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Detection of Canine Viral and Bacterial Agents Associated with Gastroenteritis by PCR and RT-PCR

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ABSTRACT

Background: The important causative agents associated with gastroenteritis include Canine Parvovirus, Canine Rotavirus, Canine coronavirus and *E. coli.* The diagnostic assay *viz.* lateral flow test, PCR, RT-PCR was used for screening of diarrheal fecal samples of dogs to detect the presence of these pathogens.

Methods: The present study envisaged the appraisal of Canine parvovirus, Rotavirus, corona virus and enterotoxigenic *E. coli* infection. Total 50 rectal swabs/fecal swab in duplicate were collected for detection of viral andbacterial enteropathogens associated with canine gastroenteritis. For virus detection by PCR a 10% fecal suspension was prepared in phosphate buffer saline (PBS) (pH 7.2). The detection of viral and bacterial agents done by as per reference methods.

Result: Out of 50 fecal smples 10(20%) samples were found positive, 2(4%) were found to be positive for Canine Rotavirus and None of sample was found positive for Canine Corona virus. Canine Parvo virus 15(30%) and 1(2%) for Canine rotavirus by PCR and RT-PCR and None of the sample was positive for Canine Corona virus. Of this Canine parvovirus, Rotavirus and *E. coli* were recovered either singly or in association with each other in the form of mixed infection. Out of 50 samples, 15(30%) and 1(2%) were found positive for Canine parvovirus, Rotavirus and 45(90%) *E. coli.*

Key words: Canine parvovirus, Gastroenteritis, Rotavirus, RT-PCR, VP2 gene, VP6 gene.

INTRODUCTION

Since the beginning of time, the dog (*Canis familiaris*) has been man's most loyal companion. In small animals, diarrhea is linked to gastroenteritis and systemic diseases. Dogs of all breeds and ages suffer from enteritis, the most common gastrointestinal ailment (Bhat *et al.,* 2013). Gastroenteritis is the most common gastrointestinal problem in dogs and it affects dogs of all ages and breeds. Anorexia, vomiting, hemorrhagic diarrhea, dehydration, lethargy, electrolyte imbalance and fever are symptoms of gastroenteritis. A severely affected animal may also lose weight or become stunted (Bhat *et al.,* 2013). Various factors like bacterial and viral infections, parasitic infestations, irritant drugs, dietary errors and ingestion of toxic materials have been reported to be associated with canine gastroenteritis (Ettinger and Feldman, 2010). Various etiological agents have been reported to be associated with canine gastroenteritis like *Canine Parvovirus* (*CPV*)*, Canine Coronavirus (CCoV), Salmonella spp, Campylobacter spp, Clostridium perfringens, Escherichia coli, Giardia spp, Toxocaraspp* and *Ancylostoma spp. Canine Parvovirus* (CPV) and *Canine Coronavirus* (CCoV), *Canine rotavirus* are the most commonly encountered viral gastrointestinal infections (Marks *et al.,* 2011; Grellet *et al*., 2014).

Gastroenteritis is a severe hazard to the kennel population, the most frequently encountered viral enteric pathogens are Canine Parvovirus (CPV) and Canine Coronavirus (CCoV) (Marks *et al.,* 2011). CPV targets cells with high replication levels (digestive crypt cells), whereas CCoV targets mature cells (villosity digestive cells) (Casseleux, 2009). When these two viral infections are together, deadly diarrhea in puppies can occur during weaning. Ortega *et al.* (2017) found that because rotavirus has a low death rate, co-infection of the CRV and CPV results in a severe case of viral gastroenteritis. In comparison to other viral etiological agents responsible for gastroenteritis in dogs, it remains a hazard to global health due to its zoonotic properties.

The present study describes the detection of *Canine Parvo virus, Canine rotavirus* and *Canine Coronavirus* along with enterotoxigenic *E. coli* infection in gastroenteric dogs by using published primers for the partial amplification of the VP2 structural gene of *Canine parvovirus*, VP6 gene of *Canine rotavirus* and M-gene of *Canine Coronavirus* using conventional PCR and RT-PCR assays. Amplification of VP2

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gene fragment and subsequent sequencing would help in detecting genetic variation between CPV-2 and its variants. PCR base assay for laboratory confirmation for Canine rotavirus byVP6 gene based RT-PCR. 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. The 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 by employing various molecular assays. The present study describes molecular detection of three important viral and one bacterial pathogens circulating in dogs associated with gastroenteritis.

MATERIALS AND METHODS

Collection and processing of fecal samples

The present study was conducted in session of 2021-2022 at the Department of Veterinary Microbiology, Kranti Sinh Nana Patil college of Veterinary Science, Shirwal dist. Satara (M.S). Total 50 rectal swab/faecal swab in duplicate were collected for virus detection and isolation of secondary bacteria from dog showing clinical signs *viz.*, emesis, anorexia, depression, haemorrhagic enteritis. For virus isolation, a 10% fecal suspension was prepared in phosphate buffer saline (PBS) (pH 7.2), mixed and centrifuged at 10000 × g for 15 min to remove coarse particles. The clear suspension was transferred to fresh tubes and stored at -20°C. For bacterial isolation and identification, rectal swabs were inoculated in nutrient broth. Depending on the staining reaction, they were inoculated in differential media and selective media.

Viral pathogen was detected in association with *E. coli* in Canine gastroenteritis cases. All the total 50 fecal samples process for confirmation of *E. coli* by phenotypic methods based on lactose-fermentation on MacConkey agar, selective isolation and metallic sheen on EMB agar, typical IMViC pattern of *E. coli viz.,* Indole and M.R. positive, V.P. and Citrate negative and morphological characters by Grams staining. The Genotypic identification of *E. coli* positive samples were further confirmed with polymerase chain reaction (PCR) using published primers targeting 16S rRNA gene of *E. coli* by Jensen *et al*. (1993) was used.

Table 1: Primers used for partial length amplification of VP6 gene of group A rotavirus.

Gene	Primers sequence (5′-3′)	Amplicon size (bp)
VP6-F	5'GAC GGV GCR ACT ACA TGG T3′	379 bp
VP6-R	5'GTC CAA TTC ATN CCT GGT GG3′	

Table 2: Details of primers specific for VP2 gene of CPV used in PCR.

Name of primer	Primer sequence	Product size	Reference
CPV-2ab-F	GAAGAGTGGTTGTAAATAATT	681 bp	Sheikh *et al.* (2017)
CPV-2ab-R	CCTATATAACCAAAGTTAGTAC		

Detection of viral agents

A 10% fecal suspension of the fecal material was prepared in 10 mM phosphate buffer saline (PBS; pH 7.2). After thorough vortexing, centrifugation was carried out at 13000 rpm for 15 min at 4°C to remove the coarse debris. Supernatant was collected in other eppendorf tube for extraction of RNA. Total RNA was extracted using Trizol extraction reagent as per the methods described by Jadhav *et al*. (2009) .The DNA was extracted from fecal samples by using Genomic DNA Method as per Sambrook and Russel (2001). Collected swabs were subjected for the isolation and identification of secondary bacteria from fecal swab were prepared as per the guidelines of Cowan and Steel (1993) and Cruickshank *et al*. (1975).

For detection of VP2 gene was standardized by using gradient PCR to procure the maximal amplification of partial length of 681 bp product of the gene was carried out using primers and conditions as optimized by Sheikh *et al.* (2017) and the cDNA synthesized is used for amplification of VP6 gene (379 bp) of Canine rotavirus by Falcone (1999) and M gene (321bp) of canine coronavirus.

Screening of the fecal samples by PCR and RT-PCR

Gradient PCR was run with the published primers (Table 1, 2, 3 and 4) for amplification of the desired gene of CPV2, CRV, CCoV and 16S rRNA gene of *E. coli.* The conventional PCR was performed in Thermal cycler (Eppendorf, Germeny) in 12.5 µl reaction containing 3 µl of template DNA, 6.25 µl of Hi-crome Master mix 2X concentration (Hi-media), 0.5 µl each of forward and reverse primer (10 pmoles concentration) and 2.25 µl of nuclease free water. Cyclic conditions for pCPV-VP2 primers included one cycle of initial denaturation at 94°C for 5 min, followed by 40 cycles of denaturation at 94°C for 30 sec, annealing at 54°C for 1 min and extension at 72°C for 1 min and final extension at 72°C for 15 min. PCR for VP6 gene was performed. The Cyclic conditions for VP6 primers included one cycle of initial denaturation at 94°C for 5 min, followed by 35 cycles of 94°C for 30 sec, annealing temperature of 50°C for 1 min and extension at 72°C for 1 min and a final extension at 72°C for 15 min. PCR for pCCoV/M was performed in 12.5 µl

Table 3: Details of the primers used for the detection of M-gene of Canine coronavirus.

Primers	Sequence (5′-3′)	Location	Expected product size (bp)	Reference
M gene-F	GTTATACAGAAGGACTAAGTCT	22	321 bp	Agnihotri *et al.* (2018)
M gene-R	GTTGAGTAATCACCAGCTTTAG	22		

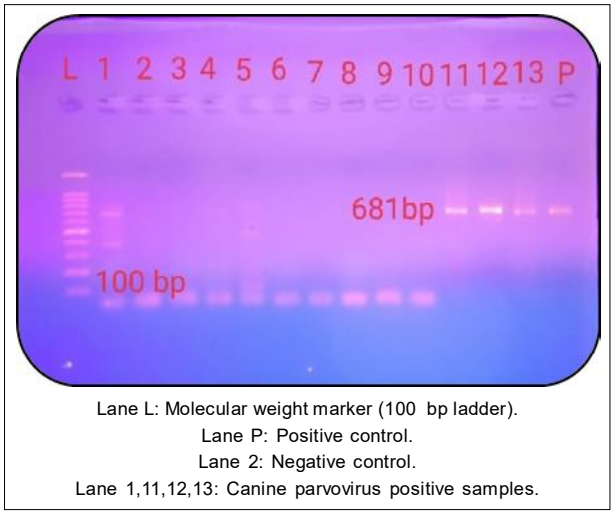
reaction as done for CPV-VP2 gene. Cyclic conditions for pCCoV/M primer set included one cycle of initial denaturation at 95°C for 5 min, 40 cycles of 94°C for 30 sec, annealing temperature of 50°C for 30sec and extension at 72°C for 1min and a final extension at 72°C for 15 min. The PCR products were analyzed in 1.5% in agarose gel electrophoresis and visualized under UV trans-illuminator. The Cyclic conditions for 16S rRNA gene of *E. coli* primers included initial denaturation at 95°C for five minutes, followed by 30 cycles with denaturation at 94°C for 40 second, annealing at 56°C for 30 seconds, extension at 72°C for 30 second with a final extension at 72°C for 7 min. The published primer sequences were used for PCR and RT-PCR. The primer sequences and nucleotide position of oligonucleotide primers are shown in Table 1, 2, 3 and 4. Amplified PCR products were visualized using agarose gel electrophoresis.

Table 4: For the amplification of 16S r RNA gene of *E. coli* following primers are used.

Primers	Primer sequence (5′-3′)	Amplicon (bp)
16S Frd (F)	CAGTCGTCTCCGAAGTTAACAA	704 bp
16S Rev (R)	CTCTACGCATTTCACCGCTAC	

Table 5: Prevalence of major enteropathogens associated with canine gastroenteritis.

Major entero -pathogens	Total number of fecal samples	No. positive (% positive)	Percentage %
Canine parvovirus	50	15	30%
Canine rotavirus	50	01	2%
Canine coronavirus	50	00	00%
E. coli	50	45	45%
Canine *parvovirus* + *rotavirus* + *E. coli*	50	03	06%



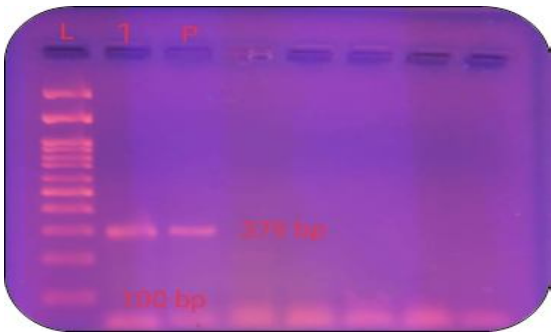
Lane L: Molecular weight marker (100 bp ladder).
Lane P: Positive control.
Lane 2: Negative control.
Lane 1,11,12,13: Canine parvovirus positive samples.

Fig 1: Detection of VP2 gene amplicon of canine parvovirus (681 bp) by PCR.

RESULTS AND DISCUSSION

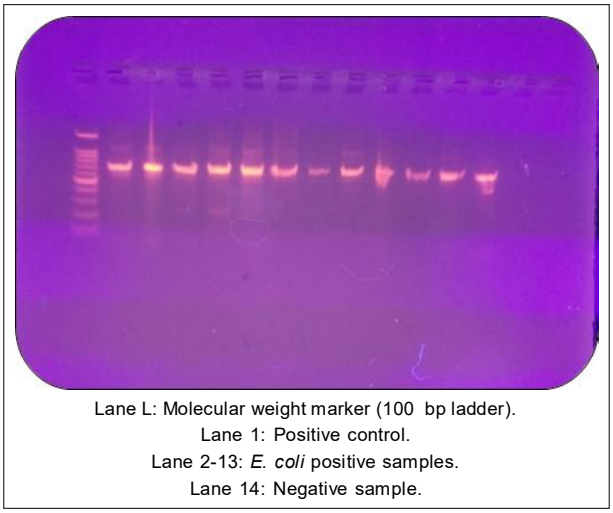
During the study period, a aggregate of 50 fecal samples 15(30%) samples were set up positive for Canine Parvo contagion by PCR with product size of 681 bp in agarose gel under UV transillumination, independently (Fig 1). Out of 50 fecal samples, 1(2%) samples were set up positive for Canine rotavirus by RT- PCR with product size 379 bp (Fig 2). The entire fecal sample also screen for presence of Canine Corona genome by RT PCR. None of the sample was positive for CCV. Out of 50 fecal sample, 45 (90%) isolates were verified as *E. coli* grounded on lactose fermenters on MacConkey agar, Metallic sheen on EMB agar, an morphological by gram staining (Fig 3, Table 1 and 5). It's veritably delicate to precisely diagnose the CPV- 2, CRV and CCV infections solely grounded on their clinical signs as utmost of the contagious and non-infectious causes of gastroenteritis produce clinical signs that are lapping in nature.

The present study findings are in agreement with earlier studies as reported by Chethan *et al.* (2022) reported that aggregate of 96 fecal samples were collected during the



Lane L: Molecular weight marker (100 bp ladder).
Lane P: Positive control.

Fig 2: Detection of VP6 gene amplicon of canine rotavirus (379 bp) by RT- PCR.



Lane L: Molecular weight marker (100 bp ladder).
Lane 1: Positive control.
Lane 2-13: *E. coli* positive samples.
Lane 14: Negative sample.

Fig 3: Amplification of *E. coli* (704 bp) of Canine faecal samples.

study period out of which 70 (72.91%) were set up positive for CPV- 2 infection. Luo *et al*. (2016) showed overall frequency of CDV, CPV and both contagions infections in pet were observed 28.5 (685); 19.6 (471) and 7.0 (168), independently in Wenzhou, China. Mager *et al.* (2020) employed conventional PCR for 15 fecal samples, 11 (22%) where set up by PCR with 681 bp. Reddy *et al.* (2015) who reported that out of 217 fecal sample 72 (33.17%) tested where positive. Our finding are in agreement with Sankalp *et al.* (2018) reported 18 frequency of CPV in Madhy Pradesh. Deovkar (2011) noted that out of 127 fecal samples 28 (22.04%) were found positive for CPV. Dorlikar *et al*. (2019) reported that out of 91 fecal sample 41 (45.05%) samples were found positive for CPV with 681 bp. The high circumstance of CPV-2 infection might be due to easy transmission of infection by faeco-oral route, resistant nature of the contagion to generally available detergents and continuity of contagion in the terrain for longer times in tropical sticky climatic condition. Flores *et al*. (2017) observed that out of 50 samples screened, 7 of puppies were inflamed with rotavirus solely, while 14 had been co-inflamed with each rotavirus and parvovirus. Tumlam *et al*. (2018) reported that Out of 50 samples none of the samples was set up positive for Canine rotavirus infection by VP6 gene base PCR. Bhargavi *et al*. (2017) studied the presence of co-infection in CPV positive 14 fecal samples from pups. Bacteriological examination shows the presence of mixed bacterial infection of *E.coli* with *Staphylococcus* spp. 3(21.43), *E.coli* with *Streptococcus* spp. 2(14.28), *Salmonella* spp. with *Staphylococcus* spp. 3(21.43), *Salmonella* spp. with *Streptococcus* spp. 1(7.14), *Klebsiella* spp. with *Streptococcus* spp. 2(14.28), *Pseudomonas* spp. with *Staphylococcus* spp. 3(21.43) in different combinations along with CPV infection.

CONCLUSION

In the present study, a total of a total of 50 fecal samples were screened and out of which, Canine Parvo virus 15(30%) and 1(2%) for *Canine rotavirus* by PCR and RT-PCR and None of the sample was positive for Canine Corona virus. Of this *Canine parvovirus, Rotavirus* and *E. coli* were recovered either singly or in association with each other in the form of mixed infection. An occurrence of CPV-2b along with canine rotavirus among the puppies with gastroenteritis is evident in the locality. In present study region and it warrants awareness among pet owners about vaccination for dogs against viral diseases. Screening of large number of samples covering wider geographic regions is required for further validation of the study. Effective vaccination program, client education and disinfection strategies will help in reducing the incidence. *E. coli* is identified to be a common enteropathogen in dogs. Various bacteria and the toxins also play an important role as a sole enteropathogen and in combination with viruses in the etiology of gastroenteritis in canines.

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Conflict of interest

All authors declared that there is no conflict of interest.

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