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Original article

Concomitant virus-induced gastrointestinal infection in dogs

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Abstract

Many viruses are involved in concomitant infections, which are prevalent in nature. In mixed infections, one or both infectious agents may be increased, reduced, or both may be increased while the other is suppressed. Canine distemper virus (CDV) and Canine parvovirus- 2 (CPV-2) are important causes of gastroenteritis in dogs. Detection of these viruses is challenging since the symptoms are very similar. CDV is a member of the *morbillivirus* genus in the *Paramyxoviridae* family, and CPV-2 is a member of the *Protoparvovirus* genus in the *Parvoviridae* family; and both predominantly affect puppies and induce gastrointestinal symptoms in dogs. The purpose of this study was to contribute to the differential diagnosis of dogs with gastrointestinal symptoms. A PCR technique with specific primers was used to identify CDV and CPV-2 infections in gastroenteric dogs, and clinical changes in the infected dogs were monitored. The VP2 structural gene of CPV and the nucleocapsid gene of CDV were partially amplified in the study. PCR amplified the partial fragments of the CDV nucleocapsid (287 bp) and CPV-2 VP2 proteins (583 bp) from feces. In total, 3 out of 36 stool samples were positive for CDV and CPV-2 in the same dogs. Gasterointestinal symptoms also supported the diagnosis of concomitant infection with CDV and CPV-2 in these dogs. Dehydration and diarrhea in dogs can be signs of various diseases, such as viral, bacterial, and parasitic infections. After the elimination of non-viral pathogens, CDV and CPV-2 should also be simultaneously investigated to establish what is causing these symptoms. This study demonstrates the potential utility of correct diagnosis for the control of viral infection in dogs, but more research with a broader use of PCR-based detections is needed to assess its impact on differential diagnosis for concomitant infections.

Keywords: canine, concomitant, distemper, gastrointestinal, parvovirus, PCR

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Introduction

CPV-2, which is divided into three subtypes (CPV-2a, CPV-2b, and CPV-2c), is a *Carnivore protoparvovirus* 1 (*Parvoviridae*, *Parvovirinae*, and *Protoparvovirus*) species. CPV-2 has four genes that are coded by two Open Reading Frames (ORFs): ORF1 codes for the non-structural proteins NS1 and NS2, and ORF2 codes for the capsid proteins VP1, VP2, and VP3 (Galvis et al. 2022). CDV is a non-segmented, negative-sense, single-stranded, and enveloped RNA virus that belongs to the genus *Morbillivirus*, family *Paramyxoviridae* (Tuteja et al. 2022, Saltık and Kale 2023). The CDV genome encodes six structural proteins and two non-structural proteins. The six structural proteins are nucleocaspid (N), phosphoprotein (P), matrix protein (M), fusion protein (F), hemagglutinin protein (H), and large protein (L). The two non-structural proteins, C and V, are encoded either by mRNA editing or by an alternate ORF in the gene that encodes P protein (da Costa et al. 2021).

CDV infection is a highly infectious, usually fatal, disease of dogs that causes respiratory and gastrointestinal disorders with frequent central nervous system involvement (Saltık and Kale 2020). CPV-2 replicates in rapidly dividing cells, especially in hematopoietic and intestinal tissues, resulting in severe immunosuppression, hemorrhagic enteritis, and myocarditis in puppies (Urbani et al. 2022). In both infections, viruses cause similar clinical symptoms in dogs, which are often non-specific and include symptoms of depression, lethargy, and fever (Tutaje et al. 2022). The affected dogs are very dehydrated as a consequence of hemorrhagic diarrhea and vomiting, which commonly occur within the first day after infection.

Concomitant canine gastrointestinal infections caused by viruses such as CDV, CPV-2, *canine enteric coronavirus* (CCoV), *canine adenovirus*-1 and -2 (CAdV-1, -2), *canid alphaherpesvirus*-1 (CaHV-1), and *canine kobuvirus* (CaKoV) often need to be co-diagnosed in dogs (Headley et al. 2018, Tuteja et al. 2022). Additionally, the presence of other pathogens makes it much more difficult to explain how the underlying mechanisms function. Although all are known as important diarrheal pathogens in dogs, CDV and CPV-2 are more prevalent (Pesavento and Murphy 2014, da Costa et al. 2021). Concomitant means multiple infectious agents coexist in the same host, which is referred to as having “mixed infections” or “co-infections” in medical terminology. Mixed infections with CDV and CPV-2 commonly occur in dogs, and both viruses could cause diarrhea, which makes differential diagnoses based on clinical symptoms difficult.

The various virus-host interactions have been difficult to explain and mainly centered on attempts to implicate disease-defined mechanisms of immunodepression, often referred to as immunosuppression, brought about by molecules induced by viruses that facilitate their own survival. Through the use of molecular tests, researchers (Frisk et al. 1999, Desario et al. 2005, Silva et al. 2014) were able to identify infectious disease agents in canines owing to the amplification of nucleic acids in both symptomatic and asymptomatic dogs. The purpose of our study was to contribute to the differential diagnosis of dogs with gastrointestinal symptoms. The differential diagnosis that is necessary in hosts infected with more than one infectious agent, CDV and CPV-2, particularly those causing gasterointestinal symptoms, will be the major focus of this research.

Materials and Methods

Sample Preparation

In this study, 36 dogs’ stool samples that were brought to the Burdur Mehmet Akif Ersoy University Faculty of Veterinary Medicine Virology Laboratory, Animal Hospital, and private veterinary clinics were studied. It was reported that all stool samples had tested positive for CPV-2 using a variety of commercially available fast test kits. As anamnesis information, the owners and veterinarians of the patients provided information on the canines to which each sample belonged. According to anemnesis, the dogs had gastrointestinal system problems, and they were of various ages, breeds, and genders.

The samples were diluted in PBS containing antibiotics in a 1:10 ratio. After fully mixing with a vortex, it was centrifuged at +4°C for 30 minutes at 3000 cycles. Following centrifugation, the supernatant was collected and transferred to sterile, 2 ml RNase-DNase-free microtubes to test for the presence of nucleic acid. It was kept at -80°C until all analyses were performed.

Viral Nucleic Acid Extraction

A commercial High Pure Viral Nucleic Acid Isolation kit (Roche, Germany), was used to efficiently purify viral nucleic acids from the supernatant of stool samples. 200 µL of supernatant was combined with 200 µL of binding buffer and 50 µL of proteinase K. After incubation, centrifugation, and filtering steps, the kit’s instructions were followed. To elute the viral nucleic acids, we added 50 µL of elution buffer to the upper reservoir of the filter tube and centrifuged the tube assembly for 2 min at 10000 x g. The purified viral nucleic acid was eluted in nuclease-free water, and they

Table 1. Oligonucleotides for PCR amplification of CDV and CPV.

Gene	Primers	Nucleotide position	Sequence (5'-3')	Size (bp)	Ref
N	N-for P1a	769-789	ACAGGATTGCTGAGGACCTAT	287	Frisk et al. 1998
	N-rev P2a	1055-1035	CAAGATAACCATGTACGGTGC		
VP2	555for	4003-4022	CAGGAAGATATCCAGAAGGA	583	Buonavoglia et al. 2001
	555rev	4585-4561	GGTGCTAGTTGATATGTAATAAACA		

were all kept at -80°C until all PCR analyses were done. Since this commercial kit allows for the simultaneous isolation of total RNA and DNA, the same extracts were used for the next analysis.

RT-PCR test for CDV

RNA templates were subjected to the efficient synthesis of first strand cDNA from RNA templates using the RevertAid First Strand cDNA Synthesis Kit (Thermo Scientific, USA) with random hexamer primer, as per the manufacturer's instruction. cDNA templates were used to amplify the targeted CDV N protein-encoding gene using PCR assay on a Thermocycler (Applied Biosystems™, USA). For the CDV N gene, primer sets and cycling conditions were used as previously described by Frisk et al. (1999), with only slight modifications. The PCR cycling conditions were pre-denaturation at 94°C for 3 min, 35 cycles of 1 min at 94°C, 2 min at 59°C, and 1 min at 72°C, and a post-extension at 72°C for 7 min.

PCR test for CPV-2

For the CPV VP2 gene, primer sets and cycling conditions were used as previously described by Buonavoglia et al. (2001), with only slight modifications. The PCR cycling conditions were pre-denaturation at 95°C for 10 min, 35 cycles of 30 sec at 94°C, 1 min at 50°C, and 1 min at 72°C, and a post-extension at 72°C for 7 min.

As negative and positive controls for both tests, ultrapure water and a commercial vaccine extract were used. All primers are shown in Table 1 together with their nucleotide positions and amplicon sizes. The PCR products were analyzed in 1.5% agarose gel electrophoresis and visualized under UV transilluminator Gel-Doc™ (BIO-RAD).

Results

This research involved a total of 16 female dogs and 20 male dogs. The majority of dogs were mixed-breeds: 58.33% (21/36), with pure-breds accounting for 41.66% (15/36) (Table 2).

Clinical anamnesis

Thirty-six puppies were evaluated to determine the pattern of clinical manifestations according to the PCR results. It was reported that the following gastero-intestinal symptoms, such as dehydration (33/36), hemorrhagic diarrhea (15/36), vomitting (18/36), and diarrhea without blood (20/36) were observed by the veterinarian and the owners. Accordingly, the most frequent gastero-intestinal symptoms were diarrhea (n=35) and dehydration (n=33) (Table 2).

All the dogs were under six months old and were various mixed-breed dogs. In addition to this, it was reported that no vaccination had ever been administered in the past. PCR was used to amplify partial fragments of the CDV N protein-encoding gene (287 bp) and the CPV-2 VP2 capsid protein gene (583 bp) from stool samples. In total, 3 were positive for CDV and 10 were positive for CPV-2 out of 36 samples (Table 3). In total, 3 out of 36 stool samples were detected as positive for both CDV and CPV-2 in the same dogs (Fig. 1).

Discussion

CPV-2 and CDV are two of the most common agents responsible for canine viral enteritis. CPV-2 infection is a fatal illness that causes hemorrhagic diarrhea in dogs of all ages and myocarditis, which leads to heart failure in puppies under the age of six months (Urbani et al. 2022). The immunodepressive effects of CDV, together with the poor immune systems of these puppies, enabled the development of concomitant infections in the same dog in the majority of cases (Beineke et al. 2009, Lechner et al. 2010, Pesavento and Murphy 2014). It is thought that CDV-induced immunodepressive effects are linked to the death or weakening of SLAM-expressing cells in the gastrointestinal tract because of their affinity for the epithelial cells and mucosal lymphocytes (von Messling et al. 2006, Carvalho et al. 2012). Pupies may be more often infected than initially assumed with various infectious disease agents such as CDV and CPV-2, and multiple organ failure may occur as a result of these infections

Table 2. Information about dogs' medical histories and biological characteristics based on anamnesis with PCR and RT-PCR results in stool samples.

No	Breed	Sex	Age (months)	Clinical symptoms*	Results	
					CPV-2	CDV
1	Mixed-breed	Female	1	D, DWH	-	-
2	Mixed-breed	Male	3	DWH, V	-	-
3	Mixed-breed	Male	2	D, DWH, V	-	-
4	English Cocker Spaniel	Female	2	D, DWH, V	-	-
5	Labrador Retriever	Female	2	D, DWH	-	-
6	Turkish Kangal Shepherd	Female	3	HD, V	-	-
7	Siberian Husky	Male	1	D, HD, V	+	-
8	Mixed-breed	Female	5	D, HD, V	+	+
9	Mixed-breed	Male	6	D, DWH	-	-
10	Turkish Kangal Shepherd	Female	2	D, HD	+	-
11	Beagle	Male	3	D, HD, V	+	-
12	German Shepherd	Male	1	D, DWH	-	-
13	Golden Retriever	Female	2	D, HD, V	+	-
14	Mixed-breed	Male	1	D, DWH	-	-
15	Mixed-breed	Male	2	D, V	-	-
16	Mixed-breed	Male	3	D, DWH	-	-
17	Mixed-breed	Female	5	D, DWH, V	-	-
18	Turkish Kangal Shepherd	Male	3	D, DWH	-	-
19	Mixed-breed	Female	3	D, DWH, V	-	-
20	Mixed-breed	Female	1	D, HD	+	-
21	Mixed-breed	Male	2	D, DWH	+	+
22	French Bulldog	Female	3	D, DWH	-	-
23	Mixed-breed	Male	1	D, HD, V	+	-
24	Mixed-breed	Male	4	D, HD	-	-
25	Mixed-breed	Male	4	D, HD	-	-
26	Mixed-breed	Female	4	D, HD, V	+	-
27	Mixed-breed	Female	4	D, DWH	-	-
28	Golden Retriever	Male	1	D, HD	-	-
29	German Shepherd	Female	3	D, DWH	-	-
30	Mixed-breed	Male	5	D, HD, V	-	-
31	Mixed-breed	Male	6	D, DWH	-	-
32	Mixed-breed	Female	2	D, DWH	-	-
33	Mixed-breed	Male	1	D, DWH, V	-	-
34	German Shepherd	Female	1	HD, V	-	-
35	Turkish Kangal Shepherd	Male	2	D, DWH, V	-	-
36	Turkish Kangal Shepherd	Male	2	D, HD, V	+	+

* Dehydration=D, hemorrhagic diarrhea=HD, diarrhea without blood=DWH, vomitting=V

Table 3. Presence of viral nucleic acid in dogs' stool samples.

	PCR result	
Viral agents	Positive	Negative
CDV	3	33
CPV-2	10	26

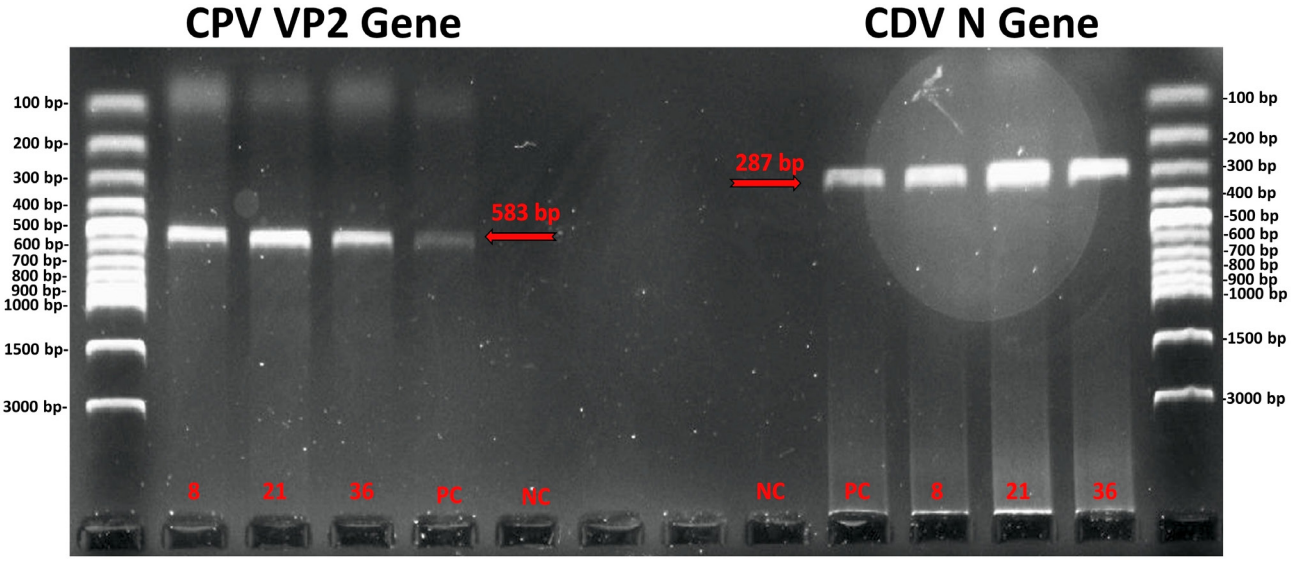

Fig. 1. DNA-stained agarose gel of PCR products that shows the result of the assay DNA marker (100 bp); PC: positive control; NC: negative control; No. 8, 21, and 36 are positive in dogs' stool samples for CDV and CPV-2.

(Headley et al 2018). As a result, it demonstrates the viruses' affinity for intestinal cells as well as their potential to induce symptoms such as dehydration, diarrhea, and vomiting in dogs. Similarily, in our study, the dogs or their stool samples were brought to veterinary clinics, suffering from dehydration, vomiting, and bloody (or not) diarrhea (Table 2).

Previous research has shown that a number of agents have a role in the development of mixed infections in dogs (Headley et al. 2015, Tuteja et al. 2022). This study documents the determination of CPV-2 and CDV infection in the same dogs that were previously unvaccinated. All of the dogs in the study had their age, breed, sex, and clinical symptoms determined and reported by either their owners or their vets based on observations of abnormal behaviors, dentition, body weight, and condition. In this study, according to the animal's anamnesis, the veterinarians of the patients provided information about all the stool samples that tested positive for CPV-2 using a variety of commercially available fast test kits. It has been reported that rapid test kits work quite well, both in terms of sensitivity and specificity (An et al. 2008). Therefore, samples that were reported to be positive with rapid kits designed for in-clinic use were preferred for use in this study. The clinical signs and symptoms were similar to parvovirus infection and were confirmed using a commercial rapid test kit, also known as an immunochromatography assay (ICA), showing parvovirus positivity. However, when we examined all stool samples for the presence of CPV-2 by PCR test, only 10/36 (27.77%) positives were obtained. We can state that there are two potential causes for this predicament. First, the rapid kits usually have low diagnostic accuracy in comparison to studies with gold-standard methods (Desario et al. 2005, Schmitz et al. 2009, Kantere et al. 2015) and second, incorrect information may have been provided by veterinarians or owners. Neverthless, the tests are used under field conditions by most veterinarians. In veterinary medicine, it is possible that some pathogens in infectious diseases of dogs, similar to those found in other animals, can be ignored or misdiagnosed. Various nucleic acid based detection techniques, which are fast, sensitive, and specific, have been developed for the confirmation of pathogens in clinical samples (Silva et al 2014, Cao et al 2019). When compared to fast kits, PCR is the most widely used diagnostic testing method for accuracy. The detection of specific amplification of a targeted DNA sequence is the basis for this method. The PCR test may be used to diagnose animal diseases with excellent performance. Obtaining a proper diagnosis or taking measures to ensure that no diagnosis is missed are both known to be out of reach financially for most owners. These facts lead to maltreatment with steroids, non-steroidal anti-inflammatory drugs, and antibiotics. Funds are spent on unnecessary treatments that wind up costing the owner much more money in the long term. In fact, our research showed that incorrect diagnoses and subsequent treatments may be avoided by vets when a proper diagnosis is made using reliable methods such as PCR. Misdiagnosis and missed diagnoses are common, particularly when it comes to similar symptoms of different pathogens, but they are not the only reasons for this. Most vets prefer quick tests but, most of the time, these tests are not sensitive or specific enough to determine the cause of even fatal diseases.

In this study, the initial diagnosis of canine parvovirus was based on anemnesis and clinical findings that are consistent with this disease. The participation of CPV-2 in relation to clinical findings was confirmed by amplifying the approximately 583-bp fragment of the VP2 gene using primers that are specific for CPV-2. However, not all samples were positive for CPV-2, and only 10 stool samples were. Because the gastrointestinal symptoms are indistinguishable from those of the other infections, it is vital to test for the other pathogens (Headley et al. 2015, Tuteja et al. 2022). In this study, CDV was preliminary diagnosed by the characteristic clinical manifestation of diarrhea associated with immunodeficiency; such findings are significant for both serological and virological aspects. Therefore, confirmation of infection was obtained from the PCR assay that successfully amplified the 287-bp fragment of CDV; similar findings have been described (Headley et al. 2018).

In this study, CDV and CPV-2 nucleic acids were detected by PCR in stool samples from unvaccinated dogs. Most viral loads can be suppressed during the late stages of the infection and/or the early presence of high CPV and CDV antibody titers in the gut lumen (Decaro et al. 2005). In this case, we may not be able to detect the etiologic agent we aim to find positive for diagnosis. It is known that for non-immunized dogs, symptoms (or no symptoms) are shed viruses from their feces during infection (Saltık and Kale 2023). Interestingly, despite the presence of CDV symptoms in these unvaccinated animals, a higher prevalence of CPV positivity was detected in this study. CDV is more unstable outside the host and hence provides fewer chances for dogs to gain immunity via exposure to contaminated environments (Lamm and Rezabek 2008). We hypothesize that this is due to the DNA virus CPV-2's prolonged existence in the environment for such a long time, which has led to an increase in natural exposure (Molnar et al. 2014).

The low number of dogs sampled could have limited some statistical comparisons by reducing the statistical power of the study. However, both viruses were found in 3 of our research samples, and the dogs that provided these samples all showed comparable signs of dehydration, vomiting, and diarrhea. The results linked to a concomitant infection in these dogs were similar to those reported clinically, indicating that CPV-2 and CDV must be considered together in the differential diagnosis of dogs with similar clinical symptoms. This was supported by the identification of these pathogens' nucleic acids by PCR in the samples of dogs, as CDV also causes gastrointestinal disease. Additionally, other infectious disease agents (Tuteja et al. 2022) may infect the gut concurrently in some of these dogs with the stated symptoms, suggesting that these pathogens should also be considered in the differential diagnosis of suspected dogs.

Conclusion

CDV also induces symptoms such as dehydration, vomiting, and diarrhea, and CPV may not be solely responsible for similar disorders. In the future, further experimental studies will be necessary to explain how these two agents interact with one another and with the host. This still continues to be a major concern, since the increase in diagnostic failure is causing the co-occurrence and spread of CDV and CPV. We can also state that PCR plays an important role in the early-stage diagnosis, especially in concomitant infections. Immunological research may also be needed to evaluate and keep track of the biological relevance of CPV and CDV genotypes that are circulating at the same time, as well as their importance for future diagnosis and possible vaccine development.

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