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Research Article

MOLECULAR DETECTION AND CLINICO-HAEMATOLOGICAL STUDY OF VIRAL GASTROENTERITIS IN DOGS

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ABSTRACT

Canine parvovirus (CPV), canine distemper virus (CDV) and canine corona virus (CCoV) are important causes of gastroenteritis in dogs. Clinical signs caused by these viruses are overlapping and therefore confirmatory diagnosis is difficult. Polymerase chain reaction (PCR) and reverse transcription polymerase chain reaction (RT-PCR) technique is widely used as a tool for the confirmation of CPV, CDV and CCoV infections which provide rapid, sensitive and accurate diagnosis of the disease. The present study was planned to detect CPV, CDV and CCoV infection in the gastroenteric dogs by designing primers for the partial amplification of the VP2 structural gene of CPV, H gene of CDV and M gene of CCoV using conventional PCR and RT-PCR assays and to determine the clinical and hematological changes in the affected dogs for the rapid initiation of treatment. Out of 50 samples screened; 25, 1 and 4 samples were found positive for CPV, CDV and CCoV, respectively on the basis of molecular detection. Majority of the dogs affected with viral gastroenteritis showed signs of dehydration, lethargy, haemorrhagic diarrhoea and vomition. Hematological findings showed lowered values of haemoglobin, PCV, thrombocyte and total leucocyte count. A significant high neutrophil count and lowered lymphocyte count was also observed in the diseased dogs as compared to the healthy dogs.

Key words: Canine parvovirus, canine distemper virus, canine coronavirus, viral gastroenteritis, PCR, RT-PCR

Various causative agents of canine viral enteritis include canine parvo virus (CPV), canine distemper virus (CDV) and canine corona virus (CCoV). Canine parvo virus is a fatal disease characterized by vomition and haemorrhagic gastroenteritis in dogs of all age (Kumar *et al*., 2010) and myocarditis subsequently leading to heart failure in puppies of less than six months of age (Ying *et al.,* 2012). Canine distemper infection is a serious, highly contagious, often fatal, disease of dogs that results in respiratory and gastro-intestinal disease with frequent involvement of the central nervous system (Deem *et al.,* 2000). The disease is characterized by a rapid onset of severe leucopenia and loss of lymphocyte proliferation ability resulting immune-suppression. In India, CDV infections are common (Ramadass and Latha, 2001). CCoV infection causes gastroenteritis in dogs and is transmitted by faecal-oral route. Although a higher mortality rate is observed in animals with multiple infections with other pathogens such as CPV-2, canine adenovirus type 1 and CDV, CCoV represents *per se* is a major infectious agent responsible for several epidemics (Decaro and Buonavoglia, 2008). Molecular diagnostic techniques like polymerase chain reaction (PCR) methods had been the most reliable techniques having high degree of sensitivity and specificity in detecting CPV from faecal samples (Sriniwas *et al*., 2013).

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The present study describes the detection of CPV, CDV and CCoV infection in gastroenteric dogs by designing primers for the partial amplification of the VP2 structural gene of CPV, H gene of CDV and M gene of CCoV using conventional PCR and RT-PCR assays. Amplification of VP2 gene fragment and subsequent sequencing would help in detecting genetic variation between CPV-2 and its variants. Studies in the past have revealed that the H gene of CDV is one of the most important antigens for inducing protective immunity against CD, and any variation of recent CDV strains may result in vaccination failure. The M glycoprotein of CCoV can elicit strong immune responses and mutations in this gene might confer some sort of growth advantage or avoids host immunity. Clinical cases of viral enteritis in dogs are on rise in spite of timely vaccinations. In addition, early and rapid diagnosis is necessary so that infected dogs can be isolated and supportive treatment can be administered to reduce morbidity and mortality. Employing various molecular assays. Sriniwas *et al*. (2013) and Singh *et al*. (2013) found the incidence of CPV to be 53.90% and 64%, respectively in different states of India. Seventy per cent seroprevalence of CDV infection had earlier been reported from South India (Latha *et al*., 2007). In a study, Deka *et al*. (2013) recorded the prevalence rate of 25.25

and 19.28% in CPV and CCoV infections, respectively in Assam by using sandwich ELISA. The present study describes molecular detection of three important viral pathogens circulating in dogs suffering from gastroenteritis and their clinico-pathological effects in the affected dogs.

MATERIALS AND METHODS

A total of 50 rectal swabs were collected from dogs of one year or less in age reported to Teaching Veterinary Clinical Complex of this University with the clinical signs compatible with gastroenteritis from October, 2015 to September 2016. The samples were collected using sterile swabs in sterile PBS (pH 7.4), vortexed and preserved at -20°C till further processing. These faecal samples were screened for the presence of viral pathogens by applying conventional PCR and reverse transcription polymerase chain reaction (RT-PCR) based assays. The genomic DNA (CPV) and RNA (CD and CCoV) were extracted using commercially available Purelink ™ Genomic DNA extraction kit (Invitrogen, USA) following the manufacturer's instructions and TRIzol reagent (Takara®) using standard protocol respectively. Primers were designed for the relevant genes (VP2, H and M gene) for CPV, CDV and CCoV, respectively. For RNA viruses, c-DNA was prepared using Revert Aid first strand synthesis kit (Thermo-scientific). DNA and c-DNA templates were amplified by standardizing the PCR/RT-PCR cycling conditions respectively. Commercially available live attenuated multivalent vaccines for CPV (Megavac 6) and CDV (Vencomax 6) served as positive control. For CCoV positive control, a gene specific for CCoV was commercially synthesized. Approximately 5ml blood was collected from cephalic/ saphanous vein aseptically. Two ml of blood in EDTA was used for hematological examination and 3 ml blood utilized for obtaining serum. The blood samples were analyzed for complete hematological examination using automated hematology cell counter (MS4s, Melet Schlosing Lab.) and serum samples were analyzed for estimation of biochemical parameters using automated random access clinical chemistry analyzer (EM Destiny 180, Erba Diagnostics Mannheim GmbH).

Screening of the Faecal Samples: Gradient PCR was run with the designed primers (Table 1) to obtain the best annealing temperatures for amplification of the desired gene of CPV2, CDV and CCoV. The conventional PCR was performed in Thermal cycler (Veriti, Applied Biosystem) in 12.5µl reaction containing 3µl of template DNA, 6.25µl of Master mix 2X concentration (Thermo-scientific), 0.5µl each of forward and reverse primer (10 pmoles concentration) and 2.25µl of nuclease free water. Cyclic conditions for pCPV-VP2 primers included one cycle of initial denaturation at 94°C for 5min, followed by 40 cycles of denaturation at 94°C for 30sec, annealing at 54°C for 1min and extension at 72°C for 1min and final extension at 72°C for 15min.

PCR for pCD/ H was performed as done for CPV-VP2 gene. Cyclic conditions for pCD/ H primers included one cycle of initial denaturation at 94°C for 5min, followed by 40 cycles of 94°C for 30sec, annealing temperature of 50°C for 1min and extension at 72°C for 1min and a final extension at 72°C for 15min. PCR for pCCoV/M was performed in 12.5µl reaction as done for CPV-VP2 gene. Cyclic conditions for pCCoV/M primer set included one cycle of initial denaturation at 94°C for 5min, 40 cycles of 94°C for 30sec, annealing temperature of 50°C for 30sec and extension at 72°C for 1min and a final extension at 72°C for 15min. The PCR products were analyzed in 1.5 % in agarose gel electrophoresis and visualized under UV transilluminator Gel-Doc™ (BIO-RAD).

RESULTS AND DISCUSSION

Out of 50 samples screened, 30 samples were found positive for viral pathogens (CPV, CCoV and CD). Of the positives, 25, 4 and 1 samples respectively were found positive for CPV, CCoV and CDV (Figs. 1, 2 and 3). Overall prevalence of CPV, CCoV and CD were found to be 50%, 8% and 2%, respectively on the basis of molecular detection. In a similar study Sriniwas *et al*. (2013) detected CPV DNA in 53.90% samples by a PCR assay targeting VP2 gene. Thomas *et al*. (2014) also reported 23 out of 44 samples to be positive for CPV from suspected dogs.

Table 1
Designed primer pair used for the amplification of viral DNA (CPV, CD and CCoV)

Virus	Primers	Sequences	Product size
Canine parvovirus	pCPV/VP2/1-19 (F)	F-ATGAGTGATGGAGCAGTTC	767bp
	pCPV/VP2/767-748 (R)	R-CTTAGTAAGTGTACTGGCAC	
Canine distemper	pCD/H/199-219 (F)	F-AAT ATGGAATTTRGCAGATTG	665bp
	pCD/ H/863-843 (R)	R-CCCACTGCGATAGTACARAC	
Canine coronavirus	pCCoV/M/FII	F-GTTATACAGAAGGACTAAGTCT	321bp
	pCCoV/M/RII	R-GTTGAGTAATCACCAGCTTTAG	

Table 2
Hematological profile of dogs diagnosed with viral gastroenteritis (Mean ± S.E.)

Parameters	Healthy control(n=10)	Dogs affected with viral gastroenteritis (n=30)
Hb (g/dl)	13.29±0.25	8.48±0.18
Hct (%)	40.81±0.60	26.14±0.48
TLC (m/mm3)	11.59±1.69	8.35±1.93
Neutrophil (%)	66.43±0.66[a]	78.32±1.76[b]
Lymphocyte (%)	26.12±0.58[a]	16.45±1.63[b]
Monocyte (%)	5.58±0.31	4.01±0.42
Eosinophil (%)	1.57±0.32	0.93±0.15
Basophils (%)	0.30±0.15	0.28±0.07
Thrombocytes (m/mm3)	449.50±17.00	206.13±16.70

Values with different superscript differ significantly (P<0.05) in a row

In this study, only one sample was found positive for the presence of CDV by RT-PCR yielding a product size of 665bp. The present finding is in accordance with Mochizuki *et al.* (1999) who also found four of the 90 rectal swabs to be positive for CDV by RT-PCR. This could be because of the incapability of the RNA virus to remain stable in the faecal material due to activities of endogenous RNases and lack of accessibility of partially degraded RNA may influence the sensitivity of RT-PCR or due to intermittent shedding of the virus in faeces. However, the use of RT-PCR with different body fluids (serum, CSF, ocular swabs, urine and whole blood) can increase the sensitivity as the CDV RNA shows a heterogeneous distribution in different body compartments (Pawar *et al*., 2011).

In a similar study to ours, Costa *et al*. (2014) reported 30 of the 250 samples to be positive (12%) for CCoV RNA by RT-PCR that targeted M gene. On the other hand targeting S gene for the detection of CCoV RNA, Sakulvera *et al.* (2003) found 12.8% faecal samples positive by using semi nested RT-PCR assay. In contrast to our observations, Pratelli *et al*. (1999) and Wang *et al*. (2016) used RT-PCR based assay targeting M gene of CCoV and reported 19.71% and 28.36 % detection of CCoV RNA, respectively in the suspected faecal samples of dogs. In Indian context there is a paucity of molecular work related to the CCoV though serological studies conducted earlier reported 19.28% prevalence of CCoV (Deka *et al*., 2013). To the best of our knowledge this is perhaps the first attempt of detection of CCoV in the diarrheic faecal samples of dogs by conventional RT-PCR.

Clinical Profile: The most common clinical sign observed were dehydration (93.33%), inappetence to anorexia (90%), haemorrhagic diarrhoea (80%), vomition without blood (76.67%), palor mucous membrane (73.33%), lethargy (70%), nausea (53.33%), fever (30%), hematemesis (23.33%) and diarrhoea without blood (20%). Less common clinical signs were pustular skin lesions (13.33%), nasal discharge (16.67%) and ocular discharge (6.67%). Sagar *et al*. (2008) also opined that the common symptoms in dogs during parvo viral infection are vomiting, bloody diarrhea and dehydration. In the present study the dog suffering from canine distemper showed clinical signs of catarrhal nasal and ocular discharge, anorexia, fever and non haemorrhagic mucoid diarrhoea. Latha *et al*. (2007) were in the view that the clinical signs such as catarrhal nasal and ocular discharges and/ or diarrhoea with or without neurological symptoms are associated with canine distemper.

The dogs having corona virus infection were showing clinical signs of anorexia, lethargy, vomition and bloody diarrhea but the frequency of vomition and diarrhea was less as compared to the other affected dogs. Present findings corroborates with the findings of Castro *et al*. (2013) and Takano *et al*. (2016).

Hematological Findings: A marked decrease in haemoglobin, PCV and mean platelet count was observed in the affected dogs (Table 2) which are in accordance with the studies earlier conducted by Sharma *et al.* (2008) and Dongre *et al.* (2015). Anaemia and thrombocytopenia in cases of CPV is observed because the virus affects bone marrow and is cytotoxic for hematopoietic cells leading to myeloid and erythroid hypoplasia during acute stages of the disease (Macintire and Smith, 1997) and severe haemorrhagic enteritis (Sharma *et al*., 2005). In the present study the affected dogs were found dehydrated as reported earlier by Bhat *et al.* (2015). This was because of more fluid loss through diarrhoea and vomition in gastroenteric dogs. Thrombocytopenia observed in the present study could be due to the loss of blood through vomitus and faeces, increased destruction and/ or aggregation, decreased production and disseminating intravascular coagulation in CPV infection (Sharma *et al.*, 2008).

A non-significant (P<0.05) decrease in total leucocyte count, significantly (P<0.05) higher neutrophils and significantly lower lymphocyte count was recorded in the affected dogs as compared to healthy dogs. A moderate to severe fall in the total leucocyte count in dogs suffering from CPV infection had also been reported by Haligur *et al*. (2009) and Behera *et al*. (2014). Macartney *et al.* (1984) observed that viral replication in rapidly multiplying bone marrow and lymphatic tissue may destroy the actively mitotic myeloid precursors and lymphoid cells resulting in a state of leucopenia. The

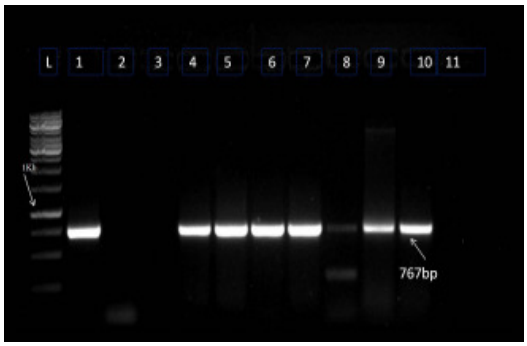

Fig 1. 767 bp product of CPV2 obtained with designed primers Lane L=1Kb ladder, Lane 1=Positive control, Lanes 4 to 10= Positive field samples, Lanes 2, 3 and 11=Negative samples

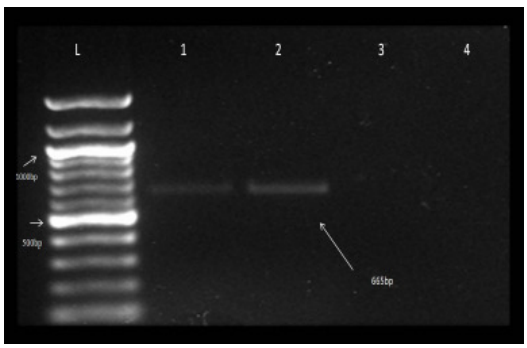

Fig 2. 665 bp product of CDV obtained with designed primers Lane L=100bp ladder, Lane 1=Positive control, Lane 2=Positive field sample, Lanes 3 and 4=Negative samples

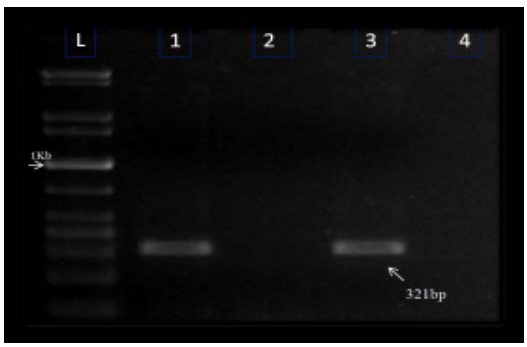

Fig 3. 321 bp product of CCoV obtained with designed primers Lane L=1Kb ladder, Lane 1=Positive control, Lane 3=Positive field sample, Lanes 2 and 4=Negative samples

changes in neutrophils and lymphocytes in this study could be due to severe inflammatory reaction caused by bacterial infections. This is in agreement with the findings of Bhat *et al*. (2015) who reported neutrophilia with the increased band cells and lymphopenia in dogs with diarrhea and vomition.

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