Full Length Article

STUDY ON THE PREVALENCE AND DIAGNOSIS OF CANINE PARVOVIRUS IN HIMACHAL PRADESH

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ABSTRACT

Diarrhoea is one of the most common clinical conditions seen in dogs caused by a variety of different etiological agents. Among common causative agents, Canine Parvovirus remains the most common cause of hemorrhagic diarrhea along with vomiting and is often accompanied by high mortality among susceptible dogs. Due to emerging strains, detection of CPV has become more challenging. Thus, a combination of molecular and immunological techniques offers a higher probability of correctly diagnosing the disease. With this objective, the study was done to detect different antigenic variants of CPV in dogs of Himachal Pradesh. A total of 238 clinical samples from affected dogs like feacal samples, urine samples and necropsied tissue samples were collected from different parts of Himachal Pradesh and processed in the laboratory. For molecular detection, the DNA of all the 238 samples were initially extracted and subjected to PCR amplification using primer pairs CPV-2, CPV-2ab, CPV-2b and CPV-2c targeting the VP2 gene. For immunological detection, all the samples were initially screened for HA activity using 1% washed pig erythrocytes. Samples showing positive HA activity were then serologically tested by HI and Dot ELISA using hyper- immune sera raised in rabbits. A total of 109 samples out of 238 (45.8 %) were found positive for CPV in PCR assay. No sample was found positive for the original CPV-2 strain, CPV-2a and CPV-2c strains out of variant. In HA test, 89 out of 238 (37.39 %) samples showed positive HA titres ranging from 1:2 to 1:2048. that were then confirmed using HI test with similar results. However, 91 out of 238 (38.23 %)samples showed positive results in Dot ELISA with formation of distinct brown-coloured spots on nitrocellulose membrane.

Keywords: *Canine parvovirus, multiplex PCR, haemagglutination tests, dot ELISA*

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INTRODUCTION

Canine parvovirus (CPV), is a small non-enveloped, single stranded DNA virus which is highly prevalent among dogs and wild carnivores. CPV causes severe gastroenteritis, myocarditis, leukopenia and other symptoms like vomiting, fever and dehydration (Murphy *et al.,* 1999 and Nandi and Kumar*,* 2010). CPV can infect dogs of any age with the most severe infection commonly seen in the age group of 2-4 months old pups that leads to high mortality and morbidity Dogonyaro, (2010). The emergence of newer antigenic strains of CPV and its rapid spread have resulted in the presentation of clinical disease in previously vaccinated animals, while the development of newer diagnostic tools has been delayed (Ogbu *et al.*, 2017). Despite the fact that newer antigenic variants of CPV have emerged, commercial vaccinations still contain the original CPV strain, weakening the immune response in animals exposed to newer strains. The development and subsequent dissemination of novel CPV variants has both evolutionary and epidemiological significance, and so field monitoring of CPV antigenic variants is critical. Thus, in this study we mainly focused on detection of different variants of CPV using both molecular and serological methods of diagnosis.

MATERIAL AND METHODS

Ethical approval

The study was conducted with due approval by the institutional animal ethics committee of DGCN, College of Veterinary and Animal Sciences, Palampur, Himachal Pradesh.

Collection of samples

Dogs presented to different veterinary hospitals of Himachal Pradesh showing predominant gastrointestinal signs like vomiting and diarrhea along with few healthy dogs were selected for conducting this study. 238 clinical samples feces, urine and necropsied tissue were collected with an antibiotic-antimycotic solution (10,000 units Penicillin, 10 mg streptomycin and 25µg amphotericin B per ml in 0.9 per cent normal saline solution) from affected dogs from different parts of Himachal Pradesh like Una, Kullu, Shimla, Kangra, Nurpur and Palampur for the study. Age, sex, breed, and immunization status were briefly documentedin order to gather information on the impact of immunization on illness occurrence as well as the susceptibility of sex, breed, and age groups.

Detection of CPV by PCR

The detection of the CPV from fecal samples was done by polymerase chain reaction (PCR) of viral DNA. CPV DNA was extracted using the conventional phenol chloroform isoamyl alcohol extraction method (Sambrook *et al.,* 2001) and were subjected to PCR for confirmation of viral DNA.

The primers used in the PCR are shown in Table 1 and they target the gene that encodes the VP2 protein. This is because the

icosahedral capsid of CPV is mainly made up of VP2, and even slight changes in its amino acid sequence can affect the virus's biological features. The positive control used in the PCR was the CPV DNA from Megavac-P, while the negative control was DNA extracted from the feces of a healthy dog.

A PCR reaction was prepared with 25 µl, including 5 µl of DNA sample. The PCR was optimized with a 25 µl reaction volume, including 5 µl of DNA, 2 µl of each primer, 3 U of Taq DNA polymerase, 1.5 mM MgCl2, 200 µM dNTP mix, and deionized water. The amplification process consisted of a denaturation step at 95°C for 1 min, followed by 40 cycles at 94°C for 30 sec and 72°C for 10 min, with specific primer annealing temperature. The final extension occurred at 72°C for 5 minutes in a thermal cycler.

First, the purified DNA from all 238 samples was amplified using CPV-2 and CPV-2ab specific PCR. Any samples that tested positive in the CPV-2ab PCR were then subjected to multiplex PCR. This multiplex PCR uses two sets of primers, CPV-2ab (F) and CPV-2ab (R) and CPV-2b (F) and CPV-2b (R), to simultaneously detect CPV-2a and CPV-2b types. The reason behind using multiplex PCR is that CPV-2b types can be amplified by both sets of primers, resulting in two distinct products at 681 bp and 427 bp. On the other hand, CPV-2a genome can only bind with the CPV-2ab primer pair, leading to the generation of one distinct product at 681 bp. If a sample tested negative with all the CPV-2, CPV-2ab, and CPV-2b primer pairs, it was further analyzed using another PCR reaction. This PCR reaction used primer pairs CPV-555 (F) and CPV-555 (R), which amplify a 583 bp fragment of the gene encoding the capsid protein VP2 of CPV-2a, CPV-2b, and CPV-2c types. The PCR products obtained with these primer pairs were then digested with the enzyme Mbo II, which recognizes the restriction site "GAAGA" (nucleotides 4062-4066 of the VP2 encoding gene) unique to CPV-2c. After digestion, the products were analyzed in a 1.2% agarose gel. If a sample contained CPV-2c, the PCR products would be cut by Mbo II, resulting in two fragments of 500 bp and 83 bp, respectively.

PCR sensitivity assay

The detection limit of the PCR was determined by using serial dilutions in nuclease free water up to 10-5 dilutions of DNA from a randomly selected CPV-2 positive sample.PCR was run for each virus dilution starting from 10-1 to 10-5 and replicates were then run on 1.2 per cent (%) agarose gel and observed under UV- trans illuminator.

Hemagglutination test

Hemagglutination is an important property of CPV(Raj *et al.,* 2010). PCR positive samples were tested for HA activity using pig erythrocytes. Blood was collected from pigs and mixed with anticoagulant solution. RBCs were washed with PBS thrice and centrifuged at 1000rpm for 5 minutes and a stock solution was prepared. Antigen was prepared by centrifuging the processed sample with chloroform. The supernatant

was collected and evaluated for HA activity. Different dilutions of RBCs were made for HA test standardization. Megavac-P vaccine was used as positive antigen and healthy dog fecal sample as negative control antigen.

Hemagglutination inhibition test (HI test)

All the samples that tested positive in the HA test were then subjected to the HI test using hyper immune serum raised in rabbits for CPV(Carmichael *et al.,*1980). 5-year-old male New Zealand white rabbits were injected with 1 mL of inactivated canine parvovirus vaccine to raise hyper immune serum against CPV. The first booster injection was given 4 weeks after the initial injection. After 2 weeks of the second immunization, test bleeding was performed and serum was isolated and tested for the presence of antibodies. A second booster injection was given after 4 weeks of the first booster injection. As detectable amounts of antibodies were produced, blood was subsequently collected, serum separated, and stored for further use. Alpha methods of hemagglutination inhibition (varying virus and constant serum) were used to identify the hemagglutinating virus as CPV. A 1:5 dilution of serum was made in PBS, and three controls were used for HI: virus control, cell control, and serum control.

Dot-ELISA

Dot-ELISA was conducted for CPV according to (Waner *et al.,* 2006). A nitrocellulose membrane sheet of 0.45 nm pore size was cut into 5 x 0.5 cm strips. Megavac-P served as positive control for CPV and a fecal sample from a healthy dog was used as negative control. Fecal samples were treated with chloroform (1/10) and centrifuged at 4000 rpm for 15 min to obtain antigen for Dot-ELISA. A positive reaction was indicated by the appearance of a brown dot at the antigen coating site.

RESULTS AND DISCUSSION

PCR has become a widely used tool for the detection of various micro organisms with high sensitivity, specificity and rapidity. In this study, specific primers were used for targeting the VP2 gene of CPV which is 64KDa NH2 terminal truncated form of VP1(84Kda) and is associated in modulating host response and viral pathogenicity (Appel *et al.,*1979).None out of the 238 samples were found to be positive for the original CPV-2 strain by PCR in the present study. The negative results in this study shows that the original CPV-2 strain is no longer circulating in the state. However, A total of 109 samples were found positive as evidenced by presence of expected 681 bp product shown in Fig.1.

All the 109 samples found positive by CPV 2ab PCR were again subjected to CPV-2ab/CPV-2b multiplex PCR using CPV-2ab and CPV-2b primer pairs for simultaneous detection of CPV-2a and CPV-2b strains. All the 109 samples (45.79 %) were found to be positive for CPV-2b antigenic variant as all the samples formed positive bands at 681 and 427 bp using multiplex PCR shown in Fig. 2 and 3. The presence of CPV-2a strain would have resulted in only the 681 bp band in multiplex PCR. Thus, no CPV- 2a strains were found in the study. In this study, CPV-

2b was found to be most prevalent antigenic variant. The remaining 129 samples out of the total 238 samples that were not positive for either CPV-CPV-2a or CPV-2b employing CPV-2ab/CPV-2b PCR were further subjected to CPV-2c PCR assay for the detection of antigenic variant CPV-2c in the samples. All the samples were found negative by CPV-2c PCR assay indicating absence of CPV-2c strain from the samples collected.

The original CPV strain which was first detected in 1978 and believed to be originated from FPLV as a host range mutant through small mutations in capsid proteins (Truyen *et al.*, 2000) which has been recently replaced by newer antigenic variant CPV 2a which was detected between 1978-79 and which was further replaced by CPV2b in 1984 (Parrish *et al.,* 1988) and CPV2c (Buonavoglia *et al.*, 2001). In the United States (Truyen U. 2006), Japan (Hirayama *et al.,* 2005), Taiwan, Brazil (Puentes *et al.,* 2012) and many other countries, CPV-2b has been responsible for the majority of CPV infections. In India, CPV-2b was discovered to be the dominant antigenic variation, with a minor proportion of CPV-2a in the Bareilly region of Uttar Pradesh (Nandi and Kumar, 2010), CPV-2b in Himachal Pradesh as well (Sharma *et al*.,2016). On the other hand, identified CPV-2a antigenic variations in Southern and Central India based on VP2 gene sequences, which contradicts this study and previous Indian result (Chinchkar *et al.,* 2006 and Srinivas Vivek, *et al.,* 2013)

The novel CPV-2c variant was first discovered in Italy (Buonavoglia *et al.,* 2001) and has since been detected in a number of additional countries(Pereira *et al.*, 2000). Nakamura *et al.,* 2004 discovered a CPV-2c strain in Vietnam, whereas Decaro *et al.,* 2006 discovered CPV-2c antigenic variations in a Spanish breeding kennel. CPV-2c was discovered to be prevalent in Italy, Germany, and Spain, as well as widespread in Portugal, France, and Belgium (Decaro *et al.,* 2007 and Decaro *et al.,* 2008). However, there are very few reports on the CPV-2c strain recovered from India. In their study, (Nandi and Kumar*,* 2010) stated that CPV-2c mutant had recently appeared as a novel strain in India.

Results of CPV positive cases based on different study population variables

Age wise distribution of CPV

A total of 65 samples (56.52 %) were found positive for CPV out of 115 faecal samples collected from age groups of 0-6 months while 31 samples (41.89 %) were positive from 74 samples from group 6-12 months and only 13 (26.53 %) samples were positive from dogs of age above 12 months or more shown in Fig.4.The maximum prevalence of CPV in the present study was observed in the age group of 0-6 months followed by 6-12 months old dogs, confirming the already established fact that CPV infection is more severe in young animals. Additional research (Tilley and Smith., 2015) claims that the majority of cases of CPV infection are identified in dogs before 6 months of age, support this age vulnerability. The main cause of the

high frequency of CPV in this particular age group is thought to be the decline or absence of maternal immunity, lower immunological competence and insufficient responses to vaccination. Additional characteristics, such as strong mitotic activity of intestinal epithelium, which provides CPV with the ability to replicate in rapidly proliferating cells.

Sex wise distribution of CPV

A total of 238 samples were collected, of which 170 samples came from male dogs and 68 samples from female dogs. In our study, 50.58 percent (%) (86/170 samples) of samples from male dogs were found to be CPV-positive, but only 33.82 percent (23/68 samples) of samples from female dogs tested positive for the virus. This showed that male dogs were more likely to have CPV infection than female dogs.

Although all breeds, ages, and sexes of dogs have been proven to be equally susceptible to CPV-2 infection (Castro *et al.,* 2007)., male dogs have been reported to have a greater incidence of CPV infection resulting to mortality (Gombac *et al.,* 2008).

Breed wise distribution of CPV

Out of 109 samples that tested positive for CPV, the breeds with the highest prevalence were German Shepherd dogs (56.7%), Labradors (54.2%), Mongrels (52.6%), and Rottweilers (50.0%). Our research showed that foreign dog breeds such German shepherd, Labrador, and Rottweiler appear to have a higher prevalence of CPV.

Results of PCR sensitivity assay for detection of CPV

The PCR demonstrated the ability to detect dilutions of CPV DNA up to 10^{-3} (Fig.5), resulting in a calculated detection limit of 0.04 ng/ µl (201.2/1000 i.e. 0.2 ng/ 5µl). The minimum detection limit of a PCR assay plays a crucial role in determining the diagnostic sensitivity of the reaction, especially in samples with a low pathogen count. By identifying the minimum detection limit of PCR for a specific pathogen, it can be utilized to diagnose clinical conditions that are still in the incubating or subclinical phase, where the pathogen quantity in the sample is minimal. In this study, the PCR was able to detect dilutions of CPV DNA up to 10^{-3} or 0.04 ng/ µl.

Results of HA tests

The HA test was carried for the detection of CPV using 1% porcine RBCs on all samples and infected cell cultured supernatants. 89 out of 238 i.e. 37.4% of samples had positive hemagglutination results with HA titers ranging from 1:2 to 1:2048. Samples with titers less than 1:32 were considered negative, while titers equal to or greater than 1:32 were taken as positive. Highly positive samples had a HA titer of 1:64 or higher, while weakly positive samples had a HA titer of 1:32. (Fig 6).

Dot-ELISA for detection of CPV

In Dot-ELISA 91 (38.2 %) samples were positive showing distinct brown colored spots in nitrocellulose membrane indicating positive reaction from fecal

samples (Fig.7). Dot-ELISA was found to be more or less comparable in efficacy to HA-HI for detecting CPV in our study. Dot-ELISAs are typical antigen–antibody reactions that are qualitative and can easily be performed on nitrocellulose membranes. The difference in sensitivity between Dot-ELISA and HA-HI may be due to the fact that HA test needs higher amount of virus to determine visible reaction while smaller quantity of virus is sufficient in Dot-ELISA to produce positive results. The sensitivity of Dot ELISA can be however, increased by using monoclonal antibodies instead of polyclonal antibodies which was used in this study.When different diagnostic tests for detecting CPV were compared, it was found that PCR was most sensitive test that was able to detect 45.79 % samples as positive while detection using either HA-HI or Dot-ELISA were almost comparable.

Out of 238 samples collected, 109 (45.79 %) samples were found positive for CPV by PCR. All CPV isolates were found to be CPV-2b antigenic variant. Original CPV-2, CPV-2a or CPV-2c could not be detected in this study. More cases of CPV were detected in 0-6 months old age group with males and unvaccinated dogs more severely affected. Infection was noticed in the exotic dog breeds The DNA detection limit for CPV was found to be 0.04 ng/µl. Both HA-HI 89/238 (37.39 %) positive samples and Dot-ELISA 91/238 (38.23 %) positive samples were found almost equal in sensitivity for detection of CPV. In conclusion, molecular tests like CPV specific PCR appears to be the more sensitive test for detection of CPV in clinical samples while Dot ELISA was found to be the more sensitive immunological test. The study also revealed that CPV-2b antigenic variant was the most prevalent strain in dogs in the state of Himachal Pradesh and so future vaccines should incorporate this strain for better protection in field conditions.

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Table.1. Different set of primers used for detection of CPV by PCR

Primers		Sequences	Virus type detection	Specific target site	Amplicon size
Forward	**Reverse**				
CPV-2(F)		5'GAAGAGTGGTTGTAAATAATA3'	Original CPV-2 strain	VP2 gene	681 bp Senda *et al*., 1986)
	CPV-2(R)	5'CCTATATCACCAAAGTTAGTA3'			
CPV-2ab(F)		5'GAAGAGTGGTTGTAAATAATT3'	CPV-2a and CPV-2b	VP2 gene	681 bp Senda *et al*., 1986)
	CPV-2ab(R)	5'CCTATATAACCAAAGTTAGTA3'			
CPV-2b (F)		5'CTTTAACCTTCCTGTAACAG3'	CPV-2b	V1/V2 gene	427 bp Pereira *et al.*, 2000)
	CPV-2b(R)	5'CATAGTTAAATTGGTTATCTAC3'			
CPV-555(F)		5'AGGAAGGATATCCAGAAGGA 3'	CPV-2c	VP2 gene	583 bp Buonavoglia et al., 2001)
	CPV-555(R)	5'GGTGCTAGTTGATATGTAATA AACA 3'			

Table.2. Annealing temperature of different primers used for CPV amplification

Primer pairs	Annealing temperature
CPV-2 (F) and (R)	57°C for 2 min
CPV-2ab (F) and (R)	55°C for 2 min
CPV-2b (F) and (R)	55°C for 2 min
CPV-2c (F) and (R)	50°C for 1 min

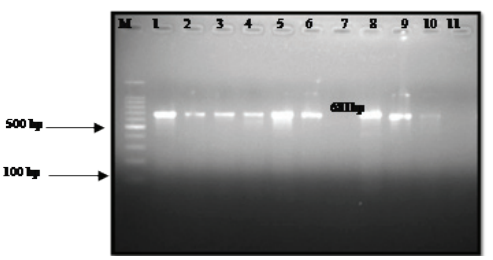

Fig.1. CPV-2ab PCR results Lane M: 100bp ladder, Lane 1: Positive control, Lane 11: Native control, Lane 2,3,45,6,8,9 and 10: Positive faecal samples, Lane 7: Nagative faecal sample

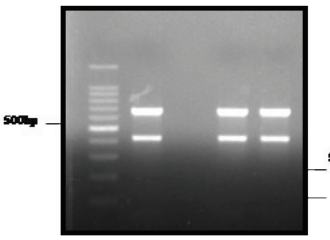

Fig.2. Multiplex PCR results of CPV-2b(1) Lane M: 100 bp ladder, Lane 1: Positive control, Lane 2: Negative control, Lane 3: CA/119, Lane 4: CA/124.

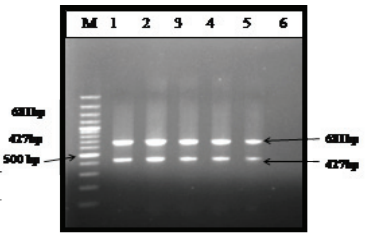

Fig.3. Multiplex PCR results of CPV-2b(2) Lane M: 100bp ladder, Lane 1: Positive control, Lane 2: CA/73, lane 3: CA/78, Lane 4: CA/85, Lane 5:CA/89, Lane 6: Negative control

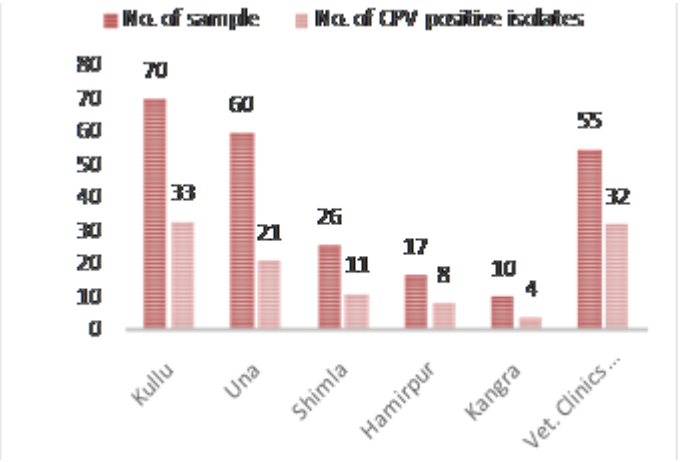

Fig. 4. Area wise distribution of samples collected to number of positive samples

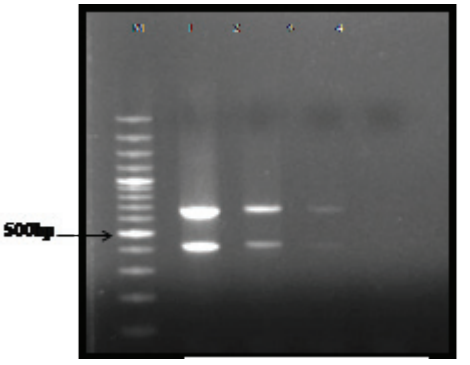

Fig. 5. Agarose electrophoresis of CPV DNA dilutions

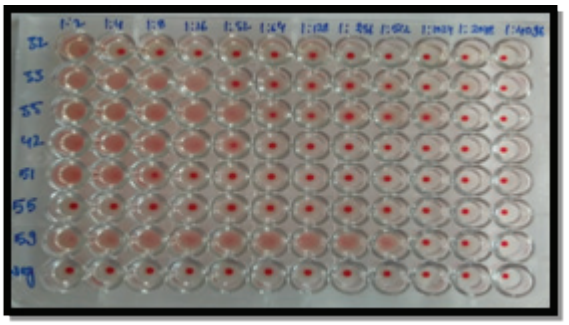

Fig. 6. Haemagglutination test of faecal samples using porcine RBC

In Row 2 and 7 are positive samples and rest are negative; Neg: negative control in well is negative control

Table.3. Results of Hemagglutination Inhibition Test

No. of samples	HA titer	HI titer (alpha method)
21	1:32	1:2
16	1:32	1:4
14	1:64	1:4
8	1:128	1:8
12	1:256	1:8
9	1:512	1:16
7	1:1024	1:16
2	1:2048	1:32
89		

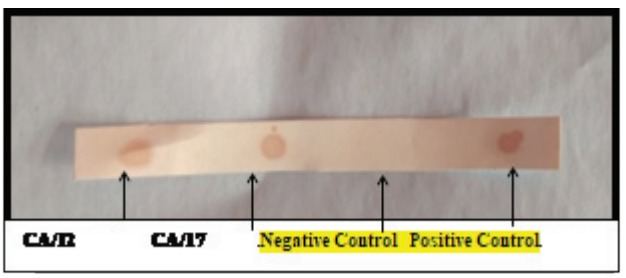

Fig. 7. Dot-ELISA on faecal samples for detection of CPV

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