Int.J.Curr.Microbiol.App.Sci (2018) 7(8): 3881-3889

EXCELLENT PUBLISHERS

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 7 Number 08 (2018)**
Journal homepage: http://www.ijcmas.com

Original Research Article https://doi.org/10.20546/ijcmas.2018.708.399

Identification of the Prevailing Antigenic Types of Canine Parvovirus in Northern and Central India

Sankalp Singh Kushwaha, Gurpreet Kaur*, Mudit Chandra and P.N. Dwivedi

Department of Veterinary Microbiology, COVS, Guru Angad Dev Veterinary and Animal Sciences University, Ludhiana-141001, Punjab, India

**Corresponding author*

Keywords

Canine Parvovirus, dogs, Antigenic types, PCR, Nested PCR, Real Time PCR

Article Info

Accepted:
20 July 2018
Available Online:
10 August 2018

ABSTRACT

CPV-2 causes hemorrhagic gastroenteritis in dogs and spreads rapidly in both domestic as well as wild population of canines. The virus sheds in large numbers in the feces, so the present study was designed to detect CPV and to identify the prevailing antigenic types of CPV using molecular techniques from rectal swabs of affected dogs. The incidence of CPV was found to be 18% and 63% by PCR and NPCR respectively. The most prevailing antigenic type as detected by Real time PCR was found to be CPV-2a. Further the study also indicated the animals vaccinated for CPV were also found positive for the disease.

Introduction

Canine parvovirus (CPV) is a single stranded DNA non-enveloped icosahedral virus with approximate diameter of 20nm belonging to the genus *Parvovirus* under the family *Parvoviridae* (Tijssen *et al.,* 1999). The phylogenetic analysis reveals that CPV originated from feline panleukopenia virus or a very closely related carnivore parvovirus of feral canids like foxes and mink (Mochizuki *et al.,* 2008). CPV-2 causes hemorrhagic gastroenteritis in dogs and spreads rapidly in both domestic as well as wild population of canines. The virus has affinity for villi of the small intestine where they replicate in the rapidly dividing epithelial cells. The virus sheds in large numbers in the feces for four to seven days post infection (Hoelzer *et al.,* 2008) and thus, feces are known to serve as a source of infection. Therefore, feces constitutes as the most suitable material for detection of CPV (Carmichael and Binn, 1981).

CPV strains have undergone a series of evolutionary selections in nature, resulting in global distribution of new variants that have replaced the original CPV-2. Currently, the three major antigenic variants of CPV-2 which are known to be distributed among the dog population worldwide are i.e. 2a, 2b and 2c (Decaro *et al.,* 2006). Isolation of CPV-2 was

done for the first time in India by Ramadass and Khader in 1982 since then several occurrence of disease have been reported from different parts of the country involving different variants of CPV (2, 2a, 2b and 2c) both in vaccinated and unvaccinated animals (Deepa and Saseendrannath, 2000; Phukan *et al.,* 2004; Biswas *et al.,* 2006). VP2 is the major capsid protein that plays an important role in the determination of antigenicity and host range of CPV.

It is also known that the mutations which affect VP2 gene are mainly responsible for evolving different antigenic variants of CPV (Phromnoi *et al.,* 2010). The early detection along with the knowledge of genetic variations of VP2 can be of immense help in identifying the emerging CPV strains. Thus the present study was designed to detect CPV and identify the prevailing antigenic types of CPV in the region under study using molecular techniques.

Materials and Methods

A total of 100 rectal swabs were collected in phosphate buffer saline (pH=7.2) from dogs exhibiting clinical signs of gastroenteritis, hemorrhagic enteritis, pyrexia etc. Samples were collected from Madhya Pradesh (n=11) [TVCC, Jabalpur (n=7); Govt. veterinary hospital, Bhopal (n=4)] and Ludhiana, Punjab (n=89) [the small animal veterinary clinics, Guru Angad Dev Veterinary and Animal Sciences University]. The samples were collected from February 2017 to June 2018. All the rectal swabs were kept at 4°C till further use. The vaccine Nobivac DHPPi (Intervet, Pvt. Ltd) was procured commercially from local market. The DNA was extracted from all the samples and the vaccine using the phenol-chloroform extraction method as described by Sambrook and Russell, 2001.

Polymerase Chain Reaction (PCR) for the detection of canine parvovirus

The primers used in PCR were as per Mizak and Rzezutka (1999). The PCR reaction was set up by adding, 5.0 µl of 10X PCR buffer (with 15 mM $MgCl_2$), 1.0 µl of forward and reverse primer (20 pm/µl) each, 1.0 µl of dNTPs mix (10 mM each), 1 U Taq DNA polymerase, 15µl of the template DNA and the reaction was made up to 50µl using nuclease free water. The rectal swab from a healthy dog was used as a negative control and a DNA from a vaccine (DHPPI) was used as a positive control.

Nested PCR (NPCR) for the detection of Canine Parvovirus

The primers used for NPCR were as per Mizak and Rzezutka (1999). NPCR reaction was set up by adding 5µl of the PCR product (from above reaction), 2.5 µl of 10X PCR buffer (with 15 mM $MgCl_2$), 1.0 µl each of forward and reverse primer (20 pm/µl), 1.0 µl of dNTPs (10 mM each), 1 U Taq DNA polymerase and the final volume was made up to 25µl by adding nuclease free water.

The rectal swab from a healthy dog was used as a negative control and a DNA from a vaccine was used as a positive control.

In both PCR and nested PCR, the reaction was put in a thermocycler (Veriti®, Life Technologies, USA) 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 min.

PCR and Nested PCR products (10 µl) were run using 1.5% agarose at 5 volts/cm with Gene Ruler ladder plus 100bp (New England Biolabs, USA). The gel was visualized and photographed using Gel documentation system (AlphaImager, USA).

Real-Time PCR for antigenic typing of

CPV

The samples which were positive for CPV by Nested PCR were subjected to Real Time PCR for antigenic typing of CPV for three antigenic type's viz. CPV-2, CPV-2a and CPV-2b. The fluorescence-probe based assays (Taqman assays) for the three antigenic types viz CPV-2, CPV-2a and CPV-2b (Table 1) were used. The primers and probe for the three antigenic types were got custom synthesized (IDT).

For the Real-Time PCR 2µl of the template DNA was added to the reaction mixture consisting of10 µl of 2X Taqman® Universal Master Mix II with UNG (Applied Biosystems), 1.0 µl of 20X Taqman® assay (for the individual antigenic type) and the final volume 20µl was made by adding nuclease free water. The PCR reaction was carried out in CFX™ 96 Real-Time System (BioRad, USA) with the thermal conditions of UNG incubation at 50°C for 2 minutes, polymerase activation at 95°C for 3 minutes and 40 cycles of denaturation at 95°C for 15 seconds and annealing at variable temperatures and time depending upon the antigenic type detected. For detection of CPV-2 the annealing was done at 52ºC for 30 seconds; for CPV-2a the annealing was done at 61 ºC for 45 seconds and for CPV-2b annealing was carried out at 57 ºC for 45 seconds. The samples which were negative for both CPV-2 and CPV-2a were subjected to detection for CPV-2b in Real-Time PCR. The DNA from vaccine (DHPPi) was used as positive control and nuclease free water was used as negative control.

Determination of end point

The samples were considered positive or negative in the Real-Time PCR depending upon the fluorescence of a particular wavelength emitted by the respective fluorophore attached to the particular probe for the three antigenic types (CPV-2, CPV-2a and CPV-2b) of CPV. Depending upon the highest and lowest relative fluorescence unit (RFU) value, the cut off value or end point was calculated by using CFX Manager Version 3.1.

Sequence analysis

The PCR products of two samples [one from Ludhiana (L50) and one from Madhya Pradesh (M1)] were got sequenced from Eurofins Genomics India Pvt. Ltd and were analysed and compared with the available CPV sequences in the gene bank using NCBI BLAST.

Results and Discussion

Polymerase Chain Reaction (PCR) and Nested PCR (NPCR) for the detection of CPV

In the present study, a total of one hundred (n=100) rectal swabs were collected from the dogs exhibiting signs of diarrhoea, gastroenteritis and haemorrhagic enteritis with pyrexia. The genomic DNA was extracted from these samples and subjected to PCR revealed that out of a total of hundred samples 18 samples were found positive by PCR yielding a product size of 1198 bp (Figure 1). Thus, in the present study the incidence of CPV was found to be 18% using PCR. Out of these 18 positive samples seven dogs had the history of vaccination for CPV.

The PCR products from the 100 rectal swabs were subjected to NPCR. Out of these 100 samples, 63 samples were positive with nested PCR yielding a product size of 548bp (Figure 2) indicating that the incidence of CPV with NPCR to be 63%. Out of these 63 positive samples, 6 samples (6/11) were from Madhya Pradesh and 57 samples (57/89) from Ludhiana, Punjab. Out of the 63 positive samples, 30 dogs had the history of being

vaccinated for Canine Parvovirus. Out of these 30 vaccinated dogs positive for CPV, three were from Madhya Pradesh and 27 from Ludhiana, Punjab.

Many workers have used PCR and NPCR for detection of CPV in rectal swabs/feces of dogs (Mochizuki *et al.,* 1993, Schunck *et al.,* 1995, Weiquan *et al.,* 2001) and have reported it to be specific, sensitive and simple method for detection of canine parvovirus in faeces of infected dogs. In India, Parthiban *et al.,* (2010) from Pondicherry reported 53.12% dogs as positive for CPV using PCR from a total of 128 faecal samples/rectal swabs. Kumar and Nandi (2010b) analyzed 129 faecal samples and found 78 were positive for canine parvovirus by PCR. In another study Singh *et al.,* (2013) screened 100 faecal samples from dogs with signs of gastroenteritis and found 63 dogs were positive for CPV. Also Kaur *et al.,* (2015) screened 100 samples from dogs suspected of CPV and found 11 samples to be positive for CPV by PCR.

From the study it was revealed that the sensitivity of NPCR was much more than PCR for detecting CPV. Similar findings indicating increased sensitivity of NPCR has been reported by various earlier workers. The results are similar to Hirasawa *et al.,* (1994), Sakulwira *et al.,* (2001) and Schmitz *et al.,* (2009) who have also stated that nested PCR being more sensitive than conventional PCR. The reason for this could be that the samples containing very few virus particles might be harbouring inhibitory substances as reported by Kumar *et al.,* (2011) leading to absence of visualization of the amplified product after a PCR which could have been resolved using a NPCR leading to visualization of NPCR product in an agarose gel. Mizak and Rzezutka (1999) used nested PCR for detection of canine parvovirus in faeces by targeting VP2 gene of CPV and reported that the sensitivity of detection of CPV in 10 stool samples by nested PCR was increased 60 per cent in comparison with the standard PCR method. In an another study conducted by Kaur *et al.,* (2011), when 65 samples from dogs subjected to PCR and NPCR yielded 3 (4.61%) and 21 (57.24%) positive reaction respectively. In a study conducted by Kaur *et al.,* (2015) demonstrated more number of samples positive by NPCR (50/100) as compared to PCR (11/100).

Real-Time PCR to detect antigenic types of canine parvovirus

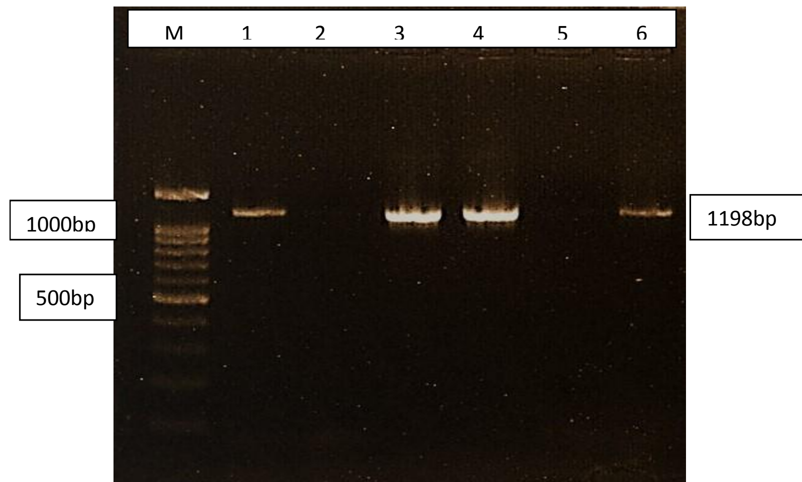
The DNA from the samples positive for CPV by NPCR (n=63) were screened individually for three different fluorescence probe-based Real-Time PCR assay viz. CPV-2, CPV-2a and CPV-2b.

Among the positive samples, 10 (10/63, 15.87%) animals were positive for CPV-2 and 39 (39/63, 61.90%) were positive for CPV-2a (Table 2). The samples which were negative for CPV-2 and CPV-2a were screened for CPV-2b and no amplification was observed for CPV-2b. Thus, from the study it was found that the most prevailing antigenic type in dog population was CPV-2a. When we examined for the presence of more than one antigenic type in a sample, it was found that nine animals were positive for both CPV-2 and CPV-2a.

Out of the ten samples positive for CPV-2, five animals had history of vaccination for CPV and out of the 39 samples positive for CPV-2a, 16 animals had the history of vaccination for CPV.

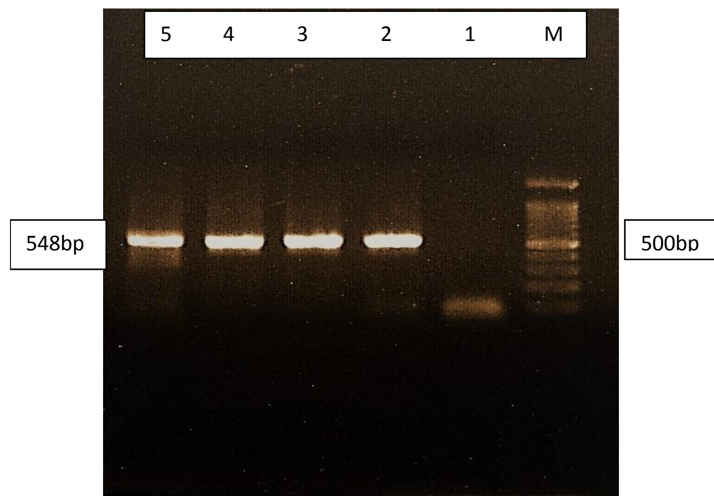
VP2, a capsid protein, is the main immunodominant protein of CPV. It is important for the determination of antigenic types based on the epitopes located on the VP2 protein region.

Fig.1 PCR for detection of canine parvovirus



Lane M: DNA ladder 100bp plus, Lane 1, 3, 6: positive samples for CPV, Lane 2: negative samples for CPV, Lane 4: Positive control, Lane 5: Negative control

Fig.2 Nested PCR for detection of canine parvovirus



Lane M: DNA ladder 100bp plus, Lane 1: Negative control, Lane 2: Positive control, Lane 3, 4, 5: positive samples for CPV

Table.1 Taqman assays for the three antigenic types of CPV

S. No.	Antigenic Type	Taqman Assay	Sequence	Reference	Position in genome	Annealing temperature (◦C)
1.	CPV-2	F	5'-AAACAGGAATTAACTATACTAATATATTTA-3'	Decaro *et al.,* 2005	-	52
		R	5'-AAATTTGACCATTTGGATAAACT-3'		-	
		Probe	5'-/6-FAM/TGGTCCTTT/ZEN/AACTGCATTAAATAATGTACC/IowaBlack/3'		-	
2.	CPV-2a	F	5'-TGACCAAGGAGAACCAACTAAC -3'	Decaro *et al.,* 2006	847-866	61
		R	5'-TGATCTGCTGGCGAGAAATATAA-3'		1013-993	
		Probe	5'/6-FAM/ACGCTGCTT/ZEN/ATCTTCGCTCTGGT/IowaBlack/-3'		867-896	
3.	CPV-2b	F	5'-ACAGGAAGATATCCAGAAGGAGA-3'	Kaur *et al.,* 2016	1216-1238	57
		R	5'-TGACCATTTGGATAAACTGGTGG-3'		1403-1381	
		Probe	5'-/HEX/TATTAACTT/ZEN/TAACCTTCCTGTAACAGATGA-/Iowa Black/-3'		1251-1280	

Table.2 Description (Age, Sex, Breed and Vaccination Status) of samples positive by Real-Time PCR

S. No.	Sample	Age (months)	Sex	Breed	Vaccination status	Real Time PCR		
						CPV-2	CPV-2a	CPV-2b
1.	M1	11	M	ND	+	+	+	-
2.	M3	1	F	Rottweiler	-	-	+	-
3.	M5	6	M	GSD	+	+	+	-
4.	L25	5	F	Golden Retreiver	+	-	+	-
5.	L27	3	F	ND	+	-	+	-
6.	L28	5	M	ND	-	-	+	-
7.	L30	2	F	Beaglehound	+	-	+	-
8.	L34	2	M	Pitbull	-_	-	+	-
9.	L43	5	F	American bullie	+	-	+	-
10.	L45	5	F	ND	-	-	+	-
11.	L46	1.5	M	GSD	-	-	+	-
12.	L50	7	F	Rottweiler	+	-	+	-
13.	L51	4	F	ND	+	+	+	-
14.	L52	4	M	Rottweiler	+	+	+	-
15.	L53	2	M	ND	-	-	+	-
16.	L54	6	F	Pug	-	-	+	-
17.	L57	1	M	Beaglehound	-	-	+	-
18.	L60	1.5	F	Cocker Spaniel	+	-	+	-
19.	L61	3	F	Boxer	-	-	+	-
20.	L63	3	M	ND	-	+	+	-
21.	L64	5	M	ND	-	-	+	-
22.	L65	1.5	M	GSD	+	-	+	-
23.	L69	3	F	Pitbull	+	-	+	-
24.	L70	2.5	F	Bully	+	+	+	-
25.	L72	2.5	F	GSD	-	+	+	-
26.	L73	3	M	ND	-	-	+	-
27.	L76	1.5	F	PakistanBully	-	-	+	-
28.	L77	1	F	Labrador	-	-	+	-
29.	L78	1.5	F	Dachshund	-	-	+	-
30.	L79	2	F	Dachshund	-	-	+	-
31.	L80	2.5	F	ND	-	-	+	-
32.	L81	3	F	ND	-	-	+	-
33.	L84	2	F	Pitbull	-	+	+	-
34.	L85	2.5	F	Boxer	-	+	+	-
35.	L87	5	M	Pitbull	-	-	+	-
36.	L89	9	F	Spitz	+	-	+	-
37.	L95	6	M	Rottweiler	+	-	+	-
38.	L96	3	F	Labrador	+	-	+	-
39.	L97	5.5	F	Pitbull	-	+	+	-
40.	L99	9	M	ND	+	-	+	-
Total	40					-	+	

(-) Negative, (+) Positive, M: Male, F: Female, GSD: German Shepherd Dog, ND: Non-Descript, Pom: Pomeranian

Thus, the mutations affecting VP2 are mainly responsible for the evolution of different antigenic variants (Mohan Raj *et al.,* 2010). It is mainly responsible for the positive selection resulting in the molecular evolution of CPV (Hoelzer *et al.,* 2008). In a study Decaro *et al.,* (2005) used real-time PCR for the diagnosis of CPV in faecal samples from dogs exhibiting diarrhoea and detected CPV-2 in 73 samples out of a total of 89 samples. Later, Decaro *et al.,* (2006) developed a minor groove binder (MGB) probe based assay to discriminate between type 2based vaccines and field strains of CPV using two MGB probes specific for CPV-2 and the antigenic variants (2a, 2b and 2c) respectively. All the antigenic variants (2a, 2b and 2c) were labelled with different fluorophores and the MGB probe assay was able to discriminate successfully between the vaccine type and the antigenic variants with good reproducibility. Also, Decaro *et al.,* (2008) characterized a strain of CPV as CPV-2c by means of real-time PCR assays using minor groove binding probes in another study.

For the antigenic typing of CPV, we used Real-Time PCR in addition to PCR because of its increased sensitivity and specificity as has been reported by various workers. Shi *et al.,* (2012) reported that the real-time PCR is a sensitive diagnostic tool that may be supplemented to conventional PCR for increased sensitivity in epidemiological and surveillance studies and confirmed that it was highly sensitive, specific and reproducible and could facilitate rapid detection and identification of CPV from different kinds of specimens. Further, Zhao *et al.,* (2013) used Real-time PCR to calculate viral loads in the CPV positive samples thus used Real Time PCR for quantitation.

In India, Kumar and Nandi (2010a) analyzed 47 fecal samples from dogs suspected of CPV- 2 using real time PCR, hem agglutination test and PCR. They observed that 24, 20 and 22 samples were found positive for CPV-2 by real time PCR, HA and PCR respectively indicating that real-time PCR is more sensitive than HA and conventional PCR. Kaur *et al.,* (2016) developed a multiplex real time PCR for antigenic typing of Canine parvovirus from rectal swabs of dogs and the most prevailing antigenic type was found to be CPV-2a.

Sequence analysis

For the sequence analysis, the PCR products of two samples (one from Madhya Pradesh, M1 and one from Ludhiana, L50) were got sequenced. After obtaining the sequences these were analysed using NCBI BLAST. On the basis of BLAST analysis it was found that the sequences had 99-100% homology with the Canine Parvovirus.

Thus from the study the incidence of CPV was found to be 18% and 63% by PCR and NPCR respectively indicating NPCR to be more sensitive. Further the study also indicated the animals vaccinated for CPV were also found positive for the disease and the most prevailing antigenic type in the samples tested by Real-Time PCR was found to be CPV-2a.

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How to cite this article:

Sankalp Singh Kushwaha, Gurpreet Kaur, Mudit Chandra and Dwivedi, P.N. 2018. Identification of the Prevailing Antigenic Types of Canine Parvovirus in Northern and Central India. *Int.J.Curr.Microbiol.App.Sci.* 7(08): 3881-3889.
doi: https://doi.org/10.20546/ijcmas.2018.708.399