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Detection and Molecular Characterization of *Canine Coronavirus* Based on Partial Membrane Gene Sequences

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ABSTRACT *Canine coronavirus (CCoV)* infection in dogs is common all over the world and progresses with gastroenteritis findings. Infection as a result of complications with secondary factors may result in death, especially in puppies. The virus, which is excreted in the feces, spreads indirectly through the contamination of food, water, and the environment. This study, it was aimed at revealing the CCoV infection and obtaining current molecular information about the infection. In addition, molecular characterization of CCoV strains circulating in the region was made based on the M (membrane protein) gene. The study material consisted of stool samples from 12 dogs with gastroenteritis findings. The amplified PCR products were subjected to sequence analysis and a phylogenetic tree was constructed by comparing them with different reference CCoV isolates from GenBank. In the phylogenetic tree, 1 of the 5 positive samples was determined to be CCoV-I, and 4 samples were determined to be CCoV-IIa. It was determined that the strains obtained were 85.4 - 97.7% similar among themselves and 82.7-98% similar to other strains obtained from GenBank. As a result of study, current molecular information about CCoV circulating in the Balıkesir region was obtained. With this study, it is thought that new research on the existence and molecular epidemiology of CCoV infection in Türkiye will make important contributions to vaccine studies and the control of infection.

Keywords: *Canine coronavirus, PCR, Phylogenetic analysis.*

ÖZ

Canine Coronavirusun Tespiti ve Kısmi Membran Gen Dizisi Temelli Moleküler Karakterizasyonu

Köpeklerin Koronavirus (CCoV) enfeksiyonu tüm dünyada yaygın olarak görülmekte ve gastroenteritis bulguları ile seyretmektedir. Sekonder etkenlerle komplikasyon sonucunda enfeksiyon, özellikle yavru köpeklerde ölümle sonuçlanabilir. Dışkı ile atılan virus, dışkının yeme, suya ve çevreye bulaşmasıyla indirekt olarak yayılır. Yapılan bu çalışmada CCoV enfeksiyonunun ortaya konması, enfeksiyona ilişkin güncel moleküler bilgilerin elde edilmesi amaçlanmıştır. Ayrıca, bölgede dolaşan CCoV suşlarının M (membran proteini) genine dayalı moleküler karakterizasyonu da yapılmıştır. Çalışma materyalini, gastroenteritis semptomları gösteren 12 adet köpeğe ait gaita örneği oluşturdu. PCR ürünleri saflaştırdıktan sonra sekans analizine tabi tutuldu ve GenBank veri tabanından sağlanan farklı referans CCoV izolatları ile karşılaştırılarak filogenetik ağaç oluşturuldu. Filogenetik analiz sonucu 5 pozitif örnekten, 1 adedinin CCoV-I, 4 adet örneğin ise CCoV-IIa olduğu tespit edildi. Elde edilen suşların kendi aralarında %85.4-%97.7 oranında, GenBank veri tabanından elde edilen diğer suşlarla aralarında %82,7-%98 oranında benzerlik olduğu tespit edildi. Çalışma sonucunda, Balıkesir bölgesinde sirküle olan CcoV'ye yönelik güncel moleküler bilgiler elde edilmiştir. Bu çalışma ile, Türkiye'de CCoV enfeksiyonunun varlığına ve moleküler epidemiyolojisine ilişkin yapılacak yeni araştırmaların aşı çalışmalarına ve enfeksiyonun kontrolüne önemli katkılar sağlayacağı düşünülmektedir.

Anahtar Kelimeler: *Filogenetik analiz, Köpek koronavirus, PZR.*

INTRODUCTION

Coronaviruses infect humans and many animal species (cattle, dogs, cats, poultry, rabbits, mice, rats, and pigs) with subclinical or moderate-to-severe infections in many organs, notably the respiratory and gastrointestinal systems, causing serious epidemics resulting in death and significant economic losses (Pratelli et al. 2001; Brownlie and Whittaker 2017; He et al. 2020). Coronaviruses have been classified into four groups (*alphacoronaviruses, betacoronaviruses, gamacoronaviruses and deltacoronaviruses*) in accordance with the virus classification made by the ICTV (International Committee

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on Taxonomy of Viruses). *Coronaviruses*, having a large (27–32 kb) genome in the order *Nidovirales*, have diversified extensively, resulting from the high frequency of RNA recombination due to RdRp (RNA-dependent RNA polymerase) (Singhal 2020).

Canine coronavirus (CCoV), first isolated in 1971, is a prevalent infection characterized by gastroenteritis in dogs (Binn et al. 1974). The clinical symptoms, which often begin with mild enteritis, are exacerbated by the addition of other pathogenic agents to the viral infection and may end in death if mutant or virulent strains spread to multiple organs (pantropic canine coronavirus). Canine respiratory coronavirus (CRCoV) is frequently diagnosed in dogs with respiratory symptoms and contributes to the infectious respiratory disease complex in dogs (Decaro et al. 2008). Although coronavirus infection with enteritis can manifest in dogs of any age, puppies are more commonly affected. Diarrhea, vomiting, dehydration, hemorrhage, and weight loss are observed, especially in infections with other pathogens (Pratelli et al. 1999; Decaro and Lorusso 2020). Deaths usually take place within 24–36 hours after the onset of infection due to severe dehydration (Buonavoglia et al. 2006).

The virus is a member of the *Alphacoronavirus* genus and contains single-stranded positive-polarity RNA (Lai et al. 2001, Haake et al. 2020). The virus has helical symmetry, is surrounded by a membrane, and has a diameter of 60-220 nm (Benetka et al. 2006). Genomic RNA has infectious characteristics (de Vries et al. 1997). The genome of the virus, whose replication cycle takes place in the cytoplasm, encodes four structural proteins: E-envelope, M-membrane, N-nucleocapsid, and S-spike protein, and two non-structural proteins, NS2 and NS4. Canine coronaviruses are divided into two subtypes, CCoV-I and CCoV-II. In addition, CCoV-II is divided into two genotypes, CCoV-IIa and CCoV-IIb (Pratelli et al. 2003).

Studies on spike and membrane proteins have reported that there may be recombinations between FCoV and CCoV. Studies have shown that CCoV-IIa may have resulted from recombination with coronaviruses of cats, and CCoV-IIb may have resulted from recombination with coronaviruses of pigs (Herrewegh et al. 1998; Pratelli 2006; Decaro and Buonavoglia 2011).

The most important mode of transmission of the disease is oral entry of the virus through stool-contaminated materials (fecal-oral route transmission). The virus excreted in feces spreads indirectly by contaminating food, water, and the environment. Asymptomatic dogs have been reported to spread the virus for a prolonged period of time without displaying any clinical signs (Yeşilbağ et al. 2004; Pratelli et al. 2022).

In this study, it was aimed to reveal the CCoV infection and obtain current molecular information about the virus. In addition, molecular characterization of CCoV strains circulating in the region was made based on the M gene.

MATERIAL AND METHODS

Permissions required for this study were obtained from Balikesir University Animal Experiments Local Ethics Committee (Dated: 27/04/2023, Numbered: 2023/3-1).

Samples

The study material consisted of fecal samples from 12 cross-breed dogs, aged between 1-6 months, exhibiting symptoms of gastroenteritis, who attended local veterinary clinics in Balıkesir province. Stool samples taken into stool containers were diluted 1/10 with PBS and centrifuged (3000 rpm, 10 minutes). The supernatant was taken into stock tubes and stored at -20 ˚C until testing.

Viral RNA extraction, Reverse- Transcriptase Polymerase Chain Reaction (RT-PCR)

Extraction of viral RNA from stool specimens was performed with a nucleic acid isolation kit (Viral RNA, DNA Preparation Kit, Jena Bioscience, Germany) in accordance with the kit's procedure. The extracted viral RNA was used as the template for complementary DNA (cDNA) synthesis. Therefore, we used a kit (Thermo Scientific RevertAid First Strand cDNA Synthesis Kit, USA) containing the Reverse Transcriptase enzyme for cDNA synthesis. The first mixture was prepared in a tube for cDNA synthesis. For this purpose, 3 µL of distilled water, 0.5 µL of random hexamer primer, and 3 µL of RNA were added to the tube and the tube was placed in the thermal cycle. After the thermal cycle temperature was 70 °C, the tubes, which were kept at this temperature for 5 minutes, were taken and immediately placed on ice. The second mixture was prepared in another tube containing 2.0 µL of 5x reaction buffer, 1.0 µL of 10 mM dNTP mix and 0.5 µL of M-MuLV reverse transcriptase enzyme in 3.5 µL was added to the tubes containing the first mixture and incubated at 48 °C for 45 minutes. For the PCR reaction, we used 3 ml (50 ng) cDNA. CCV1 (5´-TCC AGA TAT GTA ATG TTC GG-3´) and CCV2 (5´-TCT GTT GAG TAA TCA CCA GCT-3´) specific primers targeting the partial region (409 bp) of the Canine Coronavirus M (membrane protein) gene were used for RT-PCR (Herrewegh et al. 1998). For PCR amplification processes, a total of 30 µl PCR master mix was formed with the cDNA (3 µl), Tris-HCl (pH 8.8-75 mM), $NH_4(SO_4)_2$ (20 mM), $MgCl_2$ (1.5 mM), primers (10 pmol), dNTP (0.2 mM) and Taq-DNA polymerase (0.5 U) (Thermoscientific Taq DNA polimerase, USA). For the PCR, after 35 cycles; denaturation (94 °C for 30 sec), annealing (55 °C for 30 sec) and extension (72 °C for 1 min) final extension (72 °C for 10 min) was carried out. RNase-free water was used as the negative control in RT-PCR processes. The obtained PCR products were subjected to gel electrophoresis. Amplified PCR products were stained with gel red on a 2% agarose gel. Evaluation of PCR products was completed using standard 100 bp under UV light in a Gel Imaging Device.

Sequence Analysis

Amplified PCR products were purified and sequenced by BMLabosis (Ankara, Türkiye). The alignment of the raw data obtained with the Clustal W algorithm of the BioEdit version 7.0.5 program has been completed (Hall 1999). The NCBI (National Center for Biotechnology Information) BLAST (Basic Length Alignment Search Tool) search engine was used to compare the obtained sequences with other similar data in the GenBank data system. Phylogenetic analysis was done using MEGA v6.0 software. To this end, the neighbor-joining method with a Kimura-2 parameter distance matrix model in the programme MEGA version 11.0 was used. The bootstrap value was calculated as 1000 replicates (Tamura et al. 2021).

RESULTS

CcoV nucleic acid was detected in five (41.6%) of the fecal samples of 12 dogs tested for canine coronavirus. A phylogenetic tree was created using the sequences obtained from the studies and the sequences obtained

from GenBank. The phylogenetic tree showed that one out of five positive samples was CCoV-I, and four positive samples were CCoV-IIa (Figure 1). When the similarity rates were analyzed, it was found that the CCoV-IIa strains identified in this study were 93.4–97.7% similar among themselves and 82.7–98% similar to CCoV-IIs identified from Türkiye and different countries. CCoV-IIa strains identified in the study were found to be similar to one CCoV-I strain at a rate of 85.4–88.1%. When the CCoV-I strain identified in the study was compared with CCoV-I strains identified from different countries, it was found that the similarity rate varied between 94.6% and 95.7%. GenBank acceptance numbers of the samples (TR/Bal/CCV10/1-OR862749; TR/Bal/CCV10/2-OR862750; TR/Bal/H4-OR862747; TR/Bal/B10-OR862748; TR/Bal/B5-OR862751) have been assigned.

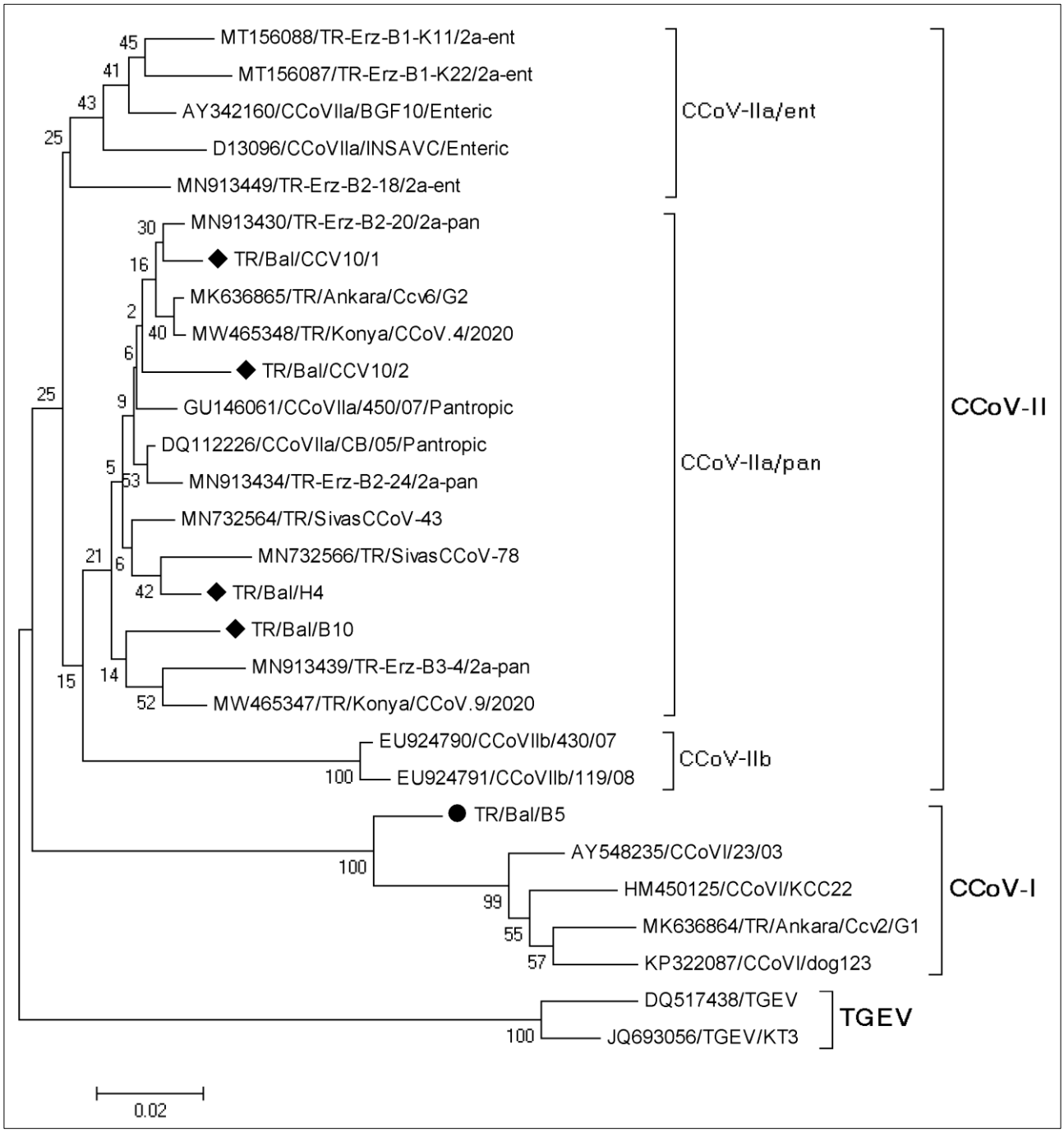

Figure 1: Phylogenetic tree constructed with nucleotide sequences based on CCoV partial M gene.

DISCUSSION AND CONCLUSION

Coronaviruses that cause infection in all species have a wide host spectrum. In susceptible species, they cause infections, sometimes subclinical and sometimes with symptoms such as hepatitis, reproductive disorders, encephalomyelitis, and nephritis, especially respiratory and digestive system diseases, and may result in death (Brownlie and Whittaker 2017; Radford et al. 2021). Coronavirus infections can cause serious epidemics. The coronaviruses, with a large RNA, are highly mutable and can expand their host range due to this feature (Brownlie and Whittaker 2017; Ozan and Tamer 2020; Lednicky et al. 2022; Li et al. 2023). Given these characteristics, it is important to conduct new studies on coronaviruses in all species. This study aims to determine the presence of the causative agent in Balıkesir province and to reveal the heterogeneity of the strains circulating in the region and

the strains obtained in national and international studies at the M gene level.

There are serological studies on the presence of canine coronavirus in Türkiye. Serological studies have shown that the presence of CCoV Ab is 74.3%, 73.4%, 96.6%, 96.5%, and 75.2% (Yeşilbağ et al. 2004; Ataseven et al. 2005; Gür and Civelek 2007; Gür et al. 2008, Avcı et al. 2016). These data reveal a high prevalence rate of infection in Türkiye.

There are studies on M and S gene regions using the RT-PCR technique in the diagnosis of CoV (Decaro et al. 2009; d Alexandry et al. 2015). The M gene region is used to determine the presence of infection since it is a protected region. Although it is a protected region, it has been found in studies that mutations in the M gene provide some opportunities for the virus to escape from the host immune system (Pratelli et al. 2003; Pratelli 2006). Likewise, in this study, RT-PCR analysis was carried out by using primers that focused on the CCoV M gene region. Thus, it was aimed to identify the mutations in the M gene and to compare the CCoV sequences obtained after sequence analysis with different sequences. The study showed that the CCoV-I and CCoV-II strains showed high similarities with the types identified in Türkiye and in other countries in previous years.

There are a limited number of studies on the virological investigation and molecular characterization of canine coronavirus in Türkiye. In their study, Yesilbağ et al., (2007) identified the presence of CCoV nucleic acid in fecal samples taken from unvaccinated dogs at the rate of 15.5% by RT-PCR. In the study, type-specific primers were used, and five of the positive samples were identified as CCoV-I. Another study found a CCoV Ag positivity rate of 14.87% by ELISA in fecal samples obtained from Adana province (Avcı et al. 2016). Similar to the present study, previous studies (Akkutay Yoldar et al. 2020; Timurkan et al. 2021; Doğan and Köse 2022) on the molecular characterization of the virus in Türkiye that identified both CCoV-I and CCoV-II have indicated that different types of the virus are circulating in Türkiye at the same time. In this study, CCoV-I and CCoV-II strains were identified in the sequences obtained after sequence analysis, and the strains and vaccine strains in Türkiye in past years had high similarities.

In reviews based on the PCR results of the presence of CCoV nucleic acid worldwide, positivity rates ranging from 15.5-57.3% have been reported in countries such as Türkiye, Italy, Japan, the USA, England, Hungary, Greece, and China (Soma et al. 2011; Dong et al. 2022). Various inactivated and modified live vaccines are used in vaccination programs undertaken in Türkiye and around the world for protection against CCoV infection in dogs (Pratelli et al. 2003). Although it has been reported that the existing vaccines are safe and attenuate CCoV replication in the intestinal system, it is not possible to prepare them adequately since they are produced by using classical virus types in vaccine production (Tizard 2020). Therefore, it is necessary to identify the circulating field isolates and incorporate them into the vaccines. Although vaccination practices vary worldwide, it is necessary to consider the circulating virus strains in the vaccine studies to be carried out, the high recombination risks in coronaviruses, and even the possibility of interspecies transmissions. It should be noted that CCoV infections may lead to more severe symptoms and high mortality when they are associated with other viral or bacterial pathogens. The study provided up-to-date information about CCoV circulating in the Balıkesir region of Türkiye. It is believed that further studies on the presence and molecular epidemiology of canine coronavirus infection in Türkiye would make significant contributions to vaccine studies and the control of infection.

CONFLICTS OF INTEREST

The authors report no conflicts of interest.

AUTHOR CONTRIBUTIONS

Idea / Concept: ZK, MÖT
Supervision / Consultancy: ZK
Data Collection and / or Processing: ZK
Analysis and / or Interpretation: ZK, MÖT
Writing the Article: ZK
Critical Review: ZK, MÖT

REFERENCES

Akkutay-Yoldar ZA, Koç BT, Oguzoglu TC (2020). Phylogenetic analysis of partial membrane protein gene of canine coronaviruses detected in Turkey. *Ankara Univ Vet Fak*, 67 (3), 265-271.

Ataseven VS, Ucar H, Akca Y (2005). Canine coronavirus antibodies in stray dogs. *Indian Vet J,* 82 (7), 782-783.

Avci O, Bulut O, Yapici O, Hasircioglu S, Simsek A (2016). Canine coronavirus infection in dogs in Turkey: Virological and serological evidence. *Indian J Anım Res*, 50 (4), 565-568.

Benetka V, Kolodziejek J, Walk K, Rennhofer M, Möstl K (2006). M gene analysis of atypical strains of feline and canine coronavirus circulating in an Austrian animal shelter. *Vet Rec*, 159 (6), 170-174.

Binn LN, Lazar EC, Keenan KP, et al. (1974). Recovery and characterization of a coronavirus from military dogs with diarrhea. *Proc Annu Meet U S Anim Health Assoc,* 78, 359-66.

Buonavoglia C, Decaro N, Martella V et al. (2006). Canine coronavirus highly pathogenic for dogs. *Emerg Infect Dis*, 12 (3), 492-494.

Brownlie J, Whittaker G (2017). Coronaviridae. In; Fenner's veterinary virology 5. Edition, Elsevier Inc, United Kingdom.

Decaro N, Buonavoglia C (2008). An update on canine coronaviruses: viral evolution and pathobiology. *Vet Microbiol;* 132:221–34.

Decaro N, Mari V, Campolo M et al. (2009). Recombinant canine coronaviruses related to transmissible gastroenteritis virus of Swine are circulating in dogs. *J Virol*, 83 (3), 1532-1537.

Decaro N, Buonavoglia C (2011). Canine coronavirus: not only an enteric pathogen. *Vet Clin N Am-Small*, 41 (6), 1121-1132.

Decaro N, Lorusso A (2020). Novel human coronavirus (SARSCoV-2): A lesson from animal coronaviruses. *Vet Microbiol*, 244, 1-18.

de Vries AAF, Horzinek MC, Rottier PJM, de Groot RJ (1997). The Genome Organization of the Nidovirales: Similarities and Differences between Arteri-, Toro-, and Coronaviruses. *Semin Virol*, 8 (1), 33-47.

Doğan F, Köse Sİ (2022). Alt solunum yolu enfeksiyonu olan köpeklerde canine coronavirusun tespiti ve moleküler karakterizasyonu. *Vet Hek Der Derg*, 93 (2), 124-132.

Dong B, Zhang X, Bai J et al. (2022). Epidemiological investigation of canine coronavirus infection in Chinese domestic dogs: A systematic review and data synthesis. *Prev Vet Med*, 209, 105792.

Gür S, Civelek T (2007). Vaka Raporu: Bir köpek barınağında Kanin Koronavirus varlığının serolojik olarak araştırılması. *Eurasian J Vet Sci,* 21, 103-106.

Gür S, Gençay A, Doğan N (2008). A Serologic investigation for canine corona virus infection in individually reared dogs in central Anatolia. *J Fac Vet Med Univ Erciyes,* 5, 67-71.

Haake C, Cook S, Pusterla N, Murphy B (2020). Coronavirus infections in companion animals: virology, epidemiology, clinical and pathologic features. *Viruses*, 12 (9), 1023.

Hall TA (1999). BioEdit: A User-Friendly Biological Sequence Alignment Editor and Analysis Program for Windows 95/98/NT. *Nucleic Acids Symp Ser*, 41, 95-98.

He HJ, Zhang W, Liang J et al. (2020). Etiology and genetic evolution of canine coronavirus circulating in five provinces of China, during 2018–2019. *Mic path*, *145*, 104209.

Herrewegh AA, Smeenk I, Horzinek MC, Rottier PJ, De Groot RJ (1998). Feline coronavirus type II strains 79-1683 and 79-1146 originate from a double recombination between feline coronavirus type I and canine coronavirus. *J Virol*, 72 (5), 4508-4514.

Lai MMC, Holmes KV (2001). Coronaviridae: the viruses and their replication. Fields virology. 4. Edition. Williams and Wilkins, Philadelphia: Lippincott.

Lednicky JA, Tagliamonte MS, White SK et al. (2022). Isolation of a novel recombinant canine coronavirus from a visitor to Haiti: further

evidence of transmission of coronaviruses of zoonotic origin to humans. *Clin Infect Dis*, 75 (1), e1184-e1187.

Li N, Bai Y, Liu R et al. (2023). Prevalence and genetic diversity of canine coronavirus in northeastern China during 2019–2021. *Comp Immunol Microbiol Infect Dis*, 94, 101956.

Ozan E, Tamer C (2020). Evcil hayvanların önemli coronavirüs enfeksiyonları. *Avrasya Sağlık Bilim Derg*, 3, 102-110.

Pratelli A, Tempesta M, Greco G, Martella V, Buonavoglia C (1999). Development of a nested PCR assay for the detection of canine coronavirus. *J Virol Methods*, 80 (1), 11-15.

Pratelli A, Martella V, Elia G et al. (2001). Severe enteric disease in an animal shelter associated with dual infections by canine adenovirus type 1 and canine coronavirus. *J Vet Med*, 48, 385-392.

Pratelli A, Martella V, Pistello M et al. (2003). Identification of coronaviruses in dogs that segregate separately from the canine coronavirus genotype. *J Virol Methods*, 107 (2), 213-222.

Pratelli A (2006). Genetic evolution of canine coronavirus and recent advances in prophylaxis. *Vet Res*, 37 (2), 191-200.

Pratelli A, Tempesta M, Elia G et al. (2022). The knotty biology of canine coronavirus: A worrying model of coronaviruses' danger. *Res Vet Sci*, 144, 190-195.

Radford AD, Singleton DA, Jewell C, et al. (2021). Outbreak of severe vomiting in dogs associated with a canine enteric coronavirus, United Kingdom. *Emerg Infect Dis*, 27 (2), 517.

Singhal T (2020). A review of coronavirus disease-2019 (COVID-19). *Indian J Pediatr,* 87 (4), 281-286.

Soma T, Ohinata T, Ishii H et al. (2011). Detection and genotyping of canine coronavirus RNA in diarrheic dogs in Japan. *Res Vet Sci*, 90 (2), 205-207.

Tamura K, Stecher G, Kumar S (2021). MEGA11: Molecular Evolutionary Genetics Analysis version 11. *Mol Biol Evol,* 38, 3022-3027.

Timurkan MO, Aydin H, Dincer E, Coskun N (2021). Molecular characterization of canine coronaviruses: an enteric and pantropic approach. *Arch Viro,* 166 (1), 35-42.

Tizard IR (2020). Vaccination against coronaviruses in domestic animals. *Vaccine*, 38 (33), 5123-5130.

Yeşilbağ K, Yılmaz Z, Torun S, Pratelli A (2004). Canine coronavirus infection in Turkish dog population. *J Vet Med*, 51, 353-355.