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ORIGINAL ARTICLE

Bovine coronavirus infections in Turkey: molecular analysis of the full-length spike gene sequences of viruses from digestive and respiratory infections

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Abstract
Bovine coronavirus (BCoV) can be spread by animal activity. Although cattle farming is widespread in Turkey, there are few studies of BCoV. The aim of this study was to evaluate the current situation regarding BCoV in Turkey. This is the first study reporting the full-length nucleotide sequences of BCoV spike (S) genes in Turkey. Samples were collected from 119 cattle with clinical signs of respiratory (n = 78) or digestive tract (n = 41) infection on different farms located across widely separated provinces in Turkey. The samples were screened for BCoV using RT-nested PCR targeting the N gene, which identified BCoV in 35 samples (9 faeces and 26 nasal discharge). RT-PCR analysis of the S gene produced partial/full-length S gene sequences from 11 samples (8 faeces and 3 nasal discharge samples). A phylogenetic tree of the S gene sequences was made to analyze the genetic relationships among BCoVs from Turkey and other countries. The results showed that the local strains present in faeces and nasal discharge samples had many different amino acid changes. Some of these changes were shown in previous studies to be critical for tropism. This study provides new data on BCoV in Turkey that will be valuable in designing effective vaccine approaches and control strategies.

Introduction

Coronaviruses, which cause infections in many species, including humans, have gained importance among zoonotic viruses in the last decade due to SARS coronavirus (SARS-CoV) and MERS-CoV infections and the recent COVID-19 pandemic caused by a novel coronavirus named SARS coronavirus 2 (SARS-CoV-2) [1–3]. In animals, coronaviruses are etiologically associated with enteric and respiratory diseases across a wide range of mammalian and avian species. Bovine coronavirus (BCoV) is mainly recognized as a cause of severe neonatal calf diarrhea, respiratory tract illnesses in calves, and winter dysentery in adult cattle, which cause economic losses to the livestock industry [4–6].

BCoV belongs to the species *Betacoronavirus 1*, which was recently assigned by the International Committee on Taxonomy of Viruses (ICTV) to the order *Nidovirales*, suborder *Cornidovirineae*, family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Betacoronavirus*, and subgenus *Embecovirus* [7]. The BCoV genome is a linear, single-stranded RNA with 31,028 bases [8]. There are five structural genes, encoding the phosphorylated nucleocapsid (N) protein, the integral membrane (M) protein, the small membrane (E) protein, the hemagglutinin/esterase (HE) protein, and the spike (S) glycoprotein [9]. The S glycoprotein is cleaved into S1 (N-terminal) and S2 (C-terminal) subunits by an intracellular protease [10]. The S glycoprotein has two main biological functions: viral attachment to target cells and fusion of viral and cellular membranes [11–13]. S1 is responsible for attaching the large receptor binding domain of the S protein to cell receptors, and it is responsible for induction of neutralizing antibodies and for hemagglutination activity. The S2 protein mediates the fusion of viral and cellular membranes by forming a spear-like stem. While the S2 subunit is highly conserved, the S1 subunit is more variable among BCoV isolates [6, 13–15]. Mutational changes in amino acid composition in cleavage sites may have a significant effect on tissue and cell tropism and pathogenicity.

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Although BCoV was reported in Turkey before 2011, the first detailed molecular study was conducted by Alkan et al. [16], who determined the partial sequence of the S gene from two faecal samples. While there have been sero-prevalence studies over many years, no detailed molecular studies have yet been conducted.

Accordingly, the first aim of this research was to determine the full-length S gene sequences of BCoV strains in Turkey, which could be valuable for designing vaccination approaches and control strategies. The study's second aim was to investigate the possible interaction of digestive and respiratory BCoV.

Materials and methods

Sampling

A total of 119 samples were obtained from cattle suspected of BCoV infection from different farms in widely distributed provinces of Turkey between 2001 and 2019 (Fig. 1). Faecal samples (n = 41) were obtained from animals aged four days to 15 months, while nasal discharge samples (n = 78) were obtained from animals aged seven days to ≥ 5 years. Details of sample information are summarized in Supplement 1.

Samples were diluted 1:10 (v/v and w/v for nasal discharge and faeces, respectively) in phosphate-buffered saline solution and centrifuged at 906 × *g* for 20 minutes at 4 °C. The supernatants were collected and stored at -80 °C.

RNA extraction and reverse transcription

Viral RNA was extracted using TRIzol® LS Reagent (Thermo Fisher Scientific), following the manufacturer's instructions. Reverse transcription (RT) was carried out using a Geneall® HyperScript^{TM} First-Strand Synthesis Kit according to the manufacturer's protocol. All RNAs and cDNAs were stored at -80 °C.

Polymerase chain reaction for the N and S gene regions of local BCoV isolates

To investigate the presence of BCoV, a nested PCR assay targeting the N gene region was carried out using primers and methods described previously [17]. All PCR reactions were performed using Thermo Scientific DreamTaq DNA Polymerase in a Biometra (Germany) thermal cycler.

For the first round of nested PCR for N gene amplification, 2.5 µl of cDNA was added to a mixture of 2.5 µl of 10X DreamTaq buffer, 0.5 µl of 10 mM dNTP mix, 1 µl of 10 µM primers (BCoV N-F/BCoV N-R), 0.25 µl of DreamTaq DNA polymerase (5 U / µl), and 17.25 µl of nuclease-free water per sample. PCR was performed according to the following protocol: an initial step of 3 minutes at 95 °C, followed by 35 cycles of 95 °C for 30 seconds, 50 °C for 30 seconds, and 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. The second round was conducted with the same master mix, using the primers nBCoV N-F/nBCoV N-R with the same thermal profile but with a different annealing temperature (58 °C). The samples showing positive results for the N gene were used for the S gene amplification (4,112

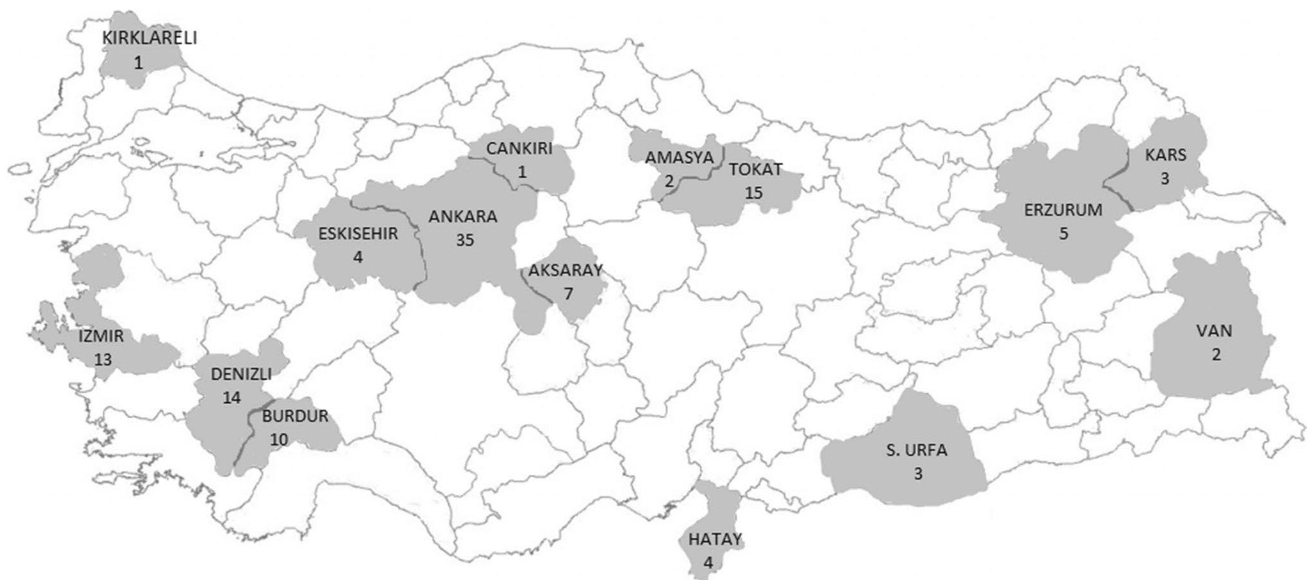

Fig. 1 Map showing the provinces where samples were collected in this study. Numbers indicate the number of samples from each location.

bp) with various primer pairs as reported previously [1, 6, 18] (Supplement 2).

PCR for amplification of the S gene region was conducted using the same master mix with different primers (Supplement 2). PCR was performed according to the following protocol: an initial step of 3 minutes at 95 °C, followed by 35 cycles of 95 °C for 30 seconds, 51-58°C for 30 seconds for the different reactions (Supplement 2), and 72 °C for 1 minute, and a final extension at 72 °C for 10 minutes. Each amplicon was analyzed on a 1% agarose gel stained with Safeview™ Classic (NextGen Life Sciences Private Limited), using 1 µl of 100-bp marker (Fermentas, Lithuania) to determine the product size.

Sequencing and phylogenetic analysis

Sequence analysis of the PCR products was performed by a commercial company (BM Labosis, BM Lab. Schist. Ltd. Sti. Ankara, Turkey). The obtained sequences were identified using BLAST (Basic Local Alignment Search Tool), provided on the NCBI (National Center for Biotechnology Information) web page. Sequences were aligned using Aliview software [19], using viral reference sequences from the GenBank database [9, 20]. Following alignment, amino acid sequences were deduced and sequence variation tables were created using MEGA X and Aliview software, based on the nucleotide sequences. The sequences were submitted to the GenBank database under accession numbers MK787427-MK787439 and MK989614-MK989619 for the N gene and S gene sequences, respectively, as shown in Table 1. Nucleotide sequence comparisons were performed using the SIAS [21] web tool. The phylogenetic relationships between partial or full-length nucleotide sequences of the S gene and the predicted amino acid sequences of the local isolates and various BCoV strains from other countries (Fig. 2; Supplement 3) were analyzed using the minimum-evolution methodology in MEGA X software. Minimum-evolution trees were computed with p-distance parameters, while confidence levels were estimated using 1,000 bootstrap replicates.

Results

Detection of BCoV

Thirty-five samples out of 119 tested positive for N gene fragments (407 bp). The gel electrophoresis results for several amplicons are presented in Supplement 4. The positivity rates for the faecal and nasal discharge samples were 21.9% (9/41) and 33.3% (26/78), respectively.

Molecular characterization of the S gene and phylogenetic analysis

S gene PCR results were evaluated for the 35 BCoV-positive samples, although not all samples were amplicon positive. As shown in Table 1, the full-length S gene sequence was obtained from five samples (3 faecal and 2 nasal discharge samples) while various partial S gene fragment sequences were obtained from six samples (4 faecal and 2 nasal discharge samples). The 488-bp S1 region, which plays a critical role in tropism and antigenicity, was amplified from 11 samples (5 full-length), using S1 primers (Supplement 2). The S1 region was therefore used for nucleotide and amino acid sequence comparisons and phylogenetic analysis. The gel electrophoresis results for some of the amplicons are presented in Supplement 5.

Table 1 Accession numbers of partial and full-length sequences of the S gene of local BCoV isolates. Because the partially sequenced region of S1 (488 bp) was located in the region amplified using the SC and SD primers, an additional accession number was not taken. Faecal samples are indicated by an asterisk (*).

BCoV isolate name	Primer pair								
	SA	SB	SC	SD	SE	SF	SG	SH	S1
BCoV/Turkey/4950/2015 *	MK989620								+
BCoV/Turkey/4945/2015 *	MK989621								+
BCoV/Turkey/U1/2014 *	MK989622								+
BCoV/Turkey/S8/2017	MK989624								+
BCoV/Turkey/T5/2018	MK989623								+
BCoV/Turkey/G76/2015 *	MK989628				-	MK989629			+
BCoV/Turkey/G83/2015 *	MK989630		MK989631	-	-	MK989632			+
BCoV/Turkey/H1/2013 *	-	-	MK989633	-	-	-	MK989634		+
BCoV/Turkey/79/2018	MK989627			-	-	-	-	-	+
BCoV/Turkey/8553/2015 *	-	-	-	-	-	-	-	-	MK989626
BCoV/Turkey/1N/2019	-	-	-	-	-	-	-	-	MK989625

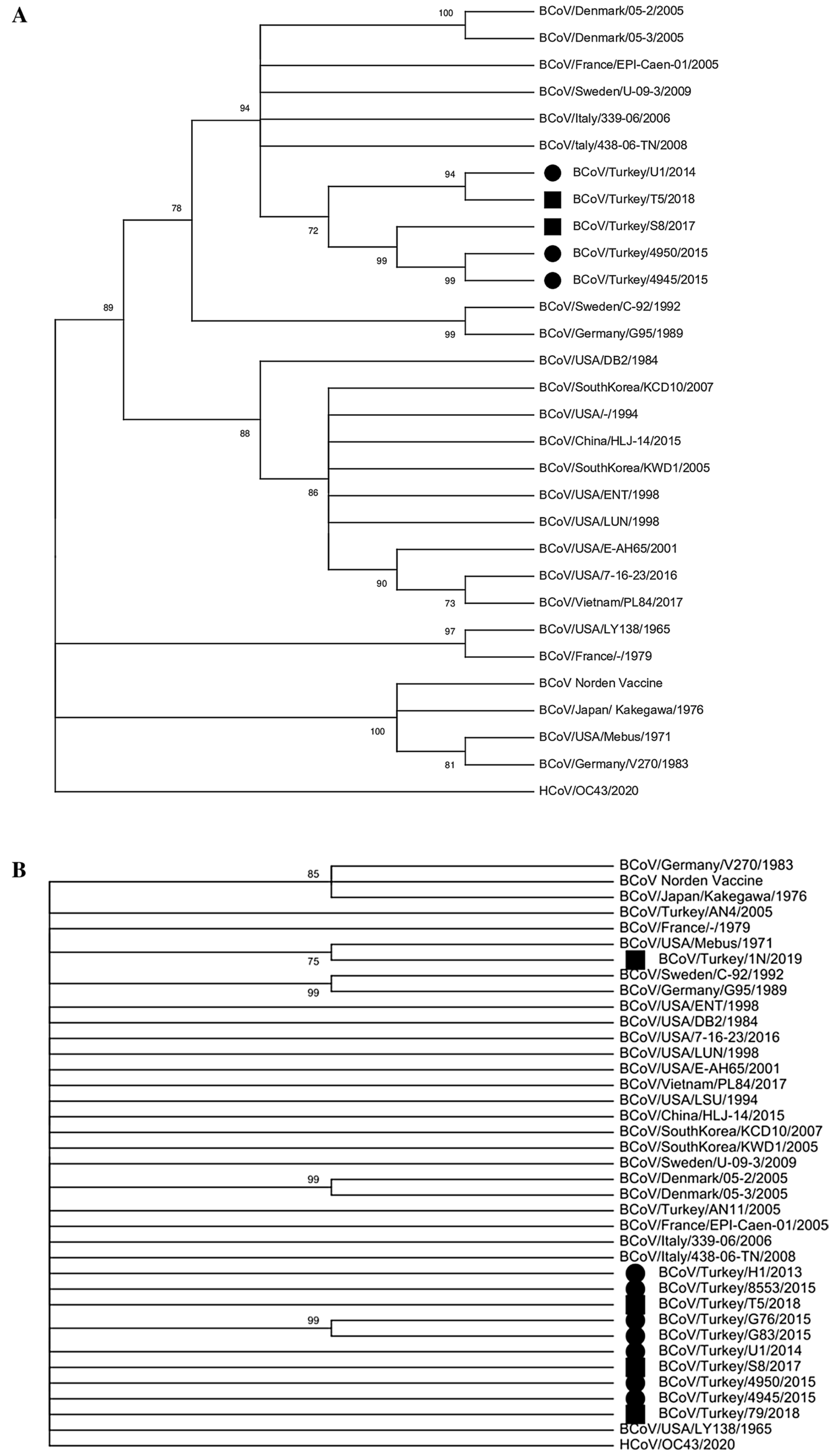

Fig. 2 Rooted phylogenetic trees constructed using full-length (A) and partial (B) S gene nucleic acid sequences of local BCoV isolates from Turkey and reference BCoV strains from other countries. The trees were constructed using the minimum-evolution method with bootstrap values calculated for 1000 replicates. Values greater than 70% are indicated. The strains from faecal and nasal discharge samples are indicated by black circles and squares, respectively.

The deduced amino acid sequences obtained from the amplicons exhibited differences when compared to the Mebus strain. Notably, all samples with one exception (BCoV/Turkey/1N/2019, accession no. MK989625) had several amino acid differences (Table 2). Most were synonymous mutations that had been reported previously in other local sequences (GQ259978 and GQ259979) [16]. No frameshift mutations, deletions, insertions, or recombinations were found in our study. In addition, there was no association between the nucleotide or amino acid changes and the type of material from which the virus originated (Table 2).

The nucleotide and amino acid sequences from the faecal samples (n = 3) were 97.6-99.0% and 96.9-98.2% identical, respectively, and those from the nasal discharge samples (n = 2) were 97.8% and 97.7% identical, respectively. The nucleotide and amino acid sequences of the full-length S genes (n = 5) were 97.4-99.0% and 96.9-98.2% identical, respectively, to each other and 96.9-97.5% and 97.3-97.9% identical, respectively, to those of the Mebus strain from faeces and the LSU strain obtained from nasal discharge. Identity rates were also calculated by SIAS for the partial sequences 488 (bp) from local strains. The partial S1 gene sequences (n = 11) showed 90.92-100% nucleotide sequence identity and 87.4-100% amino acid sequence identity to each other, and 94.78-99.11% and 96.23-97.72% nucleotide identity to the Mebus and LSU strains, respectively. The nucleotide and amino acid sequences of the faecal samples (n = 7) were 90.92-100% and 90.5-100% identical, respectively, and those from nasal discharge samples (n = 4) were 95.13-98.75% and 90-96.8% identical, respectively.

A phylogenetic tree was constructed based on full-length (4,112) and partial (488 bp) S gene sequences from local BCoVs with other randomly selected BCoV S gene sequences from around the world (Supplement 3), as shown in Fig. 2A and B. A phylogenetic tree based on amino acid

Table 2 Amino acid variations in the BCoVs S protein. Asterisks (*) represent amino acids that are identical to those of the reference sequence. The strains investigated in this study are indicated in bold.

Strain/isolate name	458	465	470	484	499	501	509	525	531	543	571	608
BCoV/USA/Mebus/1971	F	V	H	S	N	P	N	H	N	S	Y	D
BCoV/Turkey/1N/2019	*	*	*	*	*	*	*	*	*	*	*	-
BCoV/Turkey/4950/2015	*	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/4945/2015	*	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/U1/2014	S	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/G76/2015	S	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/G83/2015	S	A	D	T	S	S	T	Y	D	A	H	-
BCoV/Turkey/H1/2013	*	A	D	T	S	S	*	Y	D	A	H	-
BCoV/Turkey/8553/2015	S	A	D	T	S	S	T	Y	D	A	H	-
BCoV/Turkey/T5/2018	S	A	D	T	S	S	T	Y	D	A	H	G
BCoV/Turkey/S8/2017	*	A	D	T	S	S	*	Y	D	A	H	G
BCoV/Turkey/79/2018	*	A	D	T	S	S	T	Y	D	A	H	-
BCoV/Turkey/AN4/2005	S	A	*	T	*	S	*	*	D	*	*	-
BCoV/Turkey/AN11/2005	S	A	*	T	S	S	*	Y	D	G	H	-
BCoV/USA/LY138/1965	S	*	D	T	S	S	*	*	*	*	*	*
BCoV/China/HLJ-14/2015	S	A	D	T	*	S	*	*	D	A	*	*
BCoV/Denmark/05-2/2005	S	A	D	T	S	F	*	*	D	A	H	G
BCoV/France/-/1979	S	*	D	T	*	*	*	*	*	*	*	*
BCoV/S.Korea/KCD10/2007	S	A	D	T	S	S	*	*	D	A	*	*
BCoV/Sweden/C-92/1992	S	A	D	T	T	*	T	*	D	*	H	G
BCoV/Italy/339-06/2006	S	A	*	T	T	S	*	Y	D	A	H	G
BCoV/Japan/Kakegawa/1976	S	*	D	T	*	*	*	*	D	*	*	*
BCoV/Vietnamese/PL84/2017	S	A	D	T	S	S	H	*	D	A	H	*
BCoV/USA/LSU/1994	S	A	D	T	S	S	*	*	G	A	*	*
BCoV/Germany/G95/1989	S	A	D	T	T	*	T	*	D	*	H	G
BCoV/Denmark/05-3/2005	S	A	D	T	S	F	*	*	D	A	H	G
BCoV/France/EPICaen01/2005	S	A	D	T	S	S	*	Y	D	A	H	G
BCoV/S.Korea/KWD1/2005	S	A	D	T	S	S	*	*	D	A	*	*
BCoV/Sweden/U-09-3/2009	S	A	D	T	S	S	*	Y	D	A	H	G
BCoV/Italy/438-06TN/2008	S	A	D	T	S	S	*	Y	D	A	H	G
BCoV/Germany/V270/1983	S	*	*	T	*	*	*	*	D	*	*	*

sequences showed no differences between the local viruses (data not shown).

Discussion

In this study, faeces and nasal discharge samples were collected from cattle with suspected BCoV infection from various regions in Turkey (n = 119). Analysis by RT-PCR specific for the S gene region revealed that 21.9% (9/41) of the faecal samples and 33.3% (26/78) of the nasal discharge samples were positive. Previous studies conducted in Turkey reported significant numbers of BCoV cases alongside infections with many other enteropathogens in cases of calf diarrhea [22–28]. Furthermore, faecal-based serological studies have shown that BCoV is prevalent in cattle in Turkey [16, 26, 28–32], while two studies have shown that is associated with nasal discharge in Turkey [29, 33]. The prevalence of BCoV in our study is consistent with that reported previously in Turkey and in various other regions worldwide [5, 34–40].

Numerous studies based on partial or full-length S gene sequences have been conducted to determine and compare the biological, antigenic, and genetic characteristics of BCoV strains obtained from cattle with respiratory and enteric infections [1, 18, 41, 42] and have shown that the viruses affecting both systems are identical, with dual tropism [5, 17, 18]. The deduced amino acid sequences corresponding to the region of the S gene sequenced in the present study were 87.4-100% identical to each other and 94.78-99.11% identical to those of selected reference strains. Phylogenetic trees (Fig. 2) constructed based on partial and full-length sequences of the S gene region indicated that respiratory or digestive system origin of the samples made no significant difference in their placement in the phylogenetic tree, and the amino acid sequence identity values were similar to those reported in other studies worldwide [9, 20, 34, 35, 40, 41].

The S1 subunit of the S protein enables the virus to bind to host cell receptors, and it also stimulates neutralizing antibody synthesis and is responsible for hemagglutinin activity [12, 15, 43]. Thus, changes in this subunit could significantly affect antigenicity and pathogenicity [44]. In the present study, the S1 subunit exhibited more genetic variability than the S2 subunit (Table 2), as has been reported previously in Turkey [16] and in other countries [18, 45, 46].

Mutations make a significant contribution to the survival of infectious agents in nature. While many mutations do not notably change the structure of the virus, some may enable cross-species transmission or increase transmissibility. One of the most recent and striking examples is the SARS-CoV-2 S1 region 614 (D → G) mutation, which increases its infectivity [47]. A six-amino acid deletion (aa 526-531) found in Brazilian BCoV strains is identical to one found in human coronavirus (HCoV) OC43 [1]. We also observed multiple changes in the S amino acid sequences of local BCoV isolates. Moreover, there were some persistent changes in the S gene sequences, as seen in the amino acid variation table based on the reference strain Mebus, the study sequences, and two previously reported local viruses (GQ259978 and GQ259979). Additionally, there were similar persistent changes in different countries (Table 2), although one sample (BCoV/Turkey/IN/2019, accession number MK989625), did not show these changes but had the same partial sequence as Mebus. This may be due to vaccination shortly before sampling, although this cannot be evaluated, because no information was available. However, it should be noted that only a partial sequence fragment (488 bp) was analyzed.

Vaccination plays a crucial role in protection against infections. Among the factors affecting the success of vaccination is the level of antigenic similarity between the vaccine strain and local strains. Antigenic differences may increase due to serotype differences and variations from spontaneous mutations. Previous studies have shown that a single amino acid change in BCoV (528A→V) may cause resistance to neutralization [12]. We found no 528A → V mutations in our local BCoV sequences. Consequently, the data suggest that the present vaccines should be protective at a certain level, although further studies should be conducted to determine whether amino acid changes in the antigenic domains of local viruses could lead to resistance.

Conclusions

This is the first report of full-length BCoV S gene sequences from Turkey. The sequencing results demonstrate once again that the same BCoVs strains affect both the digestive and respiratory systems. The local strains were very similar to previously reported strains. Moreover, we found only insignificant sequence differences between the local strains and those used for vaccination. Thus, this study confirms that BCoV persists widely in Turkey and suggests that the current vaccines will remain protective at a certain level.

Supplementary Information The online version contains supplementary material available at https://doi.org/10.1007/s00705-021-05147-2.

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Declarations

Conflict of interest All authors declare that there are no financial or other relationships that might lead to a conflict of interest. All authors have seen and approved the manuscript and have contributed significantly to the work.

Ethical approval This study was approved by the ethics committee of Ankara University with document number 2017-15-127.

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