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Phylogenetic analysis of partial transmembrane protein gene of canine coronaviruses detected in Turkey

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Abstract: Canine coronaviruses (CCoVs), a member of the family *Coronaviridae*, are the causative agents of acute gastroenteritis and are genetically divided into two groups, CCoV type I and CCoV type II. The aim of this study was to detect and characterize CCoV strains in fecal samples from six dogs exhibited gastrointestinal system symptoms. To determine the presence of the CCoV RNA, samples were tested by the reverse transcription-polymerase chain reaction (RT-PCR) assay targeting the partial M gene and then sequenced. Among six samples tested, two were found positive for CCoV RNA. Phylogenetic analysis was performed by Maximum-Likelihood (ML) method and revealed that one of the obtained field sequences was classified into CCoV-I genotype; and the other positive sample grouped in CCoV-II genotype. Both genogroups demonstrated broad genetic diversity. Phylogenetic analysis of amino acid sequences shows that our CCoV field strains was closely related to Italy and Brazil strains and placed on different genogroup clades in the CCoV cluster. Sequence comparison of the partial M gene revealed nucleotide identity of 71–100% and 68–100% similarity among the 25 coronavirus strains. TR/Ccv2 (MK636864) and TR/Ccv6 (MK636865) obtained in this study demonstrated 78.5-97.5% and 71–99% nucleotide identity with other CCoV strains around the world respectively. The results of the study demonstrate, CCoV strains from different genogroups are circulating in Turkey and this is a report on the phylogenetic analysis of a CCoV in Turkey, which there is limited information.
Keywords: Canine coronavirus, dog, PCR, phylogenetic analysis.

Türkiye'de saptanan canine coronavirusların parsiyel transmembran protein geninin filogenetik analizi

Özet: *Coronaviridae* ailesinin bir üyesi olan canine coronaviruslar (CCoV'lar), akut gastroenterite yol açan ajanlar olup; genetik olarak CCoV tip I ve CCoV tip II olmak üzere iki gruba ayrılır. Bu çalışmanın amacı gastrointestinal sistem semptomları gösteren altı köpekten alınan dışkı örneklerinde, CCoV suşlarını tespit ve karakterize etmektir. CCoV RNA varlığını belirlemek için; örnekler, parsiyel M genini hedef alan, ters transkripsiyon-polimeraz zincir reaksiyonu (RT-PZR) yöntemiyle test edildi ve sonrasında sekanslandı. Test edilen altı örnek arasından iki tanesi CCoV RNA'sı yönünden pozitif bulundu. Filogenetik analiz, Maximum-Likelihood (ML) yöntemi kullanılarak gerçekleştirildi ve elde edilen sonuçlar yerel saha suşu sekanslarından birinin CCoV-I genotipinde sınıflandırıldığını; diğer pozitif örneğinse CCoV-II genotipinde gruplandırıldığını ortaya koydu. Her iki genogrup da geniş bir genetik çeşitlilik gösterdi. Aminoasit dizilerinin filogenetik analizi, CCoV kümesinde farklı genogrup sınıflarında yer alan CCoV saha suşlarımızın, İtalya ve Brezilya suşları ile yakından ilişkili olduğunu gösterdi. Parsiyel M geninin sekans karşılaştırması, 25 coronavirus suşu arasında %71-100 nükleotid özdeşliği ve %68-100 arasında nükleotid benzerliğini ortaya koydu. Bu çalışmada elde edilen TR/Ccv2 (MK636864) ve TR/Ccv6 (MK636865) suşları, dünyadaki diğer CCoV suşlarıyla, sırasıyla %78.5-97.5 ve %71-99 nükleotid özdeşliği gösterdi. Çalışmanın sonuçları, farklı genogruplara ait CCoV suşlarının Türkiye'de dolaştığını göstermektedir. Bu çalışma Türkiye'de hakkında sınırlı sayıda bilgiye sahip olduğumuz CCoV'ların filogenetik analizine ilişkin bir rapordur.
Anahtar sözcükler: Canine coronavirus, filogenetik analiz, köpek, PCR.

Introduction

Viruses in the *Coronaviridae* family, the *Nidovirales* order, are positive-sense, single-stranded and enveloped viruses, with a large genome between 27 and 31 kilobases (kb) in length. These viruses are common in a wide range of species, from birds to mammals, including cats, dogs, pigs, cattle and humans (9, 26). According to ICTV's 9th report, the *Coronavirinae* subfamily is divided into three

genera: *alpha*, *beta* and *gamma*. After the neighbour-joining method was applied to RdRp amino acid alignments, the *deltacoronavirus* group was proposed to be involved in this subfamily and expected to be a possible group (19). Canine coronaviruses (CCoVs) are classified in the *alphacoronavirus* genus with feline coronavirus (FCoV) types I and II, transmissible gastroenteritis virus (TGEV), porcine respiratory coronavirus (PRCV), porcine epidemic diarrhoea virus (PEDV) and some bat and human coronaviruses. The newly emerged canine respiratory coronavirus (CRCoV) is included in the *betacoronavirus* genus, subgroup 2a (12). CCoVs in the *alphacoronavirus* genus are classified as CCoV type I or II according to their genetic relationship with FCoV types I and II (7, 24). CCoV type I is antigenically closer to FCoV type I than to CCoV type II (24). Additionally, FCoV type II is proposed to originate from recombination between CCoV and FCoV type I (17). In a study by Pratelli et al. (24), some field strains were more closely associated with FCoV than CCoV. The S region of the CCoV helps to differentiate between CCoV-I and CCoV-II, plays a role in the production of neutralising antibodies and alters the disease pathogenesis. Further, this spike glycoprotein plays a role in attachment and fusion of the viral envelope (25, 26). As a result of the mutation between CCoV-II and TGEV in the N-terminal domain of the spike protein, two subgroups have emerged: CCoV-IIa and CCoV-IIb (8).

Two open reading frames (ORFs) were identified in the first two-thirds of the genome: ORF 1a and 1b (26). The membrane consists of spike (S), envelope pentameric membrane (E) and membrane (M) viral proteins. With these proteins, nucleoprotein (N) that is associated with the RNA genome membrane protein (M) and hemagglutinin-esterase (HE), are encoded by ORFs 2, 4, 5, and 6 and non-structural proteins are coded by ORF 1b (9, 15, 21, 22). In this study, the partial transmembrane M protein gene was amplified, aligned and analysed in the phylogenetic tree.

Coronavirus was detected for the first time in 1937, namely from chicken embryos infected with infectious bronchitis virus (3). Coronaviruses were first detected in dogs (diarrhoeic animals) from Germany in 1971 (1). CCoV is highly contagious and is the causative agent of an intestinal infection that leads to mostly mild and self-limiting diarrhoea in dogs. It is spread through contact with oral secretions or infected faeces. The coronavirus replicates inside the small intestine. Although the disease is usually mild, it may cause serious consequences in the presence of other infections such as canine parvovirus (CPV-2), canine adenovirus type 1 (CAV-1) or canine distemper virus (CDV) (7, 9).

Although studies from around the world established the presence, prevalence and evolution of CCoV infections (6, 8, 13, 28), there are only a limited number of studies about CCoV infections in Turkey (1, 16, 30). The aim of the present study was to characterise CCoV strains circulating in diarrhoeic puppies in Turkey. To the best of our knowledge, we also provide the first known report on the phylogenetic analysis of Turkish CCoV to disseminate information on CCoV's evolution.

Material and Methods

Clinical samples: Faecal samples were sent to our laboratory for diagnostic purposes from six affected dogs in Ankara, Turkey. Two of the samples were collected from puppies and the rest were from adult dogs with mild clinical signs: diarrhoea and anorexia. Specimens were placed in ice bags and transferred to the lab. Samples were kept frozen at -80°C until they were tested.

Reverse transcription-polymerase chain reaction (RT-PCR) and phylogenetic analysis: CCoV RNA was extracted from faecal samples according to the phenol:chloroform:isoamyl (25:24:1) alcohol method described by Chomczynski and Sacchi (5). The RNA pellets were eluted with 20 µl deionised water and stored at -20°C until use as a template for RT-PCR. Reverse transcription was performed with MMLV-RT (Fermentas, Lithuania) according to the manufacturer's instructions. The 20 µl reactions used random primers and included 3 µl of isolated RNA. The partial region of the gene that encodes the CCoV M protein was screened by PCR amplification using the oligonucleotide CCV1 sense (5′-TCCAGATATGTAATGTTCGG-3′) and CCV2 anti-sense (5′-TCTGTTGAGTAATCACCAGCT-3′) primers designed by Pratelli et al. (23). Cycling conditions were: 95°C for 5 min, 35 cycles of DNA denaturation at 94°C for 30 sec, primer annealing at 48°C for 45 sec and amplification at 72°C for 1 min, followed by a final extension at 72°C for 10 min. Thirty µl PCR reactions contained 5 U/µl Taq DNA polymerase (MBI, Fermentas, Waltham, MA, USA), 10 × Taq Buffer (1.25 ml, including $(NH_4)_2SO_4$, 25 mmol/L $MgCl_2$ and 18 Mohm/cm distilled water; Applichem, Darmstadt, Germany), primers and 3 µl complementary DNA (cDNA). PCR products were separated using 1% agarose gel electrophoresis. The gels contained ethidium bromide, and the separated bands were visualised with UV transillumination.

Two of the samples that gave specific amplicons were cleaned up with a Gene JET PCR purification kit (Thermo Scientific, Waltham, MA, USA), and PCR products were submitted to BM Labosis (Ankara, Turkey) for Sanger sequencing. The obtained sequences were aligned and analysed with MEGA X (20) by Neighbor-Joining method (27), (Figure 1). The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches (14). The evolutionary distances were

computed using the Tamura 3-parameter method (29) and are in the units of the number of base substitutions per site. The rate variation among sites was modeled with a gamma distribution (shape parameter = 1). This analysis involved 29 nucleotide sequences. Codon positions included were 1st+2nd+3rd+Noncoding. Partial M gene sequences were submitted to the NCBI GenBank database. The nucleotide sequences presented here were deposited in GenBank under accession numbers MK636864 and MK636865 for TR/Ccv2 and TR/Ccv6, respectively. The rates of nucleotide identities and similarities were calculated by using MatGAT 2.01 to compare between CCoV strains (4).

Results

The objective of the study was to detect the infectious agent in six diseased Turkish domestic dogs (i.e., one living in a house or on the street) with CCoV-like symptoms and to molecularly characterise and determine the genogroups in positive samples. Out of six dogs, two of them (both puppies) were positive for the CCoV partial M gene (denoted by an amplified 409 base pair [bp] product); the cleaned-up PCR products were then sequenced. BLAST searches optimised with the Megablast algorithm revealed the faeces sample sequences were 84–99% identical to various CCoV strains. Following the alignment, to find out the level of relatedness of our sequences, a phylogenetic tree was generated and compared with other CCoV strains from around the world (Table 1). We included 27 coronavirus sequences in our analyses, all of which are available in the GenBank database, and the results of the phylogenetic analysis are shown in Figure 1. Feline coronavirus cluster segregated separately from CCoV genotypes as expected. When we examined the MatGAT analysis results from all 25 coronavirus strains, sequence comparison of the partial M gene revealed 71-100% nucleotide identity and 68-100% nucleotide similarity between each Turkish strains and strains from around the world. The Turkish CCoVs obtained in this study (MK636864 and MK636865) shared 87.5% homology between each other (Figure 2). This low identity rate led to their classification in different genogroups. Sequence analysis of the M gene showed higher sequence identities (97.5% amino acid identity) with Brazilian CCoV strains (KF308997_891 and KF309017_1133) for the TR/Ccv2 cDNA sample; it was classified in the CCoV type 1 group that is shown as group 1 (G1) in Figure 1. TR/Ccv6 demonstrated greater identity with Italian strains (GU146061_450/07 and KP981644_CB/05), with 99% nucleotide identity, and it was placed into the CCoV type 2 cluster that is shown as group 2 (G2) in Figure 1.

Table 1. List of sequences used in the nucleic acid identity analysis of CCoVs

Accesion Number of virus strains	Nucleotide homologies with TR/Ccv2	Accesion Number of virus strains	Nucleotide homologies with TR/Ccv6
KF308997 891 (Brazil)	97.5%	KP981644 CB/05-IIa (Italy)	99.0%
KF309017 1133 (Brazil)	97.5%	GU146061_450/07 (Italy)	99.0%
AF502583_259/01 (Italy)	97.3%	EU924791 119/08-IIb (Italy)	98.8%
KP849472 23/03-I (Italy)	97.3%	GQ477367_ CCoV/NTU336/F (Taiwan)	97.5%
AY342160_BGF10 (UK)	91.0%	JQ404409 1-71 (USA)	96.8%
KP322080_ CCoV-I/dog99 (Brazil)	90.5%	AY899209_tn449 (USA)	95.5%
AY899209_tn449 (USA)	89.8%	AY704917 NJ17 (China)	95.0%
JQ404409 1-71 (USA)	89.5%	D13096_Insavc-1 (UK)	94.8%
GU300122_Pt3 (Brazil)	89.3%	DQ811788 TGEV Purdue (USA)	94.5%
DQ811788 TGEV Purdue (USA)	89.0%	DQ811785 TGEV Miller (USA)	94.5%
EU924791 119/08-IIb (Italy)	88.8%	AY342160_BGF10 (UK)	94.3%
AY704917 NJ17 (China)	88.5%	DQ431019_UPPS2/04 (Sweden)	94.3%
KP981644 CB/05-IIa (Italy)	88.5%	HQ339912_CoVJackal/07 (Tanzania)	91.8%
GU146061_450/07 (Italy)	88.5%	KP849472 23/03-I (Italy)	88.5%
GQ477367_ CCoV/NTU336/F (Taiwan)	88.5%	AF502583_259/01 (Italy)	88.3%
DQ811785 TGEV Miller (USA)	88.3%	KP322062_ CCoV-II/dog57 (Brazil)	88.0%
D13096_Insavc-1 (UK)	88.0%	KF308997 891 (Brazil)	87.8%
HM450124_KCC21 (Korea)	86.8%	KF309017 1133 (Brazil)	87.8%
AB086902 FIP UCD1 (Japan)	84.5%	KP322080_ CCoV-I/dog99 (Brazil)	81.0%
DQ431019_UPPS2/04 (Sweden)	83.3%	AB086902 FIP UCD1 (Japan)	80.0%
KP322062_ CCoV-II/dog57 (Brazil)	82.5%	GU300122_Pt3 (Brazil)	79.5%
HQ339912_CoVJackal/07 (Tanzania)	80.8%	HM450124_KCC21 (Korea)	78.3%
JX035833_24.5 (UK)	78.5%	JX035833_24.5 (UK)	71.0%

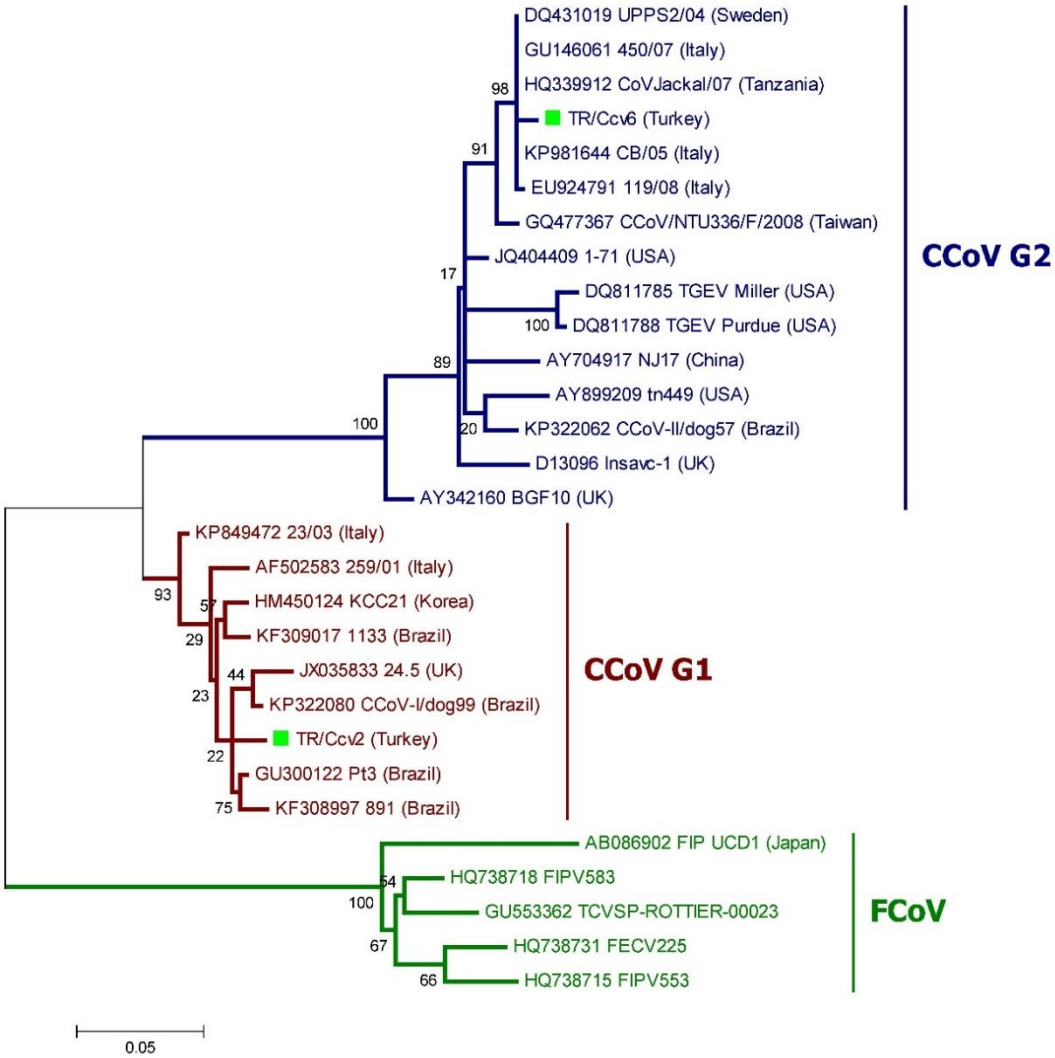

Figure 1. Molecular Phylogenetic analysis of partial M gene.

	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23	24	25
1. AB086902_FIP_UCD1_(Japan)		84.8	82.0	80.8	81.8	84.5	80.0	80.8	75.8	80.8	81.0	81.0	81.8	81.0	76.3	74.5	73.3	81.3	68.0	85.8	85.3	74.8	77.3	85.8	81.0
2. AF502583_259/01_(Italy)	84.8		91.5	88.8	90.5	97.3	88.3	88.5	84.0	88.8	89.5	89.5	89.0	89.3	88.5	87.0	81.5	90.3	78.5	97.3	97.5	83.0	89.5	97.3	89.3
3. AY342160_BGF10_(UK)	82.0	91.5		94.8	94.3	91.0	94.3	94.5	90.0	93.0	93.5	95.0	95.3	95.3	81.8	81.0	87.5	95.8	73.3	90.5	91.0	87.5	83.3	91.5	95.3
4. AY704917_NJ17_(China)	80.8	88.8	94.8		95.5	88.5	95.0	95.0	91.0	94.0	94.5	95.8	96.3	96.0	80.0	79.0	88.8	96.8	71.5	87.8	88.8	89.0	81.5	89.0	96.0
5. AY899209_tn449_(USA)	81.8	90.5	94.3	95.5		89.8	95.5	95.3	91.3	95.0	95.5	96.3	95.5	96.5	81.3	79.8	88.8	96.8	72.5	90.5	90.5	89.0	82.8	90.8	96.5
6. TR/Ccv2_(Turkey)	84.5	97.3	91.0	88.5	89.8		87.5	88.0	83.3	88.3	89.0	88.8	88.5	88.5	89.3	86.8	80.8	89.5	78.5	97.5	97.5	82.5	90.5	97.3	88.5
7. TR/Ccv6_(Turkey)	80.0	88.3	94.3	95.0	95.5	87.5		94.8	94.3	94.5	94.5	98.8	97.5	99.0	79.5	78.3	91.8	96.8	71.0	87.8	87.8	88.0	81.0	88.5	99.0
8. D13096_Insavc-1_(UK)	80.8	88.5	94.5	95.0	95.3	88.0	94.8		90.5	93.8	94.3	95.5	95.8	95.8	79.3	78.0	88.0	96.5	71.0	88.5	88.5	88.3	80.8	89.3	95.8
9. DQ431019_UPPS2/04_(Sweden)	75.8	84.0	90.0	91.0	91.3	83.3	94.3	90.5		90.3	90.3	94.5	93.3	94.8	85.3	80.5	97.5	92.5	76.8	83.5	83.5	93.8	86.8	84.3	94.8
10. DQ811785_TGEV_Miller_(USA)	80.8	88.8	93.0	94.0	95.0	88.3	94.5	93.8	90.3		99.0	95.3	95.0	95.5	80.5	78.0	87.8	95.8	71.0	88.8	88.3	87.0	81.3	89.5	95.5
11. DQ811788_TGEV_Purdue_(USA)	81.0	89.5	93.5	94.5	95.5	89.0	94.5	94.3	90.3	99.0		95.3	95.0	95.5	81.3	78.8	87.8	96.3	71.8	89.5	89.0	87.5	82.0	90.3	95.5
12. EU924791_119/08_(Italy)	81.0	89.5	95.0	95.8	96.3	88.8	98.8	95.5	94.5	95.3	95.3		98.3	99.8	80.3	79.0	92.0	97.5	71.8	89.0	89.0	88.3	81.8	89.8	99.8
13. GQ477367_CCoV/NTU336/F/2008_(Taiwan)	81.8	89.0	95.3	96.3	95.5	88.5	97.5	95.8	93.3	95.0	95.0	98.3		98.5	80.0	78.8	90.8	97.3	71.5	88.5	88.8	88.5	81.5	89.5	98.5
14. GU146061_450/07_(Italy)	81.0	89.3	95.3	96.0	96.5	88.5	99.0	95.8	94.8	95.5	95.5	99.8	98.5		80.0	78.8	92.3	97.8	71.5	88.8	88.8	88.5	81.5	89.5	100.0
15. GU300122_Pt3_(Brazil)	76.3	88.5	81.8	80.0	81.3	89.3	79.5	79.3	80.0	80.5	81.3	80.3	80.0	80.0		92.0	87.8	80.5	88.3	89.8	88.8	88.8	97.3	88.8	80.0
16. HM450124_KCC21_(Korea)	74.5	87.0	81.0	79.0	79.8	86.8	78.3	78.0	77.0	78.0	78.8	79.0	78.8	78.8	85.0		83.0	79.8	90.3	86.3	87.8	84.5	91.5	86.5	78.8
17. HQ339912_CoVJackal/07_(Tanzania)	73.3	81.5	87.5	88.8	88.8	80.8	91.8	88.0	92.3	87.8	87.8	92.0	90.8	92.3	80.0	77.0		90.0	79.3	81.0	81.0	96.3	89.3	81.8	92.3
18. JQ404409_1-71_(USA)	81.3	90.3	95.8	96.8	96.8	89.5	96.8	96.5	92.5	95.8	96.3	97.5	97.3	97.8	80.5	79.8	90.0		72.0	89.3	89.8	89.8	82.0	90.0	97.8
19. JX035833_24.5_(UK)	68.0	78.5	73.3	71.5	72.5	78.5	71.0	71.0	71.5	71.0	71.8	71.8	71.5	71.5	79.3	79.3	71.5	72.0		78.5	79.5	80.3	87.8	79.5	71.5
20. KF308997_891_(Brazil)	85.8	97.3	90.5	87.8	90.5	97.5	87.8	88.5	83.5	88.8	89.5	89.0	88.5	88.8	89.8	86.3	81.0	89.3	78.5		97.5	82.5	90.0	97.0	88.8
21. KF309017_1133_(Brazil)	85.3	97.5	91.0	88.8	90.5	97.5	87.8	88.5	83.5	88.3	89.0	89.0	88.8	88.8	88.8	87.8	81.0	89.8	79.5	97.5		82.5	89.5	97.8	88.8
22. KP322062_CCoV-II/dog57_(Brazil)	74.8	83.0	87.5	89.0	89.0	82.5	88.0	88.3	88.5	87.0	87.5	88.3	88.5	88.5	81.0	78.5	88.5	89.8	72.5	82.5	82.5		90.3	83.3	88.5
23. KP322080_CCoV-I/dog99_(Brazil)	77.3	89.5	83.3	81.5	82.8	90.5	81.0	80.8	81.5	81.3	82.0	81.8	81.5	81.5	89.5	85.5	81.5	82.0	80.0	90.0	89.5	82.5		90.0	81.5
24. KP849472_23/03_(Italy)	85.8	97.3	91.5	89.0	90.8	97.3	88.5	89.3	84.3	89.5	90.3	89.8	89.5	89.5	88.8	86.5	81.8	90.0	79.5	97.0	97.8	83.3	90.0		89.5
25. KP981644_CB/05_(Italy)	81.0	89.3	95.3	96.0	96.5	88.5	99.0	95.8	94.8	95.5	95.5	99.8	98.5	100.0	80.0	78.8	92.3	97.8	71.5	88.8	88.8	88.5	81.5	89.5	

Figure 2. Nucleic acid identities and divergences of TR/Ccv2, TR/Ccv6 and other CCoV strains.

		115	119	121	123	124	127	130	134	154	160	165	171	173	187	193	197	198	200	201	208	212	223	224	227	228	243
CCoV 2	AY342160 BGF10 (UK)	F	V	G	I	V	I	I	V	N	S	S	E	V	Y	I	M	N	D	N	V	S	Q	L	S	S	Y
	D13096 Insavc-1 (UK)	-	-	-	T	-	-	-	-	S	-	-	-	-	C	-	-	-	-	-	-	V	K	-	-	-	-
	AY899209 tn449 (USA)	-	I	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-
	JQ404409 1-71 (USA)	-	-	-	-	-	-	-	I	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-
	AY704917 NJ17 (China)	S	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-
	EU924791 119/08-IIb(Italy)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-
	KP981644 CB/05-IIa (Italy)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-
	DQ811785 TGEV Miller (USA)	-	I	-	-	-	V	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-
	DQ811788 TGEV Purdue (USA)	-	I	-	-	-	V	-	-	-	K	-	-	-	-	-	-	-	-	-	-	-	K	-	-	-	-
	*TR/Ccv6 (Turkey)	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	-	K	X	-	-	T
CCoV 1	KF308997 891 (Brazil)	-	I	-	V	I	A	-	-	-	-	-	-	T	-	M	-	-	E	H	-	-	-	-	-	-	-
	KP849472 23/03-I (Italy)	-	I	-	V	I	A	-	-	-	-	-	-	T	-	M	-	-	E	H	-	-	-	-	-	-	-
	KF309017 1133 (Brazil)	-	T	C	V	I	A	-	-	-	-	-	-	T	-	M	-	-	E	H	-	-	-	-	-	-	-
	AB086902 FIP UCD1 (Japan)	-	-	-	V	I	A	M	-	-	N	R	D	T	-	M	L	T	E	H	I	-	-	-	T	T	-
	*TR/Ccv2 (Turkey)	-	I	-	V	I	A	-	-	-	-	-	-	T	-	M	-	-	E	H	-	-	-	-	-	-	-

Figure 3. Amino acid variations-based M gene (partial) in the CCoV strains analyzed for this study.

TR/Ccv2 showed 97.3% nucleic acid identity with the Italian isolates AF502583_259/01 and KP849472 23/03-I. TR/Ccv2 showed the next highest similarity with AY342160_BGF10 (UK) and KP322080_ CCoV-I/dog99 (Brazil) isolates, with 91 and 90.5% identities, respectively. On the other hand, although TR/Ccv6 displayed greater similarity with a UK strain (AY342160_BGF10) at 94.3%, it demonstrated low nucleotide identity with Brazilian strains KP322062_ CCoV-II/dog57, KF308997_891, KF309017_1133, KP322080_ CCoV-I/dog99, and GU300122_Pt3 (88.0, 87.8, 87.8, 81.0 and 79.5%, respectively). Unlike the relationship with other Brazil strains, TR/Ccv2 showed low similarity to these two Brazilian strains (GU300122_Pt3, KP322062_ CCoV-II/dog57), one of which was classified in G1 and the other in G2, with 89.3 and 82.5% similarity, respectively.

While USA strains AY899209_tn449, JQ404409 1-71, DQ811788 TGEV Purdue and DQ811785 TGEV Miller exhibited 89.8, 89.5, 89.0 and 88.3% identity with TR/Ccv2, they were more similar to TR/Ccv6, with 95.5, 96.8, 94.5 and 94.5% identity, respectively. This finding is expected since they are also included in G2.

The identities between the strain EU924791 119/08-IIb (Italy) and TR/Ccv2 and TR/Ccv6 were 98.8 and 88.8%, respectively. The other Italian strains KP849472 23/03-I and AF502583_259/01 also showed high similarity with TR/Ccv2 and TR/Ccv6 (88.5% and 88.3% identity, respectively). The M gene sequences differed among the CCoV strains; AY704917 NJ17 (China), KP981644_ CB/05-IIa (Italy), GU146061_450/07 (Italy) and GQ477367_ CCoV/NTU336/F (Taiwan) strains all demonstrated 88.5% nucleotide identity with TR/Ccv2. Taiwanese strain GQ477367_CCoV/NTU336/F exhibited 97.5% identity with TR/Ccv6, which was greater homology than when it was compared with TR/Ccv2. Similarly, AY704917 NJ17 (China) strain shared 95.0% similarity with TR/Ccv6.

UK strain JX035833_24.5 showed the lowest identity rate with both TR/Ccv2 and TR/Ccv6 (78.5 and 71.0%, respectively). One of the other UK strains, D13096_Insavc-1, displayed 88.0 and 94.8% sequence identity between TR/Ccv2 and TR/Ccv6, respectively. DQ431019_UPPS2/04 (Sweden), HQ339912_ CoVJackal/07 (Tanzania), AB086902 FIP UCD1 (Japan) and HM450124_KCC21 (Korea) strains exhibited 94.3, 91.8, 80.0 and 78.3% nucleotide homology, respectively, with TR/Ccv6. The same strains exhibited the following identities with TR/Ccv2: 86.8, 84.5, 83.3, 82.5, 80.8 and 78.5%, respectively.

Amino acid sequence alignments of the M gene ORF from different CCoV strains with the homologous regions from TR/Ccv2 and TR/Ccv6 is shown in Figure 3. Comparisons between the Turkish strain TR/Ccv2 and the selected reference CCoV strains KF308997_891 (Brazil) and AF502583_259/01 (Italy) showed no divergence in the translation level (Figure 3). Turkish strain TR/Ccv6 showed general accordance with reference strain AY342160_BGF10 (UK). Like other CCoV strains, the glutamine (Q) at position 223 of the amino acid sequence in Turkish strain TR/Ccv6 was changed to lysine (K). The leucine (L) at position 224 was changed to an unspecified or unknown amino acid (X) and the tyrosine (Y) at position 243 was changed to threonine (T) in the TR/Ccv6 strain. These amino acid changes did not induce any critical differentiation in CCoV genogrouping.

Discussion and Conclusion

CCoVs cause disease worldwide in dogs, but they are mostly limited to the gastrointestinal tract and lead to mild infection. Since the first detection in the 1970s, several studies performed CCoV epidemiological investigations, but there is limited information about CCoVs in Turkey (6, 8, 12, 16, 18, 25, 28).

CCoVs in dogs are divided into two groups according to their genetic properties. Group 1 CCoVs are from alphacoronaviruses, and the respiratory coronaviruses that belong to group 2 are betacoronaviruses abbreviated as CRCoV. Group 1 CCoVs are also divided into two

subgroups, namely type I and type II (according to their genetic relationship with feline coronaviruses). S is the first protein that comes to mind in the immunity caused by the virus, but; antibody-dependent and complement-mediated, immune defense is carried out by M protein. (24, 25, 26). Phylogenetic analysis performed in this study for the M gene of the CCV glycoprotein that induces the immune response in infected dogs demonstrated that TR/Ccv2 and TR/Ccv6 were localised in different clusters, with a nucleotide homology of 87.5%. While TR/Ccv2 was included in group 1 from CCoV type I strains, TR/Ccv6 showed a close association with CCoV-II strains, and in particular Italian strains. TR/Ccv2 showed the highest similarity with Brazilian strains inside the CCoV type I cluster, followed by Italian isolates in this group. The lowest similarity was obtained from the UK CCoV strain (JX035833_24.5_UK). The TR/Ccv6 strain, which is among the type 2 CCoVs, displayed the lowest similarity with the same UK strain located in G1, similar to TR/Ccv2 but with more divergence. The strain had the highest nucleotide identity with Italian strains and also showed high identity with Taiwanese, USA and Chinese strains. Some CCoV strains (DQ431019_UPPS2/04_ Sweden, KP322062_CCoV-II/dog57_Brazil and JX035833_24.5_UK) even showed lower similarity with TR/Ccv2 than with the feline coronavirus strain, with a ratio below 84% in nucleic acid identity and divergence analysis performed by MatGAT 2.01. The same analysis produced a similar result for TR/Ccv6 and GU300122_Pt3_Brazil, HM450124_KCC21_Korea and JX035833_24.5_UK strains, all of which exhibited less than 80% similarity with the Turkish isolate. It is known that CCoV types in G1 are closer to FCoV type 1 than CCoV types in G2. As mentioned above, the fact that some CCoV strains in G1 have lower similarities with TR/Ccv2 and TR/Ccv6 is an expected result due to the difference between the two groups. However, the UK strain in G1 also showed low similarity with our TR/Ccv2 strain alike in G1 (24). The amino acid sequence comparison performed against the M gene did not show any changes that could lead to genogroup differentiation, although there were some amino acid changes in the Tr/Ccv6 strain.

Although CCoVs have been identified in Turkish dogs by serologic and virologic methods, including enzyme-linked immunosorbent assay (ELISA), immunofluorescence and PCR (1, 16, 30), this study performs phylogenetic analysis of Turkish CCoV strains. Obtaining information about the CCoV evolutionary pattern that is largely unknown in Turkey makes is important to conduct molecular characterisation studies. Based on the previous studies, CCoVs are circulating in different geographical regions of Turkey (Marmara-Mediterranean and southeastern Central Anatolia Regions). The information that does exist about the presence and prevalence of the virus raises the need to take measures for prevention of the diseases by creating an immune response. It is crucial to take control of CCoVs, which cause serious diseases that can result in death in dogs when combined with other canine pathogens. One of the most effective ways to protect against this infectious viral agent is vaccination. Although there are vaccines against CCoV strains, differences in the genome among strains from diverse geographical areas play a role in the immune response. As with the attenuated vaccine that contains the Italy strain developed by Decaro et al. (11) that can stimulate the immune system, the local Turkish strains will also make progress in the fight against the disease. Therefore, field strains are needed to generate local vaccines. To obtain and analyse the field strains, phylogenetic analysis should be done on samples taken from the same or different Turkish regions, and serological surveys should be conducted in order to obtain more detailed information throughout the country. According to the results of this study; in the phylogenetic comparison with circulating CCoV strains in the world, the presence of CCoVs from both genogroups was determined in our country. In this context; the vaccine strain compositions used in the immunization should include viruses from both genogroups. However, it is not known whether adequate immunity is available with vaccines since vaccination programs do not routinely control the protective antibody titer in vaccinated puppies and the presence of maternal antibodies is not investigated at the beginning of the program. We believe that there is a need for studies to assess the value of CCoV vaccines in providing adequate immunity.

The M gene region of local field CCoV strains was partially analysed and is presented here as a preliminary study for the phylogenetic analysis of Turkish CCoVs. Although CCoV-related infections have been identified in different Turkish geographical areas, vaccine development studies have not been conducted using Turkish local strains. Further studies that use more samples and scan more regions will provide a background for genetic differences. This endeavour will contribute to the desired immune response in dogs. This molecular study in faecal samples of affected dogs provides up-to-date data on the CCoV occurrence in Turkey. However, prospective studies that reveal the full-length Turkish CCoV-I and CCoV-II strain genome sequences, as in the study of Decaro et al. (10) with the Italian isolates, will help researchers and clinicians understand CCoV epidemiology and pathogenesis in Turkey. Although the result of partial M gene phylogenetic analysis of strains in this study obtained from Turkey with the circulating strains in the world does not provide information about the level of immunogenic response which is important in struggling against this infection, but reveals that

commercial vaccines used in our country should contain both strains.

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Ethical Statement

This study does not present any ethical concerns.

Conflict of Interest

The authors declared that there is no conflict of interest.

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