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Molecular Detection and Partial Membrane Gene Sequence-Based Characterization of Canine Coronavirus in Diarrheal Shelter Dogs in Sivas, Türkiye

Türkiye, Sivas'taki İshalli Barınak Köpeklerinde Köpek Koronavirüsünün Moleküler Tespiti ve Kısmi Membran Gen Dizisine Dayalı Karakterizasyonu

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ABSTRACT

Canine coronavirus (CCoV) infection is widespread among dogs worldwide and is a known cause of gastroenteritis. It is particularly lethal in puppy populations. The virus spreads easily in environments where dogs are densely populated, such as animal shelters, and is shed through feces. This study aimed to detect the presence and molecular characteristics of canine coronavirus (CCoV) in shelter dogs with diarrhea at the Sivas Municipality Animal Shelter. Fecal samples were collected from 150 shelter dogs (127 adults and 23 puppies) showing diarrheal symptoms. RT-PCR analysis was performed using two primer sets targeting the CCoV M gene (CCV1/CCV2 and CCV1a/CCV2). The CCV1/CCV2 primer pair showed that 38% (57/150) of diarrheic dogs tested positive for CCoV. The positivity rate was 31.50% (40/127) in adult dogs and 73.91% (17/23) in puppies. Typing was performed using the second primer set (CCV1a/CCV2) based on the M gene. Of the 57 positive samples, 6 (10.53%) were CCoV Type II and 51 (89.47%) were CCoV Type I. RT-PCR sequence analysis, bioinformatics evaluation, and phylogenetic assessment were conducted. These findings indicate that CCoV Type I was responsible for the majority of coronavirus infections causing diarrhea in the studied dog population.

Keywords: Diarrhea, dog coronavirus, M gene, phylogenetic analysis

ÖZ

Köpek koronavirüsü (CCoV) enfeksiyonu dünya çapında köpekler arasında yaygındır ve gastroenteritin bilinen bir nedenidir. Özellikle yavru köpek popülasyonlarında öldürücüdür. Virüs, hayvan barınakları gibi köpek yoğunluğunun yüksek olduğu ortamlarda kolayca yayılır ve dışkı yoluyla yayılır. Bu çalışma, Sivas Belediye Hayvan Barınağı'ndaki ishalli barınak köpeklerinde köpek koronavirüsünün (CCoV) varlığını ve moleküler özelliklerini tespit etmeyi amaçlamıştır. İshal semptomları gösteren 150 barınak köpeğinden (127 yetişkin ve 23 yavru) dışkı örnekleri toplandı. CCoV M genini (CCV1/CCV2 ve CCV1a/CCV2) hedef alan iki primer seti kullanılarak RT-PCR analizi gerçekleştirildi. CCV1/CCV2 primer çifti, ishalli köpeklerin %38'inin (57/150) CCoV için pozitif olduğunu gösterdi. Pozitiflik oranı yetişkin köpeklerde %31,50 (40/127) ve yavru köpeklerde %73,91 (17/23) idi. Tiplendirme, M genine dayalı ikinci primer seti (CCV1a/CCV2) kullanılarak gerçekleştirildi. 57 pozitif örnekten 6'sı (%10,53) CCoV Tip II ve 51'i (%89,47) CCoV Tip I olarak belirlendi. RT-PCR dizi analizi, biyoenformatik değerlendirme ve filogenetik değerlendirme yapıldı. Bu bulgular, CCoV Tip I'in incelenen köpek popülasyonunda ishale neden olan koronavirüs enfeksiyonlarının çoğundan sorumlu olduğunu göstermektedir.

Anahtar Kelimeler: Filogenetik analiz, ishal, köpek coronavirus, M gen

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INTRODUCTION

The global dog population is estimated at approximately 700 million, of which around 300 million are stray dogs. This corresponds to roughly one dog for every ten people.[1] It is widely accepted that the dog was the first domesticated animal, originating from the domestication of wolves in the Middle East approximately 12,000 years ago.[2] Gastroenteritis is one of the most common health problems affecting dogs.[3-5]

Coronaviruses (order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae*) are enveloped, positive-sense single-stranded RNA viruses with the largest known RNA genomes, ranging from 27 to 31 kb. The *Orthocoronavirinae* subfamily comprises four genera: *Alphacoronavirus*, *Betacoronavirus*, *Deltacoronavirus*, and *Gammacoronavirus*. CCoV belongs to the *Tegacovirus* subgenus within the *Alphacoronavirus* genus, alongside feline coronavirus (FCoV) and transmissible gastroenteritis virus of pigs (TGEV).[6] The CCoV genome contains four open reading frames (ORFs) encoding the structural proteins spike (S), envelope (E), membrane (M), and nucleocapsid (N), as well as several ORFs translating into non-structural proteins, including RNA-dependent RNA polymerase polyprotein precursors.[7-9] CCoV was first described in 1971 in association with diarrheal outbreaks in dogs.[10] It is widely accepted as the cause of canine gastroenteritis, which is characterized by symptoms such as anorexia, lethargy, diarrhea, and vomiting. These symptoms typically last up to two weeks. Although clinical signs are usually mild and mortality is low, the virus is highly contagious.[11, 12] It is prevalent worldwide, having been identified or isolated in Europe, the United States, Asia, and Australia.[12-17]

MATERIALS AND METHODS

Sampling

In this study, 150 rectal swab samples were collected from dogs at the Sivas Municipality Animal Shelter between 2019 and 2020. The samples comprised 127 adult dogs (over 1 year old) and 23 puppies (2–4 months old), all exhibiting symptoms of gastroenteritis and diarrhea. Following collection, specimens were transported to the laboratory and stored at −80°C prior to RNA extraction. (Ethics Committee Decision No: 65202830-050.04.04-34, Date: 15.05.2025)

RNA Isolation

Swab samples were diluted 1:10 with 1 M phosphate-buffered saline, then centrifuged at 3,500 rpm for 10 minutes to remove coarse particles. A 200 µl aliquot of the resulting supernatant was used for extraction with a commercial Viral Nucleic Acid Extraction Kit (Vivantis Technologies, Malaysia) following the manufacturer's instructions. The extracted nucleic acids were stored at −80°C until further analysis.

Reverse Transcription Polymerase Chain Reaction (RT-PCR)

cDNA synthesis was performed in a total volume of 20 µl, containing 5 µl of RNA extract, 10 mM deoxynucleoside triphosphates (dNTPs), 2.5 µl of 10× RT buffer (50 mM Tris-HCl, pH 8.3 at 25°C; 75 mM KCl; 3 mM $MgCl_2$; and 10 mM DTT), 50 ng of random hexamers, 40 U of RNasin, and 200 U of M-MuLV Reverse Transcriptase RNase H (Vivantis, Germany). Reverse transcription was carried out at 37°C for 1 hour. The resulting cDNA samples were amplified using the CCV1/CCV2 and CCV1a/CCV2 primer sets described by Pratelli et al.[18] (Table 1).

Table 1. Oligonucleotide primers used for the detection and sequencing of a partial region of the M gene of CCoV

Primer name	Sequence (5'-3')	Target gene	Amplicon size
CCV1*	TCCAGATATGTAATGTTCGG	M gene of Canine coronavirus	410 bp
CCV1a*	GTGCTTCCTCTTGAAGGTACA		239 bp
CCV2*	TCTGTTGAGTAATCACCAGCT		

*Pratelli et al.[18]

PCR was performed in a final volume of 50 µl, using 5 µl of the reverse transcription (RT) reaction mixture as template. The PCR mixture contained 5 µl of 10× PCR buffer, 10 mM dNTPs, 10 pmol/µl of each forward and reverse primer, and 5 U of Taq DNA polymerase (Vivantis, Germany).

Molecular detection of the partial M gene of CCoV was conducted using the primer sets listed in Table 1. The PCR conditions were as follows: initial denaturation at 95°C for 3 minutes; 40 cycles of denaturation at 94°C for 40 seconds, annealing at 50°C for 40 seconds (primer set 1) or 52°C for 30 seconds (primer set 2), and extension at 72°C for 1 minute; followed by a final extension at 72°C for 10 minutes.

Sequencing and Phylogenetic Analysis

PCR amplicons were purified using the Gel and PCR Clean-Up System (Promega, Madison, WI) and sequenced with the Cycle Sequencing Kit (Applied Biosystems, Foster City, CA) on an automated sequencer (ABI 3100; Applied Biosystems, Foster City, CA). All sequenced products were used for phylogenetic analysis. We compared partial M gene sequences with reference sequences obtained from the

National Center for Biotechnology Information (NCBI) database. Sequence alignment and phylogenetic analysis based on a 410 bp partial nucleotide sequence of the M gene were performed using Geneious Prime 2025.1.2 software.[19]

RESULTS

This molecular investigation was based on the detection of 410 bp and 239 bp amplicons specific to the M gene of the CCoV genome, which were amplified using the primer sets listed in Table 1. The CCoV results for a total of 150 dog fecal samples with diarrhea, tested with the PCR primer sets, are summarized in the table below (Table 2).

Among the 127 adult dogs, 40 fecal samples (31.50%) tested positive for the 410 bp partial M gene of CCoV. In contrast, 17 fecal samples (73.91%) from puppies were positive. The 57 samples positive with the CCV1/CCV2 primer pair were further analyzed by PCR using the CCV1a/CCV2 primer pair. Of these, 51 yielded a Type I-specific 239 bp product, while 6 samples failed to produce a positive result and were classified as Type II.

In this study, partial sequence data from 14 samples (410 bp M gene), obtained using the primer pair described by Pratelli et al.[18], were compared both among themselves and with existing sequences in GenBank. For this purpose, a phylogenetic tree was constructed using the Tamura-Nei Neighbor Joining substitution model.[20] Of these sequences, 8 (SivasCCoV-4, 20, 26, 46, 60, 67, 76, 102) were confirmed as CCoV Type I. Meanwhile, 6 sequences (SivasCCoV-3, 16, 43, 65, 78, 116), which did not yield a 239 bp product using the Type I-specific primer pair, were classified as CCoV Type II (Figure 1).

According to the consensus sequence, this mutation involved a TTT→CTC change at the first and third nucleotide positions of the codon encoding the 128th amino acid, resulting in a phenylalanine-to-leucine substitution (F→L). This change was observed in six of the eight CCoV Type I isolates: SivasCCoV-26, 46, 60, 67, 76, and 102 (MK507576–MK507581). A similar amino acid substitution was also detected in the Turkish isolate TR-Erz-B1-K34 (MN913446) (Figure 2).

Table 2. Overall PCR results for the detection of canine coronavirus in both adult dogs and puppies.

	Total Samples	Total	CCoV Type I	CCoV Type II
Adults	127	31.50% (40/127)	95.0% (38/40)	5.0% (2/40)
Puppies	23	73.91% (17/23)	76.47% (13/17)	23.53% (4/17)
Adults + Puppies	150	38.00% (57/150)	89.47% (51/57)	10.53% (6/57)

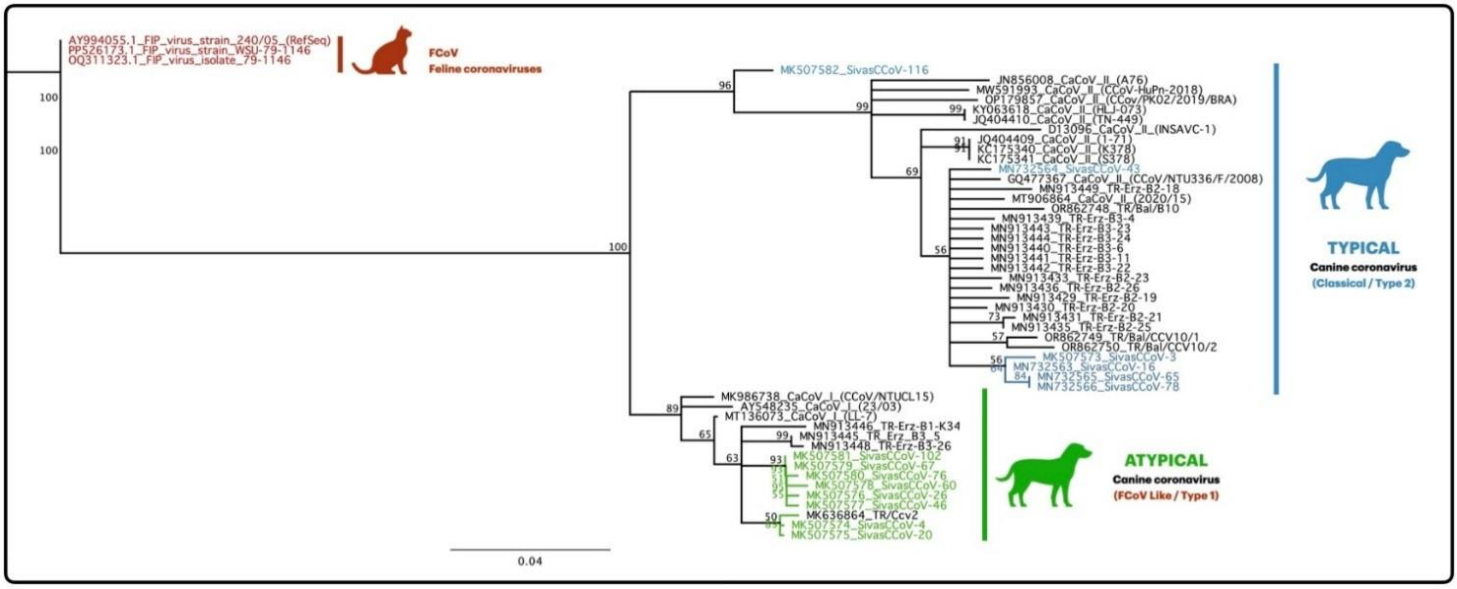

Figure 1: A consensus tree based on a 410 bp partial M gene sequence of CCoV. The phylogenetic tree was constructed using the Tamura-Nei Neighbor-Joining substitution model[20] and bootstrapped 1000 times. Feline infectious peritonitis virus (FIPV) isolates were used as an outgroup.

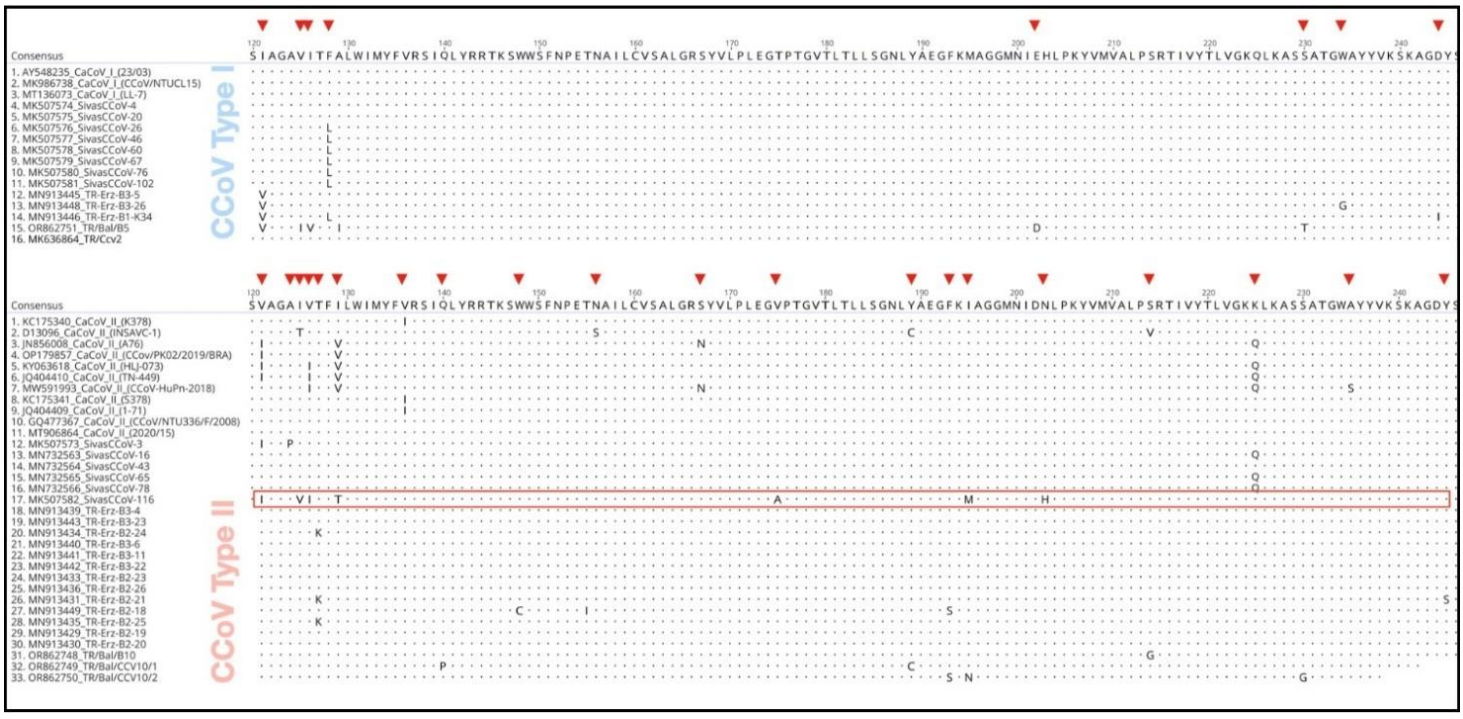

Figure 2. Amino acid differences detected in the partial M gene sequences of CCoV isolates obtained in this study, along with other sequences retrieved from the GenBank database. (–) indicates identical amino acids. Amino acids with mutations are marked with red arrows. (The SivasCCoV-116 isolate, which exhibits seven mutations, is highlighted in red.)

A significantly higher number of mutations were detected in the six isolates identified as CCoV Type II compared to the Type I isolates. The first mutation involved a GTT→ATC substitution at the first and third nucleotide positions of the codon encoding amino acid 121, resulting in a valine-to-isoleucine change (V→I). This mutation was observed in two of the six Type II isolates, SivasCCoV-3 and 116 (MK507573 and MK507582). A similar amino acid substitution was also detected in the US isolates A76 (JN856008) and TN-449 (JQ404410), the Chinese isolate HLJ-073 (KY063618), and the Brazilian isolate CCoV/PK02/2019/BRA (OP179857).

In the SivasCCoV-3 (MK507573) isolate, a GCA→CCA substitution at the first nucleotide position of the codon encoding the 124th amino acid resulted in an alanine-to-proline change (A→P). This mutation was unique, as it was not detected in any other isolate in study, nor in those reported from Türkiye or other countries.

In the SivasCCoV-116 (MK507582) isolate, a nucleotide substitution (ATT→GTT) at the first position of the codon encoding the 125th amino acid resulted in an isoleucine-to-valine change (I→V). This mutation was unique and was not detected in any other isolate. However, in the UK isolate INSAVC-1 (D13096), a different mutation was identified at the second nucleotide position of the same codon, leading to an isoleucine-to-threonine substitution (I→T) via an ATT→ACT change.

In the SivasCCoV-116 isolate (MK507582), a nucleotide substitution at the first position of the codon encoding amino acid 126 resulted in a valine-to-isoleucine change (V→I), corresponding to a GTT→ATT transition. Interestingly, this substitution (ATT→GTT; I→V) is the exact reverse of the mutation observed at position 125. The same mutation was also identified in the Chinese isolate HLJ-073 (KY063618), the US isolate TN-449 (JQ404410), and the Malaysian isolate CCoV-HuPn-2018 (MW591993).

In our SivasCCoV-116 (MK507582) isolate, a nucleotide substitution at the second position of the codon encoding the 129th amino acid (ATA→ACA) resulted in an isoleucine-to-threonine change (I→T). In contrast, the American isolates A76 (JN856008) and TN-449 (JQ404410), the Brazilian isolate CCoV/PK02/2019/BRA (OP179857), the Chinese isolate HLJ-073 (KY063618), and the Malaysian isolate CCoV-HuPn-2018 (MW591993) exhibited a different mutation at the first nucleotide position of the same codon, leading to an isoleucine-to-valine substitution (I→V) via an ATA→GTA change.

In the SivasCCoV-116 (MK507582) isolate, codon substitutions resulted in three unique amino acid changes: valine to alanine (V→A) at position 175 via a GTG→GCG mutation; isoleucine to methionine (I→M) at position 195 via an ATT→ATG mutation; and isoleucine to histidine (I→H) at position 203 via an ATT→CAT mutation. None of these mutations were observed in the other 48 sequences

analyzed. However, in the Turkish isolate TR/Bal/CCV10/2 (OR862350), the mutation at position 195 involved a different substitution— from isoleucine to asparagine (I→N) through an ATT→AAT change, rather than to methionine.

The final mutation identified in our CCoV Type II isolates was a lysine-to-glutamine substitution (K→Q) at amino acid position 225, resulting from an AAA→CAA mutation. This change was detected in the SivasCCoV-16, 65, and 78 isolates (MN732563, MN732565, and MN732566), as well as in the American isolates A76 (JN856008) and TN-449 (JQ404410), the Chinese isolate HLJ-073 (KY063618), and the Malaysian isolate CCoV-HuPn-2018 (MW591993) (Figure 2).

DISCUSSION

The primary objective of this study was to investigate the presence of CCoV in diarrheic cases frequently affecting shelter dogs in Sivas, Türkiye, and to assess its contribution to diarrhea in both adult dogs and puppies. Additionally, we analyzed the genetic diversity of CCoV isolates identified in Türkiye based on the M gene and examined their relationships with previously characterized strains. CCoV was detected in 38% (57/150) of diarrheic dogs. The detection rate was 31.5% (40/127) in adult dogs and increased to 73.91% (17/23) in puppies, suggesting a significant role of CCoV in puppy diarrhea. These rates were higher than those reported in studies from Türkiye, the United Kingdom, Portugal, Spain, and Japan[14, 21], but lower than those observed in Italy, Hungary, and Greece.[22, 23] Notably, the 73.91% positivity rate in puppies was considerably higher than the 15.5% reported by Yeşilbağ et al.[21] in Türkiye.

Of the limited number of studies focusing on the molecular characterization of canine coronavirus via M gene analysis in Türkiye, Type II has generally been reported as the predominant type.[21, 24–27] Nevertheless, in our study, 51 out of 57 samples (89.5%) were identified as Type I using the RT-PCR assay with primer pairs described by Pratelli et al.[18] These findings differ from most previous studies conducted in Türkiye. Moreover, such a pronounced predominance of Type I has not been reported in global studies.[22, 28–30]

The M gene region of coronaviruses is highly conserved and plays a crucial role in triggering robust immune responses. Consequently, it is often targeted in the detection of viral infections. However, despite its conserved nature, studies have demonstrated that mutations in the M gene may enable the virus to evade the host immune system.[27, 31–33]

Accordingly, RT-PCR analysis targeting the CCoV M gene was conducted in this study. This approach enabled the detection of nucleotide and amino acid variations, and the resulting CCoV sequences were compared with available GenBank data. Both CCoV Type I and Type II isolates identified in this study showed high similarity to previously characterized strains from Türkiye and other regions worldwide.

Our findings showed that the eight Type I isolates exhibited high nucleotide identity (99.2%–100%) with the Italian 23/03 (AY548235)[34], Taiwanese LL7 (MT136073), and Chinese CCoV/NTUCL15 (MK986738) strains. Notably, SivasCCoV-4 and -20 shared identical amino acid sequences, while the remaining six isolates (SivasCCoV-26, -46, -60, -67, -76, and -102) exhibited a single amino acid substitution from Phenylalanine to Leucine (F→L) at position 128. In a study by Akkutay Yoldar et al.[24] in Türkiye, the TR/Ccv2 isolate (MK636864) showed 99.2%–100% amino acid similarity with our sequences. Similarly, Timurkan et al.[25] reported 97.6%–99.2% similarity between our isolates and the Tr-Erz-B3-5 (MN913445), Tr-Erz-B3-26 (MN913448), and Tr-Erz-B1-K34 (MN913446) strains in GenBank. In another study conducted in Türkiye[27], similarity rates between our Type I isolates and the Tr/Bal/B5 isolate (OR862751) ranged from 94.5% to 95.3%.

The rate of amino acid changes was significantly higher in the six Types II CCoV isolates than in the Type I isolates. For instance, the SivasCCoV-116 isolate exhibited seven amino acid substitutions, resulting in identity rates ranging from 93.8% to 100%. These mutations occurred at positions 121 (Valine→Isoleucine, V→I), 125 (Isoleucine→Valine, I→V), 126 (Valine→Isoleucine, V→I), 195 (Isoleucine→Methionine, I→M), and 203 (Asparagine→Histidine, N→H). Despite these alterations, the isolate was still classified as Type II based on phylogenetic analysis (Figure 1). When compared with isolates from other countries, identity rates ranged from 92.2% to 100%. The lowest similarity was observed between SivasCCoV-116 and the UK isolate INSAVC-1 (D13096)[35], while the highest was between SivasCCoV-43 (MN732564) and the Taiwanese CCoV/NTU336/F/2008 (GQ477367) and UK 2020/15 (MT906864)[36] isolates. In comparisons with Turkish isolates, identity rates ranged from 92.1% to 100%. The lowest similarity was observed between SivasCCoV-116 and TR-Erz-B2-18 (MN913449), whereas the highest was between SivasCCoV-43 (MN732864) and nine Turkish isolates (MN913429, MN913430, MN913433, MN913436, MN913439–MN913443).[25] In another study conducted in Türkiye[27], identity rates between our isolates and three Turkish isolates ranged from 92.4% to 99.2%.

In conclusion, this study demonstrated the presence of both

Type I and Type II CCoV in adult and puppy dogs with diarrhea at the Sivas Municipality Animal Shelter. This finding was based on a partial molecular analysis of the conserved M gene. However, a clear predominance of CCoV Type I was observed. The isolates identified exhibited unique amino acid substitutions not previously reported in the literature. These findings enhance understanding of the genetic diversity of CCoV isolates circulating in Türkiye and have implications for future vaccine development efforts.

Ethics Committee Approval: Ethics committee approval was received from Sivas Cumhuriyet University Animal Experiments Local Ethics Committee (Date: 15.05.2025, Approval No: 65202830-050.04.04-34).

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