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RESEARCH ARTICLE

Evaluation of the Performance of Diagnostic Methods of Canine Parvovirus-2 and Canine Enteric Coronavirus Infections under Different Storage Conditions and Determination of Molecular Characterization

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Köpek Parvovirus-2 ve Köpek Enterik Coronavirus Enfeksiyonlarının Tanı Yöntemlerinin Farklı Saklama Koşulları Altındaki Performansının Değerlendirilmesi ve Moleküler Karakterizasyonunun Belirlenmesi

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Öz

Amaç: Bu araştırma, köpeklerde CPV ve CCoV enfeksiyonlarını hızlı kit ve PCR ile karşılaştırmalı olarak tespit etmek ve Konya bölgesindeki bu enfeksiyonların moleküler karakterizasyonunu belirlemek amacıyla gerçekleştirilmiştir. Ayrıca, enfeksiyon tanısı için taze veya dondurulmuş-çözülmüş dışkı sonrası tanı testlerinin duyarlılık ve özgüllük oranlarının belirlenmesi amaçlanmıştır.

Gereç ve Yöntem: Dışkı örnekleri, barınakta ishal semptomları gösteren, aşılanmamış, 0-12 aylık 50 köpekten toplandı. Örnekler CPV ve CCoV yönünden hızlı test ve PCR testi ile incelendi. Örnekler dondurma-çözdürme işleminden sonra tekrar aynı testler ile kontrol edildi.

Bulgular: CPV, hızlı test ve PCR ile sırasıyla 2 ve 29 taze dışkı örneğinde, CCoV ise 14 ve 28 örnekte pozitif olarak teşhis edilmiştir. Dondurma-çözme prosedüründen sonra CPV pozitif örneklerde değişiklik olmazken, CCoV 10 örnekte ve 28 örnekte hızlı test ve PCR ile pozitif olarak teşhis edilmiştir. CPV tanısında herhangi bir farklılık olmamasına rağmen, CCoV tanısında hızlı testin duyarlılığı dondurma-çözdürme prosedüründen sonra azalmıştır. Ayrıca CPV pozitif örneklerde sadece CPV-2b tipi tespit edilirken, CCoV pozitif örneklerde moleküler olarak hem GI hem de GII alt tipleri tespit edilmiştir. Sonuç olarak, hızlı testlerin CPV ve CCoV enfeksiyonlarının doğru teşhisi için duyarlı olmadığı görülmüştür.

Öneri: Virolojik enfeksiyonların doğru teşhisi için moleküler tanı yöntemlerinin seçilmesinin ve taze numunelerin kullanılmasının önemi vurgulanabilir

Anahtar kelimeler: CPV, CCoV, filogenetik analiz, metot karşılaştırılması

Abstract

Aim: This research was carried out to detect CPV and CCoV infections in dogs in comparison with rapid kit and PCR and to determine the molecular characterization of these infections in Konya region. Besides, it was aimed to determine the sensitivity and specificity rates of the diagnostic tests after fresh or freeze-thawed stool for infection diagnosis.

Materials and Methods: Faecal samples were collected from 50 unvaccinated, 0-12 months old dogs with diarrhoea symptoms at the shelter. The samples were analysed for CPV and CCoV by rapid test and PCR test. After freeze-thawing, the samples were checked again with the same tests.

Results: CPV was positively diagnosed by rapid test and PCR in 2 and 29 fresh stool samples, respectively, and CCoV in 14 and 28 samples. CPV positive samples did not change while CCoV was diagnosed as positive in 10 samples and 28 samples by rapid test and PCR, respectively, after the freeze-thaw procedure. Although there were no differences in the diagnosis of CPV, the sensitivity of the rapid test in the diagnosis of CCoV decreased after the freeze-thaw procedure. In addition, only CPV-2b type was detected in CPV positive samples and both GI and GII subtypes were detected in CCoV positive samples as molecular. In conclusion, it was observed that rapid tests are not sensitive for accurate diagnosis of CPV and CCoV infections.

Conclusion: The importance of choosing molecular diagnostic methods and using fresh samples for accurate diagnosis of virological infections can be emphasized.

Keywords: CPV, CCoV, phylogenetic analysis, method comparison

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Introduction

Viral gastroenteritis is a common clinical condition in dogs. It was reported that 40-60% of this condition is of viral origin. For the last 50 years, the most common cause of viral gastroenteritis in dogs is canine parvovirus (CPV), but studies have reported that different viruses cause diarrhea in dogs (Khatri et al 2017, Caddy 2018). These are defined as Canine rotavirus (CRV), Canine enteric coronavirus (CCoV), Canine norovirus, Canine astrovirus, Canine distemper virus (CDV) and Canine circovirus (Caddy 2018).

Canine Parvovirus type 2 (CPV-2) is one of the most important enteric viral pathogens for dogs. This virus is observed very frequently, especially in places where dogs are kept together and in large numbers, such as shelters and pet shops. It also has a high mortality rate. Sudden death can be observed in unprotected puppies 2-3 days after clinical signs are observed (Khatri et al 2017). Although CPV-2 can cause infection in dogs of all ages, it is more common in dogs aged between six weeks and six months (Miranda et al 2016b). Canine Parvovirus type 2 is in the family *Parvoviridae*, genus *Protoparvovirus.* It is also classified in Carnivore protoparvovirus 1 together with Feline panleukopenia virus (FPV), Mink enteritis virus (MEV), Raccoon parvovirus (RPV) and (Khatri et al 2017). In the 1980s, a new strain of CPV-2, CPV-2a, was identified due to differences in amino acid sequence on the VP2 protein. In the following years, the virus mutated rapidly, and new antigenic variants, CPV-2b, and CPV-2c, emerged (Gupta et al 2016). This gene region is important for molecular characterization as these antigenic variants are formed due to differences in amino acid sequence on the VP2 protein (Gupta et al 2016).

Coronaviruses (family *Coronaviridae*) are enveloped, single-stranded, positive-stranded RNA viruses that cause infections in many mammals, including humans and birds, often associated with mild enteritis and respiratory (Alfano et al 2020). Notable domestic animal coronaviruses include canine enteric coronavirus (CCoV), feline infectious peritonitis virus (FIPV), equine enteric coronavirus (ECoV), ferret systemic coronavirus (FRSCV), ferret enteric coronavirus (FRECV), and canine respiratory coronavirus (CRCoV), feline enteric coronavirus (FECV), and alpaca enteric coronaviruses (Haake et al 2020). The Canine Coronavirus (CCoV), family Coronaviridae, genus Alphacoronavirus, species Alphacoronavirus-I) membrane (M) protein is the most detected structural protein. Based on the analysis of M- , S- protein-coding genes, CCoV strains are divided into two genotypes CCoV-I and CCoV-II (Decaro et al 2011, Navarro et al 2017). CCoV is generally accepted as the etiological agent of small intestine infections and may lead to gastroenteritis (Decaro et al 2011).

Various studies have been conducted on CPV and CCoV in the world and Turkey (Avci et al, 2015b, Navarro et al 2017, Akkutay et al 2020a, Akkutay et al 2020b, Dik et al 2021). These studies reveal the importance and prevalence of these infections in dogs. This study aims the determination of the presence of CPV and CCoV infections in dogs in the shelter environment by rapid test and PCR; the comparison of the frequently used rapid tests with a molecular method such as PCR; whether fresh or frozen stool samples affect the sensitivity and specificity of diagnostic methods; and to determine the circulating types in this region by making phylogenetic analyzes of these factors.

Material and Methods

Faecal samples were collected between April and September 2020 from 50 dogs aged 0-12 months, that did not have a vaccination history for the factors examined and had clinical symptoms such as especially severe diarrhea and bloody diarrhea in Konya metropolitan municipality stray animal shelter and rehabilitation centre. First of all, freshly collected diarrhoeic samples of dogs tested by employing CPV and CCV rapid test kits obtained from a commercial company. After then, DNA (QIAamp DNA Mini Kit Cat No. 51304) and RNA extraction (QIAmp Viral RNA Mini Kit Cat. No.52906) procedures were performed for PCR tests. A portion of stool samples were stored in -20 °C for 3 weeks, after which they were thawed, and all samples were subjected to rapid testing and extraction again.

PCR analyzes were performed after the extraction processes of all samples, both fresh and after freezing and thawing. After viral DNA extraction for CPV, primers specific to the VP-2 gene region (5'-CTTTAACCTTCCTGTAACAG-3', 5'-CATAGTTAAATTGGTTATCTAC-3') (Pereira, et al., 2000) were used, and PCR was performed with the following cycling conditions: 94°C for 3 min, 33 cycles of DNA denaturation at 94 °C for 45 sec, primary annealing at 50 °C for 45 sec and amplification at 72 °C for 1 min, followed by a final extension at 72 °C for 10 min. For CCoV, primers specific to the partial region of the gene encoding the M protein (5'-TCC AGA TAT GTA ATG TTC GG-3', 5'-TCT GTT GAG TAA TCA CCA GCT-3') (Pratelli, et al., 1999) were used, PCR was performed with the following conditions: 60 °C for 15 min, 98 °C for 30 sn, 33 cycles of DNA denaturation at 98 °C for 10 sec, primer annealing at 55 °C for 45 sec and amplification at 72 °C for 30 sn, followed by a final extension at 72 °C for 2 min. The resulting PCR products were separated using 1.5% agarose gel electrophoresis. Moreover, separated bands were visualized by UV transillumination.

For phylogenetic analysis, samples were determined to be CPV positive by PCR (555forc CAGGAAGATATCCAGAAGGA, 555revc GGTGCTAGTTGATATGTAATAAACA) were subjected to re-PCR with primers. Bidirectional sequencing of the PCR products of the selected samples for sequencing was applied.

The obtained data were compared on the NCBI service's Basic Local Alignment Search Tool (BLAST) web program (https://blast.ncbi.nlm.nih.gov). The validated sequences were then used in phylogenetic analyses. Indexes converted to FASTA format were aligned via AliView software. The bootstrap value was calculated (1000 replications) with the Maximum Likelihood method in the MEGAX program and a pedigree was created according to the Neighbor-Joining (Saitou et al 1987) model.

Statistical analysis

The study computed sensitivity and specificity values for the test data according to the methodology specified by Martin et al. (1987). Statistical evaluation of diagnostic methods using fresh samples and Freezing and Thawing samples was evaluated with the chi-square test ($P < 0.005$).

Results

In this study, stool samples collected from 50 dogs with clinical signs such as severe diarrhea and bloody diarrhea were examined for CPV and CCoV using rapid test and PCR methods. Among these, samples negative for both CPV and CCV in all tests are not given in the table (Table 1). Collected samples were tested twice, both fresh and after freeze-thaw. When the tests above were performed while the samples were fresh, the rapid test and PCR results for CPV were determined as 2 and 29, respectively, while these results for CCoV were determined as 14 and 28, respectively. When the same samples were analyzed with the same tests after freezing and thawing; the results were unchanged for CPV, while 10 samples were determined as positive by rapid test and 28 samples by PCR for CCoV (Table 1). The sensitivity and specificity rates of the Rapid test performed using

Table 1. Rapid test and PCR results of the samples used in the study in terms of CPV and CCoV and typing and GenBank Accession numbers

	Sample No	CPV/Rapid Test		CCoV/Rapid Test		CPV/PCR		CCoV/PCR		CPV Type GenBank Accession number	CCoV Type GenBank Accession number
		Fresh	Freeze-Thaw	Fresh	Freeze-Thaw	Fresh	Freeze-Thaw	Fresh	Freeze-Thaw		
1	2	-	-	-	-	+	+	+	+	CPV/2b MW465344	CCoV GI MW465349
2	4	+	+	+	+	+	+	+	+	CPV/2b MW465345	CCoV GII MW465348
3	5	-	-	-	-	+	+	+	+		
4	9	+	+	+	-	+	+	+	+	CPV/2b MW465343	CCoV GII MW465347
5	10	-	-	-	-	+	+	+	+		
6	11	-	-	-	-	+	+	+	+		
7	15	-	-	-	-	+	+	+	+		
8	17	-	-	+	+	+	+	+	+		
9	21	-	-	-	-	+	+	-	-		
10	23	-	-	+	+	+	+	+	+		
11	24	-	-	-	-	+	+	+	+		
12	25	-	-	+	+	+	+	+	+		
13	26	-	-	-	-	+	+	+	+		
14	27	-	-	-	-	+	+	-	-		
15	28	-	-	-	-	+	+	+	+		
16	29	-	-	+	+	+	+	+	+		
17	30	-	-	-	-	+	+	+	+		
18	31	-	-	+	+	+	+	+	+		
19	33	-	-	+	+	+	+	+	+		
20	37	-	-	+	+	+	+	+	+		
21	38	-	-	-	-	+	+	+	+		
22	40	-	-	-	-	+	+	+	+		
23	42	-	-	+	+	+	+	+	+		
24	43	-	-	+	-	+	+	+	+		
25	44	-	-	-	-	+	+	-	-		
26	45	-	-	+	+	+	+	+	+		
27	46	-	-	-	-	+	+	+	+		
28	47	-	-	-	-	+	+	+	+	CPV/2b MW465346	
29	48	-	-	+	-	-	-	+	+		
30	49	-	-	+	-	+	+	+	+		
31	50	-	-	-	-	-	-	+	+		
Total		2	2	14	10	29	29	28	28		

Table 2. Specificity and sensitivity results of comparison of PCR with immunochromatography (IC) of CPV and CCoV for fresh and Freeze- Thaw samples.

		PCR	
		+	-
IC TEST	+	2[a]	0[b]
	-	27[c]	2[d]
Sensitivity (%)=6,89			
Specificity (%)=100			
(1)			

		PCR	
		+	-
IC TEST	+	14[a]	0[b]
	-	14[c]	3[d]
Sensitivity (%)=50			
Specificity (%)=100			
(2)			

		PCR	
		+	-
IC TEST	+	10[a]	0[b]
	-	18[c]	3[d]
Sensitivity (%)=35,71			
Specificity (%)=100			
(3)			

a = True Positive (TP), b = False Positive (FP), c = False Negative (FN), d = True Negative (TN). Sensitivity (%) = TP/(TP+FN) × 100, Specificity (%) = TN/(TN+FP) × 100.
1. Both Fresh and Freeze-thaw results of CPV, 2. Test results of CCoV fresh samples, 3. Test results of CCoV freeze-thaw samples

Table 3. Statistical data of rapid test and PCR results for fresh and freeze-thaw samples for CPV and CCoV.

	CPV		CCoV	
	Fresh samples	Freezing and Thawing samples	Fresh samples	Freezing and Thawing samples
Rapid Test	2	2*	14	10#
PCR	29	29*	28	28#

*: It indicates that there is no difference between fresh and freeze-thaw for CPV (P<0.005). #: It indicates that there is no difference between fresh and freeze-thaw for CCoV (P<0.005).

fresh samples were evaluated for both infections and these rates were found to be 6.89% and 100% for CPV and 50% and 100% for CCoV, respectively (Table 2). As a result of the rapid test and PCR test applied after the samples were freeze-thawed; when the sensitivity and specificity of the rapid test were evaluated, no difference was observed for CPV. In contrast sensitivity for CCoV was 35.71% (Table 2). As a result of the chi-square test performed to determine the effect of using fresh samples and freeze-thaw on the tests, it was determined that there was no statistically significant effect in both diseases (Table 3).

As a result of the phylogenetic analysis performed to determine the molecular characterization of CPV and CCoV infections, four samples were determined as CPV/2b, 1 sample was classified as CCoV/GI and 2 samples were classified as CCoV/GII (Figure 1-2).

Discussion

Studies conducted in the world have reported that Canine Parvovirus and Canine Corona virus are important etiological agents of diarrhea for both domestic and wild dogs (Navarro et al 2017). In the current study, the presence of CPV and CCoV agents was comperatively determined by rapid test and PCR in dogs with diarrhea diarrhea symptoms in the shelter environment. As a result, sensitivity of the rapid test was quite low for both infections (Table 2).

Since the diagnosis of infection can't be made definitively based on the clinical signs observed in parvoviral enteritis and CCoV infections, various laboratory methods have been developed to detect the viral particle in the feces of infected dogs. PCR and rapid tests for virus diagnosis are widely used in many countries (Yoon et al 2018, Dik et al 2021). Especially in places where dogs are cared for and fed together, such as shelters and dog breeding farms, fast and reliable diagnosis of dogs with gastrointestinal system diseases brought to pet clinics is of great importance for preventing the virus's spread and for appropriate treatment. Although rapid tests are widely used due to their fast and easy results, it was reported by various researchers that they are sometimes insufficient for the correct diagnosis of the disease (Schmitz et al 2009, Miranda et al 2016a, Dik et al 2021). Schmitz et al (2009) stated that the specificity of rapid tests (92.5-100%) was high, but sensitivity (15.8-26.3%) was quite low. In their study Tinky et al (2015) compared the diagnostic potential of rapid test and PCR used in diagnosing CPV from diarrheic dog faeces found the sensitivity of rapid test as 72.73% specifity as 92.86 compared to PCR. They compared to data obtained with the McNemar statistical test and reported that there was no statistically significant difference between the two tests (p>0.05). Miranda et al (2016a), reported that 61 (56%) of the 260 stool samples collected from dogs showing clinical signs were positive when evaluated by rapid test, while 198 (76.2%) of 260 samples were positive for CPV-2 by PCR. In a study to compare PCR and rapid test in the detection of CCoV, the sensitivity of the rapid test was reported as 93.1% and the specificity as 97.5% (Yoon et al 2018). Dorlikar et al (2019) in their study for the detection of CPV, when they evaluated 91 stool samples collected from dogs by PCR and rapid test, they determined the sensitivity of the rapid test as 78.08% and the specificity as 96%. Dik et al (2021), determined the sensitivity of the rapid test as 36.6% and the specificity as 100% in the detection of CPV-2, and that the results of the rapid test and PCR were statistically different.

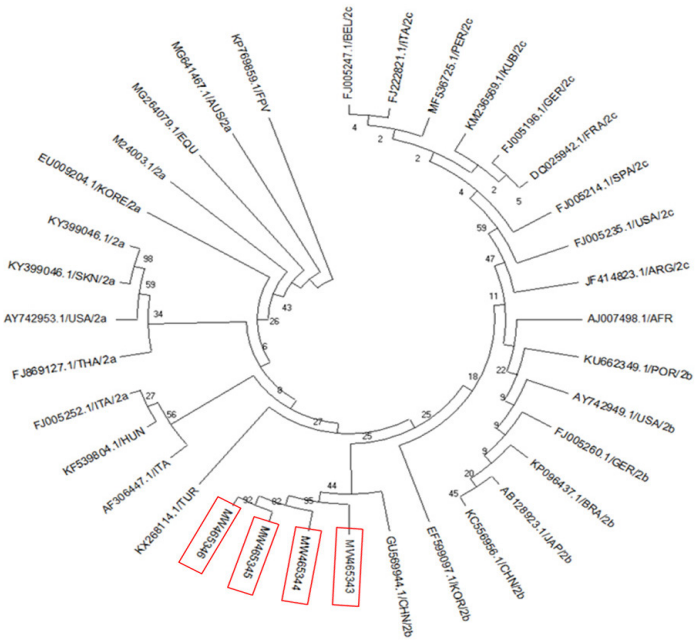

Figure 1. Phylogenetic tree of VP2 gene nucleotide sequences of CPV-2 strains obtained from the GenBank database and Central Anatolia in Turkey.

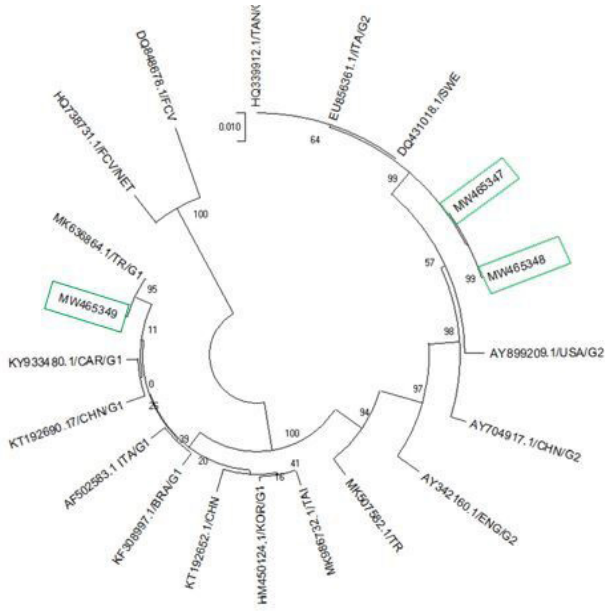

Figure 2. Phylogenetic tree of M gene nucleotide sequences of CCoV strains obtained from the GenBank database and Central Anatolia in Turkey.

When the data obtained in this study and the results of previous studies are evaluated together, it can be concluded that although the specificity of the rapid test is at the desired level, it is insufficient in terms of sensitivity.

In the current study, when the reason for the low sensitivity obtained in rapid tests was examined, it was evaluated whether there is an effect of the samples being fresh or freeze-thawed, and it was determined that there was no statistical difference between fresh samples and freeze-thaw (Table 3). In a study conducted by Kantere et al (2015), rapid test and PCR method were compared in terms of CPV-2 and it was reported that the rapid test was less sensitive, the amount of CPV-2 decreased in stool as the sampling time was delayed, the viral antigens decreased due to intestinal antibodies and accordingly less sensitive and the sensitivity of the rapid test decreased after the sample was frozen and thawed at -20°C. However, it was determined that the current research results do not fully comply with the stated research results. It is thought that this situation may have been shaped due to the delays in the sampling time and the increase in the number of antibodies accordingly. Shao et al (2012), it was

stated that genomic DNA degrades as the number of freeze-thaw cycles increases, and the degradation is directly related to the large size of the DNA. They reported that increasing the stocked DNA samples from 10 mg/mL to 100 mg/mL had some protective effect on DNA stability. They reported that DNA degradation of the samples was minimal at up to three freeze-thaw cycles, but as the number of freeze-thaw cycles increased, the DNA size profile indicated that the DNA was progressively fragmented. In the study conducted by Avci et al (2015a), which was conducted to determine the stability of Bovine viral diarrhea virus (BVDV) RNA stored at different temperatures, they stated that exposure to different temperatures did not affect the stability of BVDV RNA as a result of ELISA and RT-PCR performed after the BVDV RNA was stored at 4, 21 and 37 °C for one month. Tennant et al (1994) stated that as a result of their test with coronavirus, the virus titer decreased from 105.3 DKID50 to 105 DKID50 after 6 times of freezing and thawing. They also stated that it is important to dilute the stool samples by 1/10 to preserve the infectivity of the virus.

There several subtypes of CPV and CCoV that cause gastroenteritis in dogs. Phylogenetic analyzes are needed to identify these subtypes. The existence of CPV and CCoV subtypes has been demonstrated by studies conducted in our country and the world (Yi et al 2016, Navarro et al 2017, Barros et al 2018, Akkutay et al 2020a, Akkutay et al 2020b, Haake et al 2020). As a result of the studies conducted, it was reported that CPV subtypes vary according to the sampling region and time (Dik et al 2022). In this study, as a result of the phylogenetic analysis performed to determine the CPV and CCoV subtypes in the samples determined positive by PCR, when evaluated in terms of VP2 gene region for CPV, while all samples were in the CPV-2b subgroup, as a result of the diagnosis made by targeting the CCoV M gene, it was determined that the samples were in the CCoV/GI and CCoV/GII subgroups. When the various studies reported are examined, although CPV-2a is common in Europe (Battilani et al 2019) and the USA (Giraldo et al 2020), CPV-2b has been identified as the dominant variant circulating in Brazil (Gogone et al 2020), Japan (Takano et al 2021) and Australia (Saei et al 2017). In many countries such as China (Chen et al 2021) and Chile (Alexis et al 2021), 2c has recently replaced 2a and 2b variants. While the results of studies conducted in our country between the years 2000-2010 reported that the dominant variant in Turkey was CPV-2a, (Yılmaz et al 2007, Timurkan et al 2015), in studies conducted in 2018 and later (Akkutay et al 2020a, Dik et al 2022) the active variant has been reported to be CPV-2b. In the present study, similar to previous studies, all samples were determined as CPV-2b. Temizkan ve Temizkan (2023) determined the 18.75% (6/32) of the samples were CPV-2a, 78.13% (25/32) were CPV-2b, and 3.12% (1/32) were CPV-2c. In their study, Abayli et al (2022) identified 48 of 68 samples as CPV-2b, while CPV-2a and CPV-2c were not detected. Hasırcıoğlu ve Aslım (2022), out of 30 samples found positive by differential PCR, 30 (100%) were found positive for CPV-2a, 27 (90%) for CPV-2b and 29 (96.6%) for CPV-2c. When studies in terms of CCoV were evaluated, it was reported that CCoV G1 and CCoV G2 generally progress together at similar rates (Ntafis et al 2012, Costa et al 2014 , Barros et al 2018). Phylogenetic data on CCoV in Turkey is limited. In two studies reported in Turkey, both CCoV 1 and CCoV 2 were reported to be circulating (Akkutay et al 2020b, Timurkan et al 2021). The presence of G1 and G2 in the Konya region was determined in the data obtained in the current study (Figure 2).

Conclusion

As a result, it was determined that there is a widespread presence of CPV-2 and CCoV in the shelter in Konya province and these infections are a great threat to dogs in the crowd area. Rapid and accurate diagnosis of infections in dogs is important to prevent the spread of these viruses. Although the rapid tests used in the world and our country seem like a solution to save the day (such as preventing the use of wrong antibiotics), it continues to have question marks about the reliability of the results. Considering this situation, it was concluded that although the rapid tests, which are widely preferred, give rapid results, they are insufficient to give accurate results. For this reason it would be more accurate to prefer a molecular diagnostic method in addition to using fresh samples in the acute phase of the disease for accurate diagnosis. Also, it is thought that these data will contribute to the determination of the CPV-2 and CCoV subtypes that are circulating in Turkey and to the vaccine studies planned in the future.

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Conflict of Interest

The authors did not report any conflict of interest or financial support.

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Ethical Approval

All procedures and animal care complied with the guidelines of the Selcuk University Veterinary Faculty Ethics Committee (Ethical approval number 2020/53 on 11/06/2020).