et al.* 2006), United Kingdom (Decaro *et al.* 2007), South America (Pérez *et al.* 2007) and North America (Kapil *et al.* 2007).

The study on real time PCR typing of canine parvovirus

types in regions of India, viz. Punjab, Assam, Jammu, Chandigarh and Delhi revealed that CPV 2a is the most prevalent in the regions under study. Few samples were positive for more than one antigenic type simultaneously. Also, real time PCR is a sensitive method for detection of canine parvovirus.

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