

Molecular Detection Based Epidemiology of Canine Parvovirus and Canine Coronavirus Infection in Diarrheic Dogs in Haryana

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ABSTRACT

Diarrheic faecal samples of dogs were screened for the presence of canine parvovirus (CPV) and canine coronavirus (CCoV) using polymerase chain reaction (PCR) and reverse transcriptase polymerase chain reaction (RT-PCR) respectively followed by analysis of positive cases to find their relationship with breed, age, sex and vaccination status. Faecal samples were collected from dogs having vomition and diahorrea. PCR amplification of DNA and RNA (c-DNA) templates extracted from the faecal samples was conducted using the designed primers against the relevant genes (VP2 and M) of the two viruses respectively. Out of a total 50 screened faecal samples, twenty five were found positive for CPV and 4 samples for CCoV infection by developed PCR/RT-PCR. Out of 25 CPV positive dogs, 23 dogs were from Hisar district and one dog each was reported from Fatehabad and Jind district of Haryana. Out of four positive CCoV dogs one dog was reported from Fatehabad and three other were reported from Hisar. The disease was found to be more common in mixed breed, male pups of less than 6 months of age. The percent prevalence of positive cases in unvaccinated or inadequately vaccinated dogs was found to be 96.55%. PCR and RT-PCR based detection revealed 50% and 8% prevalence of CPV and CCoV respectively. It may be concluded that CPV and CCoV infections are very much prevalent in the state and their prevalence is very high in dogs not vaccinated. Thus, there is a need to strengthen vaccination against these etiological agents.

Keywords: Canine parvovirus, canine coronavirus, gastroenteritis, PCR, prevalence

Canine viral enteritis (CVE) is highly contagious and is characterized by sudden and acute onset of vomition and diahorrea. It occurs worldwide and is still evolving (Shackelton *et al.*, 2005). Important causative agents of CVE include canine parvovirus (CPV), canine distempervirus (CDV) and canine coronavirus (CCoV) (Decaro *et al.*, 2007). Canine parvovirus infection causes vomition, haemorrhagic gastroenteritis in dogs (Nandi and Kumar, 2014) and myocarditis followed by heart failure in puppies less than six months of age (Ying *et al.*, 2012). In India, CPV is an emerging disease and poses a serious threat to canine population despite of routine vaccination (Sanjukta *et al.*, 2011).

Canine coronavirus (CCoV) infection is an emerging disease in dogs (Pratelli *et al.*, 2000). It causes a mild to moderate enteritis in dogs characterized by high morbidity and low mortality but highly virulent CCoV-II strains also cause fatal disease in puppies (Ntafis *et al.*, 2010). In India, serological studies revealed the presence of antibodies to this virus in dogs, indicating its spread in the canine population (Deka *et al.*, 2013).

Molecular diagnostic techniques like PCR had been the most reliable techniques having high degree of sensitivity and specificity in detecting CPV from faecal samples (Srinivas *et al.*, 2013). RT-PCR based assays targeting M gene are sensitive methods for the detection of CCoV in the diarrheic faecal samples of dogs (Costa *et al.*, 2014; Wang *et al.*, 2016).

There is a paucity of information related to the viral origin of gastroenteritis in dogs in Haryana, therefore the present study describes the prevalence of the two viruses (CPV and CCoV) circulating in the diarrheic dogs, on the basis of molecular detection methods and their association with age, sex, breed and immune status of dogs.

MATERIALS AND METHODS

Sample collection

This study was conducted on fifty dogs of one year or less age suffering from vomition and diarrhoea presented to Veterinary Clinical Complex, College of Veterinary Sciences, Lala Lajpat Rai University of Veterinary and Animal Sciences, Hisar, Haryana. Rectal swabs were collected using sterile swabs and were added to 1 ml of sterile PBS (pH 7.4), vortexed and preserved at -20°C till further processing. Information related to the breed, age sex, vaccination and deworming status of dogs along with the complete history of clinical illness was simultaneously recorded. No ethical approval was required as the dogs included in the study were all clinically affected and were brought to the clinics for the diagnosis and treatment.

Preparation of faecal samples

A total of fifty faecal samples were collected and screened for viral pathogens by applying conventional PCR and RT-PCR based assays. The genomic DNA (CPV-2) and RNA (CCoV) were extracted respectively using commercially available DNA extraction kit (Pure link Genome extraction kit, Invitrogen, USA) and TRIzol reagent (Takara®) as per manufacturer's instructions respectively. The commercial vaccine against CPV, Megavac 6 (Indian Immunological, India) was used as positive control after similar DNA extraction procedure. Genome specific CCoV gene construct from available NCBI Gen Bank was commercially synthesized (Bio Link ®) which was used as a positive control for CCoV. c-DNA of the extracted faecal RNA samples was prepared using "Revert Aid first strand c-DNA synthesis Kit" by Thermo-scientific ® (K1622). Briefly, master mix was prepared consisting of 4µl RT Buffer (5X); 2µl dNTP's; 1µl Random Hexamers; 1µl RT Enzyme; 1µl Ribolock and 6µl NFW. Initially 5µl of RNA template was heated at 65 °C for 5 minutes to melt the secondary structures within the template and snap chilled on ice. Final Reaction Volume (20µl) consisted of 15 µl master mix and 5µl snap chilled template was initially preheated for primers annealing at 25°C for 10 min, followed by reverse transcription step carried out at 42°C for 60 minutes in a programmable 96 well thermal cycler (Veriti). The reverse transcriptase was heat inactivated at 70°C for 5 min. The cDNA was stored at -20°C till further use.

Primer designing and optimization of PCR

Specific primers were designed for the partial amplification of VP2 gene of CPV-2 and M gene of CCoV. PCR was standardized using commercial vaccine, Megavac 6 for CPV-2 and a genome specific construct was designed for CCoV. To standardize the optimum annealing temperature for maximum amplification of VP2 gene and M gene gradient PCR was run using positive control with annealing temperature ranging from 48°C to 54°C for CPV-2 and from 48°C to 56°C for CCoV. The sequences of CPV-2 and CCoV forward and reverse primers are given in Table 1. Maximum amplification for VP2 gene of CPV-2 was obtained at annealing temperature of 54°C and for M gene of CCoV maximum amplification was obtained at 50°C as visualized in 1.5% agarose gel.

Polymerase chain reaction was performed using extracted DNA and c-DNA templates obtained from faecal samples with vaccine as a positive control for CPV-2 and gene construct as positive control for CCoV; keeping negative control without any template DNA. A reaction volume of 12.5 μ l was prepared consisting of 3.0 μ l of template DNA/ c-DNA, 6.25 μ l of PCR Master Mix (Thermo scientific ®), 0.5 μ l each of forward and reverse primers (10 p mol concentration) and 2.25 μ l of Nuclease free water. The reaction mixture was prepared in 200 μ l PCR tubes. The amplification was performed in a thermocycler (Veriti-Applied Biosystems) with reaction conditions for VP2 gene and M gene as given in Table 2. The amplified

Target gene	Designed primer pair	Sequence 5'-3'	Length	Amplicon size
VP2 gene	pCPV/VP2/1-19 (F)	ATGAGTGATGGAGCAGTTC	19	767bp
	pCPV/VP2/767-748(R)	CTTAGTAAGTGTACTGGCAC	20	
M gene	pCCoV/M/FII	GTTATACAGAAGGACTAAGTCT	22	321bp
	pCCoV/M/RII	GTTGAGTAATCACCAGCTTTAG	22	

Table 1: Primers used for the amplification of VP2 (CPV) and M gene (CCoV)

Table 2: PCR	cycling	conditions	for am	plification	of VP2	(CPV-2)) and M	gene (CCoV)
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Stage	CPV-2	CCoV
Initial Denaturation	94°C for 5 min.	94°C for 5 min.
Start Loop	40 cycles	40 cycles
Denaturation	94°C for 30 sec.	94°C for 30 sec.
Annealing	54°C for 1 min. (40 cycles)	50°C for 30 sec.(40 cycles)
Elongation	72°C for 1 min.	72°C for 1 min.
Closed Loop		
Final Elongation	72°C for 15 min.	72°C for 15 min.

PCR products were analyzed on 1.5% agarose gel and visualized under UV transilluminator provided in gel documentation system (BIO-RAD).

Epidemiological analysis of positive cases

PCR and RT-PCR based confirmed positive cases were analyzed on the basis of their breed, age, sex and vaccination status.

RESULTS AND DISCUSSION

Out of 50 samples screened using conventional PCR and RT PCR, 29 samples were found positive for the viral pathogens (CPV and CCoV). Agarose gel 1.5% revealed a single and uniform band of 767 bp and 321bp size (Fig. 1 and Fig. 2) for CPV and CCoV respectively.

In the present study, out of 29 positive samples, 25 (86.20 per cent) and 4 (13.79 per cent) were found positive for CPV and CCoV respectively. Total percent prevalence of CPV was observed to be 50 per cent and 8 per cent prevalence was found for CCoV. An overall prevalence of CPV and CCoV infection in gastroenteric dogs was found to be 58 per cent. Srinivas *et al.* (2013) reported 53.90 per cent incidence of CPV in five different states of southern India by PCR assay which is partially in accordance with the present study. On the other hand a comparatively less prevalence of 40.85 per cent and 42.90 per cent of

CPV infection in suspected dogs was found by PCR and sandwich ELISA methods respectively by Behera et al. (2015) and Phukan et al. (2010). Singh et al. (2013) in their study found a higher incidence of CPV infection (64 per cent) using PCR based studies. In contrast to the present study the prevalence rate of CPV and CCoV infections were recorded as 25.25 and 19.28 per cent using sandwich ELISA by Deka et al. (2013). Sakulvera et al. (2003) found 62.8 per cent and 12.8 per cent samples positive for CPV-2 and CCoV, respectively using semi nested PCR and semi nested RT-PCR based assays, while only 40 per cent samples were found positive for CPV by conventional PCR assay (Bhat et al., 2012). Pinto et al. (2012) reported only 29.2 per cent (42/144) prevalence of CPV-2 in diarrheic dogs, while Wang et al. (2016) observed 28.36 per cent (57/201) faecal samples of diahorreic dogs positive for CCoV on the basis of RT-PCR studies targeting M gene. Variations in the prevalence observed in different studies are difficult to explain because of several factors including study area and different study assays employed; having variable sensitivity and specificity etc. Maximum occurrence of CPV and CCoV was found in the mixed breed dogs (44.83 per cent) (Table 3) followed by Labrador (27.59 per cent), Rottweiler (10.34 per cent) and Pit Bull (10.34 per cent). German shepherd and Doberman breeds were found to have only 3.45 per cent occurrence. This could be due to more reporting of these breeds in the clinics or because of the preference of owners for these breeds in the area of study.





Fig. 1: Agarose gel electrophoresis of 767 bp size PCR product of positive CPV samples, Lane L: 1Kb ladder, Gene Ruler (Qiagen). Lane 1: negative control, Lane 2: positive control. Lane 3, 4, 5 and 7 to 14: positive samples. Lane 6, 15 to 19: negative samples



Fig. 2: Agarose gel electrophoresis of 321bp PCR product of positive CCoV samples, Lane L: 1Kb plus ladder, Gel Pilot (Qiagen). Lane 1: negative control, Lane 4: positive control. Lane 11, 13: positive samples. Lane 2, 3, 5 to 10, 12 &14: negative samples

Breed	No. of cases (n=50)	No. of positive cases by PCR/RT- PCR (n=29)	Per cent positive cases w.r.t total positive cases
Mixed breed	18	13	44.83
Labrador	11	08	27.59
Rottweiler	07	03	10.34
Pitbull	05	03	10.34
German Shepherd	04	01	3.45
Pug	02	00	0
Doberman	01	01	3.45
Gaddi	01	00	0
Golden Retriever	01	00	0
Total	50	29	100

Table 3: Breed wise distribution of dogs affected with viral gastroenteritis

Breeds of Pug, Gaddi and Golden Retriever did not show any occurrence of CPV and CCoV based on molecular tests. No specific comments can be made on breed susceptibility as the population density of breeds varies from one geographical area to another (Archana et al., 2009). Behera et al. (2015) reported that mixed breeds and Labrador breed were equally susceptible to CPV infection, followed by other breeds such as Rottweiler, Spitz, Dalmatian, Pug, Great Dane and Neapolitan mastiff. The findings of the present study are in accordance with the observations of McCaw and Hoskins (2006) and Morais and Costa (2007) that medium and large breeds were more susceptible to CPV infection. On the other hand, Banja et al. (2002) recorded highest susceptibility to haemorrhagic gastroenteritis (HGE) in German shepherd breed followed by Spitz, Labrador and Doberman. In a similar study, Thomas et al. (2014) reported maximum prevalence of CPV infection in Labrador breed. Similarly, Deka et al. (2013) observed that German spitz breed was maximum affected with CPV and CCoV infection.

Maximum occurrence of viral gastroenteritis was found in the pups between the age group of 1 to 3 months (62.07 per cent) (Table 4) followed by the pups between 3 to 6 months (27.59 per cent). Dogs between the age group of 6 months to one year were found to have 10.34 per cent occurrence. 26 out of 29 dogs (89.65 per cent) suffering from viral gastroenteritis were in the age group of 1 to 6 months.

Table 4: Age	wise	distribution	of viral	gastroenteritis
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Age group	No. of cases examined (n = 50)	No. of positive cases by PCR/ RTPCR (n=29)	Percent positive cases w.r.t total positive cases
1 to \leq 3m	27	18	62.07
$>3m$ to $\leq 6m$	16	08	27.59
>6m to ≤1yr	07	03	10.34

16 dogs out of 25 positive dogs with CPV were in the age group of 1-3 months and 6 dogs were between the age group of 3 to 6 months (Data not shown). Two each of CCoV affected dogs were in the age group of 1 to 3 months and 3 to 6 months. Increased intestinal epithelial turnover caused by changes in microflora, diet (weaning) and diminishing maternal antibody level are the predisposing factors to CPV infection in pups (Decaro *et al.*, 2004 and Prittie, 2004). The canine transferring receptor is expressed at high density on actively dividing cells, and this enhances the pathogenesis of parvovirus infection in young pups as the virus needs mitotically active tissues for multiplication (Parthiban et al., 2010). Present findings are in conformity with the earlier observations that dogs less than 12 months of age were at increased risk (90.8%) of developing the haemorrhagic gastroenteritis (Alves et al., 1998 and Castro et al., 2013). Pups below six months of age are more affected with acute canine parvo viral enteritis (Behera et al., 2015; Singh et al., 2013; Srinivas et al., 2013; Thomas et al., 2014; Xu et al., 2013). The prevalence of CCoV in the faeces of diarrheic dogs aged 2-4 months in the present study is in accordance with the earlier findings (Costa et al., 2014; Parthiban et al., 2010; Soma et al., 2011). No correlation between CCoV infection and age, sex or breed of dogs was observed, but higher infection rate in dogs younger than one year was observed with symptoms of diarrhoea (Takano et al., 2015). Similarly, highest incidence in the age group of 7-12 months followed by 1-6 month, 13 months and above was reported by Phukan et al. (2010). In the present study, prevalence of CPV and CCoV in male and female dogs was observed to be 65.52 per cent and 34.48 per cent respectively (Table 5). Present findings suggested that male dogs were having high prevalence of CPV and CCoV gastroenteritis which simulate with the earlier observations of Behera et al. (2015); Sakulvera et al. (2003); Thomas et al. (2014) and Parthiban et al. (2010).

Table 5: Sex wise distribution of viral gastroenteritis

Sex	No. of cases examined (n = 50)	No. of positive cases by PCR/ RTPCR (n = 29)	Per cent positive cases w.r.t. total positive cases
Male	32	19	65.52
Female	18	10	34.48

The incidence of roaming and dominance aggression directed towards other dogs is comparatively high in males (Varshney, 2001). However, no significant sex variation in haemorrhagic gastroenteritis caused by canine parvovirus infection was noticed earlier (Banja *et al.*, 2002 and Singh *et al.*, 2013). The high prevalence of CPV and CCoV in male dogs might be due to more exposure and certain behavioral pattern in males and also due to selective preference of keeping male dogs by pet owners

Vaccination	No. of cases examined (n = 50)	No. of positive cases by PCR (n = 29)	Per cent positive cases w.r.t. total positive cases
None	39	25	86.21
Incomplete	08	03	10.34
Complete	03	01	3.45

Table 6: Vaccination vs non vaccination distribution of viral gastroenteritis

(Deka *et al.*, 2013 and Anderson, 1980). In the present study prevalence of viral gastroenteritis in dogs which were either not vaccinated or inadequately vaccinated was 96.55 per cent (Table 6).

Veterinary Clinical Complex, Hisar is the referral centre to which the cases are being brought from several adjoining districts of Haryana. Two dogs out of twenty five CPV positive dogs were from Fatehabad and Jind districts. Amongst CCoV positive dogs, one dog out of four positive dogs was brought from Fatehabad district. Rest twenty six positive dogs were from Hisar district of Haryana. The gastroenteric dogs were randomly selected for the study and thus the results show that vaccination against common viral diseases is not being followed in one or more districts of Haryana. Out of 29 positive dogs, 24 dogs that suffered from CPV infection were not vaccinated which is in agreement with earlier findings of Deka et al. (2013) and Houston et al. (1996) who observed that unvaccinated dogs were mostly affected by parvovirus gastroenteritis. Poor deworming and vaccination status might have contributed to the occurrence of clinical disease in affected dogs. In the present study 13 out of 29 positive dogs were not dewormed (44.83 per cent) which supports the earlier observation. Susceptibility to CPV-2 infection often coincides with the time when puppies are separated from dams as the level of protective immunity or maternal immunity declines (Cavalli et al., 2008). In the present study the diarrheic dogs had no vaccination history against CCoV infection which indicated that there is a lack of awareness amongst pet owners for vaccination in this area. Castro et al. (2013) reported 48 per cent detection of CCoV and/or CPV in the household pet puppies and emphasized that these pathogens have a significant etiological role in acute diarrhea in pet puppies even in areas where owners have access to veterinary care, and CPV/ CCoV vaccination is widely used. In the present study only 1 out of 29 positive dogs was completely

vaccinated (3.33 per cent) with multivalent vaccine. The occurrence of CPV in vaccinated dog was partially in agreement with observations of Behera *et al.* (2015) and Thomas *et al.* (2014) that 13.64 per cent vaccinated dogs suffered from CPV. However more number of samples from vaccinated diarrheic dogs should be screened and characterized further to know about the reasons of vaccine failure.

CONCLUSION

PCR is a sensitive technique for diagnosing the parvo viral and corona viral enteritis. In the present study, 58% suspected cases were confirmed positive for these pathogens by PCR and RT-PCR. The disease was found to be more common in mixed breed, male pups of less than 6 months of age. Disease reported in non vaccinated dogs suggested that intensive vaccination programme and awareness campaign is required amongst the pet owners of Haryana in order to bring down the prevalence of these dreadful viral diseases in the state.

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