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Paper

Comprehensive genetic analysis of the first near-complete genome of bovine coronavirus and partial genome of bovine rotavirus in Türkiye through metagenomics

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Abstract

Obtaining the complete or near-complete genome sequence of pathogens is becoming increasingly crucial for epidemiology, virology, clinical science and practice. This study aimed to detect viruses and conduct genetic characterization of genomes using metagenomics in order to identify the viral agents responsible for a calf's diarrhoea. The findings showed that bovine coronavirus (BCoV) and bovine rotavirus (BRV) are the primary viral agents responsible for the calf's diarrhoea. The current study successfully obtained the first-ever near-complete genome sequence of a bovine coronavirus (BCoV) from Türkiye. The G+C content was 36.31% and the genetic analysis revealed that the Turkish BCoV strain is closely related to respiratory BCoV strains from France and Ireland, with high nucleotide sequence and amino acid identity and similarity. In the present study, analysis of the S protein of the Turkish BCoV strain revealed the presence of 13 amino acid insertions, one of which was found to be shared with the French respiratory BCoV. The study also identified a BRV strain through metagenomic analysis and detected multiple mutations within the structural and non-structural proteins of the BRV strain, suggesting that the BRV Kirikkale strain may serve as an ancestor for reassortants with interspecies transmission, especially involving rotaviruses that infect rabbits and giraffes.

Keywords

Bovine coronavirus, Bovine rotavirus, Calf diarrhoea, Metagenomics, Near-complete genome

Introduction

Neonatal calf diarrhoea (NCD) is a challenging problem for cattle industry globally in consequence of calf death, yield and economical losses (Azkur and Aksoy, 2018; Lorenz *et al.*, 2011). Bovine coronavirus (BCoV) and bovine rotavirus (BRV) are the leading causative viral agents of NCD. BCoV is classified as the member of family *Coronaviridae*, subfamily *Orthocoronavirinae*, genus *Betacoronavirus*, subgenus *Embecovirus*, and species *Betacoronavirus 1* (ICTV, 2021). The genome of coronaviruses consists of 27–31 kilobase (kb) pairs single-stranded positive-sense RNA which encodes numerous non-structural proteins, hemagglutinin-esterase protein (HE), spike glycoprotein (S), small envelope protein (E), membrane protein (M), and nucleocapsid protein (N). The proteins from the rest of ORFs are functionally less-characterized non-structural proteins (Brian and Baric, 2005; Suzuki *et al.*, 2020). The complete genome sequence analyses of BCoVs in domestic and wild ruminants revealed both nucleotide/amino acid (aa) homologies, and also genetic recombination and putative interspecies transmission in bovine coronaviruses (Alekseev *et al.*, 2008; Bidokhti *et al.*, 2013; Zhu *et al.*, 2022). Furthermore, intra-host and intra-isolate quasispecies diversity has been demonstrated in BCoVs (Zhang *et al.*, 2007). Despite many full-length and/or near-complete BCoV genome sequences are available from other countries, there is no study reported complete or near-complete genome analysis of BCoV from Türkiye yet.

BRV is classified in order *Reovirales*, family *Sedoreoviridae*, genus *Rotavirus*, and species *Rotavirus A* (ICTV, 2021). Rotaviruses are a group of viruses characterized by a segmented double-stranded RNA genome. The genome,

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ranging from 16 to 21 kb in length, encodes six structural and six non-structural proteins. The genetic diversity among rotaviruses is defined by the sequence variations in each of the 11 genome segments. The genotypes are assigned using a specific nomenclature based on the VP7-VP4-VP6-VP1-VP2-VP3-NSP1-NSP2-NSP3-NSP4-NSP5/6 gene segments. (Matthijnssens *et al.*, 2008, 2011). Rotaviruses have broad host range including many animal species and human, and due to its segmented genome, reassortment has an important role on host diversity, evolution that led to emerge of novel rotavirus A (RVA) genotypes which can have interspecies transmission including zoonotic infections (Fritzen *et al.*, 2020; Fukuda *et al.*, 2023; Lestari *et al.*, 2023; Martella *et al.*, 2010; Pathak *et al.*, 2022; Tsugawa and Hoshino, 2008). There are few studies conducted in Türkiye that exhibit full-length or partial sequences of bovine RVAs (Aksoy and Azkur, 2023; Alkan *et al.*, 2010; Karayel *et al.*, 2017; Karayel-Hacioglu *et al.*, 2022; Timurkan, 2012). However, reassortment dynamics of rotaviruses require constant screening of the circulating RVAs in animal in any countries.

Metagenomics is a cutting-edge technology in the field of virology that has the potential to revolutionize our understanding of viruses and their impact on both the environment, human and animal health. It enables the simultaneous detection of multiple viruses, the characterization of their genomes, and the determination of full or partial viral genome sequences (David *et al.*, 2022; Lizarazo *et al.*, 2019; Yandle *et al.*, 2023). In this study, the faeces sample of a calf with diarrhoea was analysed by metagenomics in order to detect viral agents in the sample and obtain viral genome sequences for the first time from Türkiye.

Material and methods

The sample

The sample that tested positive for BRV and BCoV by reverse transcription-polymerase chain reaction in our previous study (Aksoy *et al.*, 2021) was used for metagenomic analysis in this study. The sample was from a 5-day-old female Simmental calf that had diarrhoea for 2 days and was reared in Kirikkale, a province in central Anatolia, Türkiye, and died due to diarrhoea.

Shotgun Metagenomics

The RNA extraction was carried out with a commercial kit (High Pure Viral RNA kit, Roche, Germany) and the reverse transcription was conducted as described previously (Aksoy *et al.*, 2021). Briefly, RNA and dimethyl sulfoxide were incubated in 95°C for 5 minutes. Then sterile distilled water and random hexamer primer (PM-301S, Jena Bioscience) were added to the mixture and incubated in 70°C for 5 minutes. Finally, dNTPs (DN001-0250, GeneDirex), RT buffer and reverse transcriptase (M0253, New England Biolabs) were added to the mixture, and incubated in 25°C for 10 minutes, 37°C for 1 hour and 70°C for 5 minutes. The cDNA sample of the faeces was analysed by metagenomics (Massive Bioinformatics, Türkiye). Briefly, using 45 µL (1.5 µg) of cDNAs, DNA repair with NEBNext End Repair/dA-tailing Module (New England Biolabs). For the adapter ligation step, a total of 0.2 pmol of prepared DNA ends were added to a 50 µL Blunt/TA ligase master mix (New England Biolabs) mixture, and 20 µL of adapter mixture was added, followed by incubation at room temperature for 10 minutes. The final purification step for obtaining the cDNA library was completed using Adapter Bead Binding buffer (provided in the SQK-LSK108 kit) and 0.5X Agencourt AMPure XP beads (Beckman Coulter) kits. Sequencing mixture (14 µL DNA library), loading beads (25.5 µL) and running buffer mix (35.5 µL) were prepared using R9.4 flow cell priming and loaded to the R9.4.1 flow cell (Oxford Nanopore Technologies, UK). Sequencing was carried out for 48 hours using MinION™ control software and MinKNOW™ version 0.46.1.9 (R9.4).

Bioinformatic Analysis and Phylogenetics

After sequencing, the raw data was processed using the guppy v3.1.5 software, which included base-calling and de-multiplexing, resulting in the conversion of the data from fast5 to fastq format. Barcode and adapter sequences were then removed using the Porechop v0.2.3 software, and a further trimming of 45 bases from both ends of the sequences was performed to eliminate universal primers and tags. Subsequently, reads with a length less than 200 bp were filtered out and excluded from the analysis. The cleaned sequences were subsequently analysed using a custom-designed workflow on the Genious Prime 2019.2.1 platform. The sequences were aligned against viral genome databases at NCBI and evaluated through *de novo* alignment to determine the consistency of the resulting contigs and to detect corresponding organisms in the database.

To carry out multiple alignments of consensus genomes of bovine rotavirus and bovine coronavirus, and genome sequences obtained from GenBank for each of the viral genome, the MUSCLE algorithm was applied using the MEGA

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10.2.6 software. Relevant sequences of bovine rotavirus and bovine coronavirus, representing a diverse range of genotypes and sources such as those from different species including humans, wild type or tissue-cultured, etc. were retrieved from BLAST for all sequences.

Phylogenetic analyses of the BCoV strain and BRV segments were conducted and the trees were constructed using neighbor-joining method, p-distance nucleotide substitution model and bootstrap analysis with 500 replicates in MEGA 10.2.6 software. The amino acid (aa) identity and similarity of the viral proteins were determined by the Sequence Identity and Similarity (SIAS) tool and aa sequences were compared using average identity and similarity to other aa sequences in the matrix. The aa substitutions in the related viral proteins were identified using the option for determination of aa variable sites in MEGA 10.2.6 software.

Genome sequences of the BCoV (Bovine coronavirus strain Kirikkale) and five segments (VP1, VP2, VP6, NSP4, and NSP5/6) of the BRV (RVA/Cow-wt/TUR/KIRIKKALE/2019/G6P[5]) were deposited in GenBank under the accession numbers of OQ161705 and OQ215302-OQ215306, respectively (Table I).

VIRUS	GENE(S)	GENBANK ACCESSION NUMBERS
Bovine coronavirus	Near-complete genome	OQ161705
Bovine rotavirus	VP1, partial	OQ215302
Bovine rotavirus	VP2, complete	OQ215304
Bovine rotavirus	VP6, complete	OQ215305
Bovine rotavirus	NSP4, complete	OQ215306
Bovine rotavirus	NSP5/6, complete	OQ215303

Table I The GenBank accession numbers of the genome sequences of bovine coronavirus and bovine rotavirus strains reported in the present study.

Recombination Analysis of Bovine Coronavirus strain

Recombination analysis carried out for near-complete BCoV genome sequences that are retrieved from GenBank (n=145, including BCoV strain Kirikkale genome) by using Recombination Detection Program (RDP) v.4.101 software (Martin *et al.*, 2015). The primary scan for recombination events were carried out with selecting RDP, GENECONV, Chimaera and 3Seq algorithms, and then all available algorithms (RDP, GENECONV, Bootscan, MaxChi, Chimaera, SiScan, 3Seq) were used for confirmation of recombination events (Franzo *et al.*, 2020). Recombination events were considered valid only when identified by at least two independent algorithms, demonstrating statistical significance with a *p*-value less than 0.001 and selecting Bonferroni correction.

Selective Pressure Analysis of Bovine Coronavirus strain

The S genes of the BCoV strain Kirikkale and other BCoV strains that shared high aa identity and similarity with our strain (Table II) were used for selective pressure analysis (n=25). Sequences were retrieved from GenBank and aligned at the codon level using MEGA software. Selective pressures acting on the S protein were investigated using the SLAC, FEL, FUBAR and MEME methods implemented in the DataMonkey web server (Datamonkey Adaptive Evolution Server, 2023; Delport *et al.*, 2010; Kosakovsky Pond and Frost, 2005; Murrell *et al.*, 2013). A *p*-value threshold of less than 0.05 was used as the significance level for SLAC, MEME and FEL methods, and a posterior probability higher than 0.9 was used for FUBAR method.

Codon Usage Analysis of Bovine Coronavirus strain

The EMBOSS Cusp software (EMBOSS: cusp, 2023) was employed for the determination of the total G + C genomic content as well as the G + C content at the first, second, and third codon positions of the BCoV strain Kirikkale and other selected BCoV strains.

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Results

Metagenomics

Bovine coronavirus (BCoV) and bovine rotavirus (BRV) were the leading viral sequences from the data obtained from metagenomics of diarrheic calf faeces. Beside BCoV and BRV, numerous bacteriophage genome sequences were acquired partially (data not shown).

The genome of the BCoV strain (named BCoV strain Kirikkale) was almost fully recovered through metagenomic analysis, marking the first near-complete genome sequence of a BCoV in Türkiye. The BCoV genome is 31035 bp in length and HE, S, E, M, N structural genes, and ORF1ab polyprotein, ns2, ns4.8, ns4.9, ns12.7 non-structural genes were identified (Figure 1).

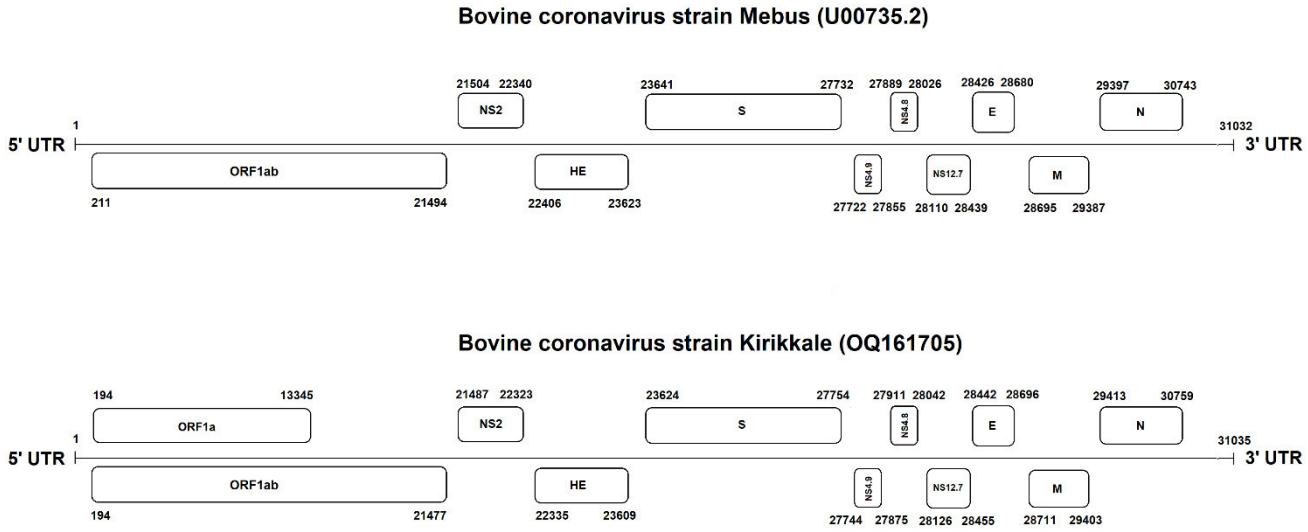

Figure 1 Schematic and comparative genome organization of the bovine coronavirus strain Kirikkale (OQ161705) and the reference strain Mebus (U00735.2). UTR: untranslated region, ORF: open reading frame, NS: non-structural, HE: hemagglutinin esterase, S: spike, E: envelope, M: membrane, N: nucleocapsid.

The BRV strain in this study was named RVA/Cow-wt/TUR/KIRIKKALE/2019/G6P and five out of 11 viral segments could be sequenced by metagenomics. For the five BRV genomic segments, VP2, VP6, NSP4, and NSP5/6, complete sequences were generated while for the VP1 only partial sequence was obtained.

Phylogenetic Analysis of the Bovine Coronavirus strain

The phylogeny of the nucleotide (nt) sequence of the BCoV strain Kirikkale showed that the highest nt identity was with the French respiratory BCoV strains (ranging from 97.85% to 97.76%) and the French enteric BCoV strain (97.74%). The respiratory BCoV strains from Ireland have high nt identity between 97.67% and 97.55% with the BCoV Kirikkale strain. BCoV strains from other ruminants such as sambar deer (up to 97.28%), waterbuck (up to 97.27%), white-tailed deer (up to 97.27%), sable antelope (97.12%), alpaca (97.0%) from USA were found to be genetically close to the present BCoV strain. The other closest BCoV strains were cattle from either Japan or USA. Interestingly, human enteric coronavirus strains shared high nt identity with the BCoV strain Kirikkale up to 96.93% (Figure 2, Table S1).

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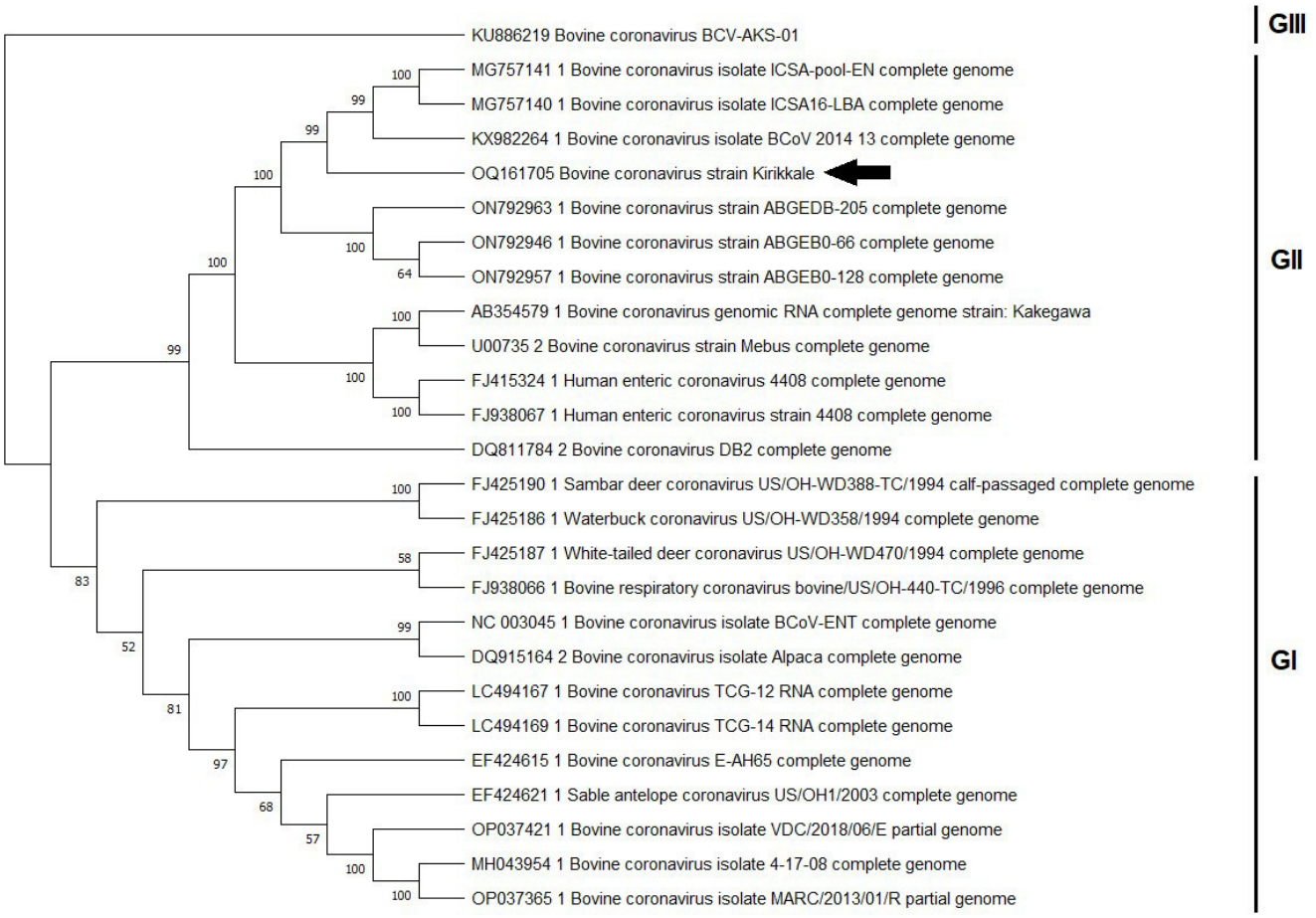

Figure 2 The phylogenetic tree of a near-complete genome sequence of the bovine coronavirus strain Kirikkale and other selected bovine coronaviruses and human enteric coronaviruses. The arrow indicates the BCoV strain Kirikkale which was obtained from the present study. GI: genogroup I, GII: genogroup II, GIII: genogroup III (Bahoussi et al., 2022).

Analysis of Deduced Amino Acid Sequences of Bovine Coronavirus strain

The five structural proteins of the BCoV strain Kirikkale, HE, S, M, E, and N proteins, were identified and analysed in order to determine aa sequence identity and similarity and aa substitutions when compared to reference BCoV Mebus and other coronavirus strains. The amino acid sequence of HE (424 residues) closely resembles that of French respiratory BCoV strains and Irish respiratory BCoV strains, and it exhibits the lowest amino acid identity and similarity when compared to the Japanese BCoV strain Kakegawa (Table II). In the HE protein sequence, 13 aa substitutions were detected in comparison to the BCoV Mebus strain (Table S2).

The M protein sequence (230 residues) of the Kirikkale strain shares 100% aa identity and similarity with French and Irish respiratory BCoV strains and the human enteric coronavirus 4408 strain from Germany (Table II). Compared to the Mebus strain, the Kirikkale strain's M protein has two aa substitutions (I38V and I58L). Notably, the I58L substitution is consistently found in association with French, Irish, and other Turkish BCoV strains, as well as with the human enteric coronavirus from Germany (Table S2).

Phylogeny of the E protein (84 residues) sequence showed that the BCoV Kirikkale strain has 98.8% aa identity and similarity with respiratory and enteric BCoV strains, wild ruminant coronaviruses and human enteric coronaviruses (Table II). The E protein was the most conserved aa sequence and when compared to Mebus strain; the Kirikkale strain showed only one aa substitution (G53V) which was also exhibited by all other coronaviruses that selected in this study (Table S2).

The BCoV Kirikkale strain's N protein (448 residues) sequence was found to be highly similar to that of French and Irish BCoV strains, human enteric coronaviruses, the BCoV DB2 strain, and wild ruminant coronaviruses (Table II). The N protein of the Kirikkale strain exhibits six amino acid substitutions compared to the Mebus strain N protein sequence: F15S, A49V, L53Q, N354S, K435R, and Y441F. Notably, N354S and K435R are unique to the French, Irish, and Turkish BCoV strains and are not found in other analysed coronavirus strains (Table S2).

The S protein (1376 residues) sequence identity and similarity of the Kirikkale strain were close to French and Irish

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BCoV strains (Table II). There are many aa substitutions in Kirikkale strain when compared to the Mebus strain S aa sequence. There were also 13 aa insertions detected in the Kirikkale strain and interestingly, the insertion at position 549 aa was also present in French respiratory BCoV strains, but not present in any other coronaviruses (Table S2).

For the non-structural proteins of the BCoV strain Kirikkale, Orf1a, ns2, ns4.8, ns4.9, ns12.7 proteins were identified and analysed in aa level. The Orf1a protein (4383 residues) showed close relationship with all selected coronaviruses (Table III). Five mutations common in French, Irish and the current Turkish BCoV strains were I241V, E950D, V1291I, I2025N, M2094I which are not exhibited by any other selected coronaviruses (Table S2). The aa identity and similarity results of the ns2 (278 residues), ns4.8 (43 residues), ns4.9 (43 residues), and ns12.7 (109 residues) proteins indicated that the Kirikkale strain was very close to French enteric BCoV and French respiratory BCoV strains (Table III). Comparison of the Kirikkale strain to the Mebus strain revealed 14 aa substitutions in the ns2 sequence, and interestingly, two of these mutations (M205K and V275F) were common to the French, Irish, and the current Turkish BCoV strains. Furthermore, Irish respiratory BCoV strains and the present Turkish BCoV strain exhibited the same K217N mutation commonly which is not present any other coronaviruses. The ns4.8 protein of the Kirikkale strain has 10 amino acid substitutions compared to the Mebus strain sequence, with eight of these mutations being common to both the Kirikkale strain and the French BCoV strains. There were four aa substitutions determined in the ns4.9 protein of the Kirikkale strain (I15T, L24P, I26L, M40T) and all of them were in common with French respiratory and enteric BCoV strains. The ns12.7 protein exhibited only one substitution (A25V) in comparison to Mebus strain and this mutation was commonly shared by the French BCoV strains, but not any other selected coronaviruses (Table S2). All protein accession numbers associated with Tables II and III are given in Table S3.

Recombination Analysis of Bovine Coronavirus

We conducted an analysis of all complete and near-complete genome sequences of BCoV viruses available in the GenBank database (n=145) to investigate the presence of recombination events. To accomplish this, we employed seven different algorithms included in the Recombination Detection Program 4 (RDP4) package: RDP, GENECONV, BootScan, MaxChi, Chimaera, SiScan, and 3Seq. Our comprehensive recombination analysis revealed the detection of 45 potential natural recombination events that occurred between BCoV isolates. However, 10 out of 45 events were positive in more than two independent algorithms and were accepted as recombination (LC494144, LC494150, LC494179, ON142320, ON792956, OP004056, OP298992, OP296992, OP866728, OP866729). BCoV Kirikkale strain does not exhibit recombination because it was positive only in a single algorithm (Table S4). The phylogenetic tree following recombination analysis of the near-complete genomes of BCoV strains was conducted in MEGA software (Figure 3).

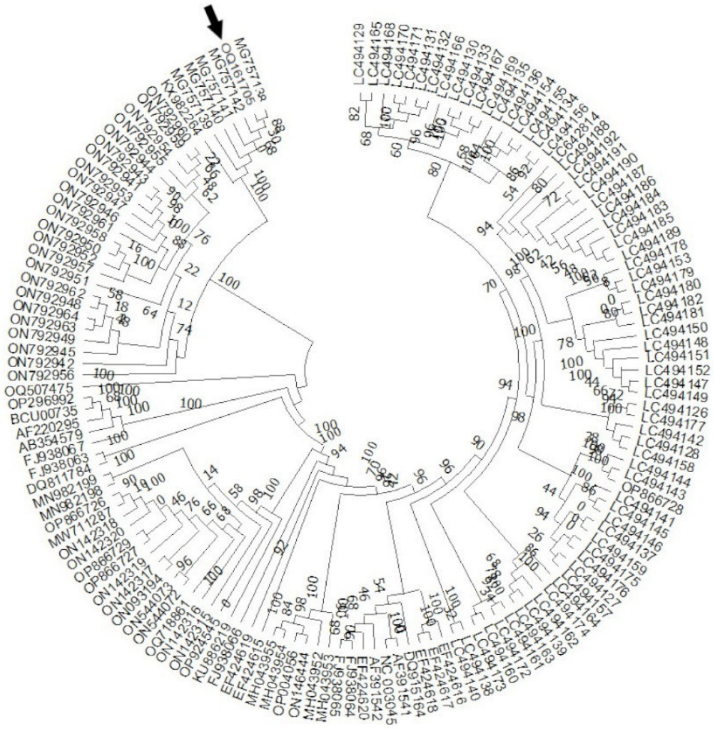

Figure 3 The phylogenetic tree of the complete genomes of BCoV strains and near-complete genome of the BCoV strain Kirikkale. The tree was conducted in MEGA software following recombination analysis in the RDP4 software. The bovine coronavirus strain Kirikkale is shown by black arrow.

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Table II The amino acid (aa) identity and similarity of the structural proteins of the bovine coronavirus strain Kirikkale (OQ161705) with other coronavirus strains. The identity and similarity were determined by SIAS (SIAS, 2023).

The coronavirus strains that are compared to the BCoV Kirikkale strain (OQ161705)	Hemagglutinin Esterase		Spike		Membrane		Envelope		Nucleocapsid	
	AA Identity	AA Similarity	AA Identity	AA Similarity	AA Identity	AA Similarity	AA Identity	AA Similarity	AA Identity	AA Similarity
Bovine coronavirus/ Respiratory/ France (MG757141.1, MG757140.1)	92.45-92.92%	92.68-92.92%	87.2%	87.42%	100%	100%	98.80%	98.80%	99.77%	99.77%
Bovine coronavirus/ Enteric/ France (KX982264.1)	91.98%	92.45%	86.84%	87.06%	99.56%	99.56%	98.80%	98.80%	99.77%	99.77%
Bovine coronavirus/ Respiratory/ Ireland (ON792946.1, ON792957.1, ON792963.1)	92.45%	92.45%	86.62-86.91%	86.91-87.20%	100%	100%	98.80%	98.80%	98.66-99.55%	98.88-99.77%
Bovine coronavirus/ Enteric/ USA (NC_003045.1, EF424615.1, MH043954.1, OP037421.1)	91.98-92.45%	92.45-92.68%	85.24-85.68%	85.97-86.40%	98.26-99.13%	98.69-99.56%	98.80%	98.80%	98.21-98.66%	98.66-98.88%
Bovine coronavirus/ Respiratory/ USA (FJ938066.1, OP037365.1)	91.74-91.98%	91.98-92.45%	85.61-85.68%	86.19-86.26%	98.26-99.13%	99.13-99.56%	97.61-98.80%	97.61-98.80%	97.99-98.66%	98.43-98.88%
Bovine coronavirus/ Enteric/ Japan (LC494169.1)	91.50%	91.74%	85.53%	86.19%	99.13%	99.56%	98.80%	98.80%	97.99%	98.43%
Bovine coronavirus/ Respiratory/ Japan (LC494167.1)	91.74%	91.98%	85.46%	86.04%	99.13%	99.56%	98.80%	98.80%	97.99%	98.43%
Human coronavirus/ Enteric/ Germany (FJ415324.1)	91.74%	92.45%	85.39%	86.11%	100%	100%	98.80%	98.80%	98.66%	99.10%
Human coronavirus/ Enteric/ USA (FJ938067.1)	91.74%	92.45%	85.46%	86.19%	99.56%	99.56%	98.80%	98.80%	98.66%	99.10%
Wild ruminant coronaviruses*/ Enteric/ USA (FJ425190.1, FJ425186.1, FJ425187.1, EF424621.1)	92.45%	92.68%	85.75-86.04%	86.40-86.55%	99.13-99.56%	99.56-100%	96.42-98.80%	96.42-98.80%	98.43-99.10%	98.88-99.33%
Bovine coronavirus isolate Alpaca/ Enteric/ USA (DQ915164.2)	92.21%	92.45%	85.24%	85.90%	99.13%	99.56%	ND**	ND**	98.43%	98.88%
Bovine coronavirus DB2/ Enteric/ USA (DQ811784.2)	92.45%	92.68%	85.68%	86.19%	99.56%	100%	98.80%	98.80%	99.10%	99.33%
Bovine coronavirus Kakegawa/ Enteric/ Japan (AB354579.1)	91.03%	91.50%	84.81%	85.24%	98.26%	99.13%	97.61%	98.80%	98.66%	99.33%
Bovine coronavirus Mebus/ Enteric/ USA (U00735.2)	91.74%	92.21%	84.30%	84.81%	99.13%	100%	97.61%	97.61%	98.43%	98.88%

*Sambar deer coronavirus, waterbuck coronavirus, white-tailed deer coronavirus, sable antelope coronavirus; ** Bovine coronavirus isolate Alpaca sequence data on GenBank (DQ915164.2) does not contain envelope protein. The identities and similarities above 99% are indicated as bold. All protein accession numbers associated with the table are provided in Table S3. AA: Amino acid, ND: Not determined.

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Table III The amino acid (aa) identity and similarity of the non-structural proteins of the bovine coronavirus strain Kirikkale (OQ161705) with other coronavirus strains. The identity and similarity were determined by SIAS (SIAS, 2023).

The coronavirus strains that are compared to the BCoV Kirikkale strain (OQ161705)	ORF1a		NS2		NS4.8		NS4.9		NS12.7	
	AA Identity	AA Similarity	AA Identity	AA Similarity	AA Identity	AA Similarity	AA Identity	AA Similarity	AA Identity	AA Similarity
Bovine coronavirus/ Respiratory/ France (MG757141.1, MG757140.1)	95.66%	95.80%	93.52%	93.88%	95.55%	91.11%	100%	100%	99.08%	99.08%
Bovine coronavirus/ Enteric/ France (KX982264.1)	95.43%	95.57%	93.52%	93.88%	97.77%	93.33%	100%	100%	99.08%	99.08%
Bovine coronavirus/ Respiratory/ Ireland (ON792946.1, ON792957.1, ON792963.1)	95.07-95.18%	95.34-95.43%	92.80-93.52%	93.16-93.88%	ND	ND	ND	ND	ND	ND
Bovine coronavirus/ Enteric/ USA (NC_003045.1, EF424615.1, MH043954.1, OP037421.1)	95.04-95.23%	95.39-95.45%	91.72-92.44%	92.44-93.16%	71.11-73.33%	71.11-75.55%	46.51-51.16%	46.51-53.48%	97.24-98.16%	98.16-99.08%
Bovine coronavirus/ Respiratory/ USA (FJ938066.1, OP037365.1)	94.95-95.14%	95.25-95.43%	92.44-92.80%	93.16%	71.11%	68.88%	48.83-53.48%	51.16-55.81%	97.24-98.16%	98.16-99.08%
Bovine coronavirus/ Enteric/ Japan (LC494169.1)	ND	ND	92.08%	92.80%	73.33%	75.55%	41.86%	44.18%	98.16%	99.08%
Bovine coronavirus/ Respiratory/ Japan (LC494167.1)	ND	ND	92.08%	92.80%	73.33%	75.55%	41.86%	44.18%	97.24%	99.08%
Human coronavirus/ Enteric/ Germany (FJ415324.1)	ND	ND	91%	91.36%	80%	80%	83.72%	88.37%	98.16%	98.16%
Human coronavirus/ Enteric/ USA (FJ938067.1)	94.93%	95.25%	91%	91.36%	80%	80%	83.72%	86.04%	98.16%	98.16%
Wild ruminant coronaviruses*/ Enteric/ USA (FJ425190.1, FJ425186.1, FJ425187.1, EF424621.1)	95.14-95.16%	95.36-95.41%	92.08-92.80%	92.44-93.16%	68.88-71.11%	66.66-73.33%	51.16-86.04%	53.48-88.37%	96.33-98.16%	99.08%
Bovine coronavirus isolate Alpaca/ Enteric/ USA (DQ915164.2)	95%	95.25%	92.8%	93.16%	ND	ND	ND	ND	97.24%	98.16%
Bovine coronavirus DB2/ Enteric/ USA (DQ811784.2)	95.25%	95.43%	92.44%	92.80%	75.55%	75.55%	90.69%	93.02%	98.16%	99.08%
Bovine coronavirus Kakegawa/ Enteric/ Japan (AB354579.1)	ND	ND	91.36%	91.72%	73.33%	73.33%	90.69%	93.02%	99.08%	99.08%
Bovine coronavirus Mebus/ Enteric/ USA (U00735.2)	ND	ND	91.36%	91.72%	73.33%	73.33%	90.69%	93.02%	99.08%	99.08%

*Sambar deer coronavirus, waterbuck coronavirus, white-tailed deer coronavirus, sable antelope coronavirus; ** The GenBank sequence data of these strains do not contain all non-structural proteins. The identities and similarities above 99% are indicated bold. All protein accession numbers associated with the table are provided in Table S3. AA: Amino acid, ND: Not determined.

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Selective Pressures of S protein of Bovine Coronavirus

SLAC and FEL analyses of the S proteins revealed 22 and 60 sites under purifying selection, respectively, and there are no diversifying selection codon sites determined with these methods.

Five episodic diversifying selection (codon sites 179, 684, 891, 963, and 1065) were detected with MEME with p-value threshold of 0.05 (Table IV).

FUBAR analysis of S proteins detected 11 pervasive diversifying selection and 123 pervasive purifying selection with posterior probability of 0.9. The pervasive diversifying selection sites were at codon sites 113, 115, 179, 253, 499, 501, 509, 510, 571, 578, 1260 (Table IV and Figure 4).

	FUBAR		MEME	
Position	dN-dS	Posterior probability	dN-dS	*p*-value
113	11.265	0.950	ND	ND
115	15.721	0.955	ND	ND
179	21.966	0.941	4.67	0.04
253	11.293	0.931	ND	ND
499	14.594	0.955	ND	ND
501	12.029	0.963	ND	ND
509	23.687	0.982	ND	ND
510	7.289	0.906	ND	ND
571	12.121	0.948	ND	ND
578	6.584	0.900	ND	ND
684	ND	ND	5.06	0.04
891	ND	ND	7.78	0.01
963	ND	ND	5.55	0.03
1065	ND	ND	9.05	0.00
1260	10.693	0.927	ND	ND

Table IV The codons of the spike protein have been identified to undergo statistically significant diversifying selection through FUBAR and MEME methods. dN-dS: the difference between non-synonymous and synonymous substitution rates, ND: not determined with the specific method.

Codon Usage of Bovine Coronavirus

In order to acquire codon usage of the BCoV strain Kirikkale and some other BCoV strains that are close to our strain, the near-complete genomes were analysed in EMBOSS Cusp program. The G+C content of the BCoV strain Kirikkale was 36.31%, and G+C content at the first, second and third positions were 33.66%, 40.77%, and 34.51%, respectively. A further analysis was carried out to find out the G+C contents and codon usage of some other BCoV strains that were genetically close to Kirikkale strain. The closest strains, French (MG757141.1) and Irish (ON792946.1) respiratory BCoV strains, have 37.09% and 36.96% G+C content, respectively. French enteric BCoV strain (KX982264.1) has 37.03% and the Mebus strain (U00735.2) has 37.02% G+C content.

The codon usage preferences of the aforementioned strains were also analysed. The most frequent two codons in the BCoV strain Kirikkale were UUU (Phe) and UGU (Cys) (Table V). The most frequent codon was UUU in all analysed BCoV strains. The codon usage preference of the BCoV strain Kirikkale was the same with the French respiratory and enteric BCoV strains (UUU and UGU) (Table S5).

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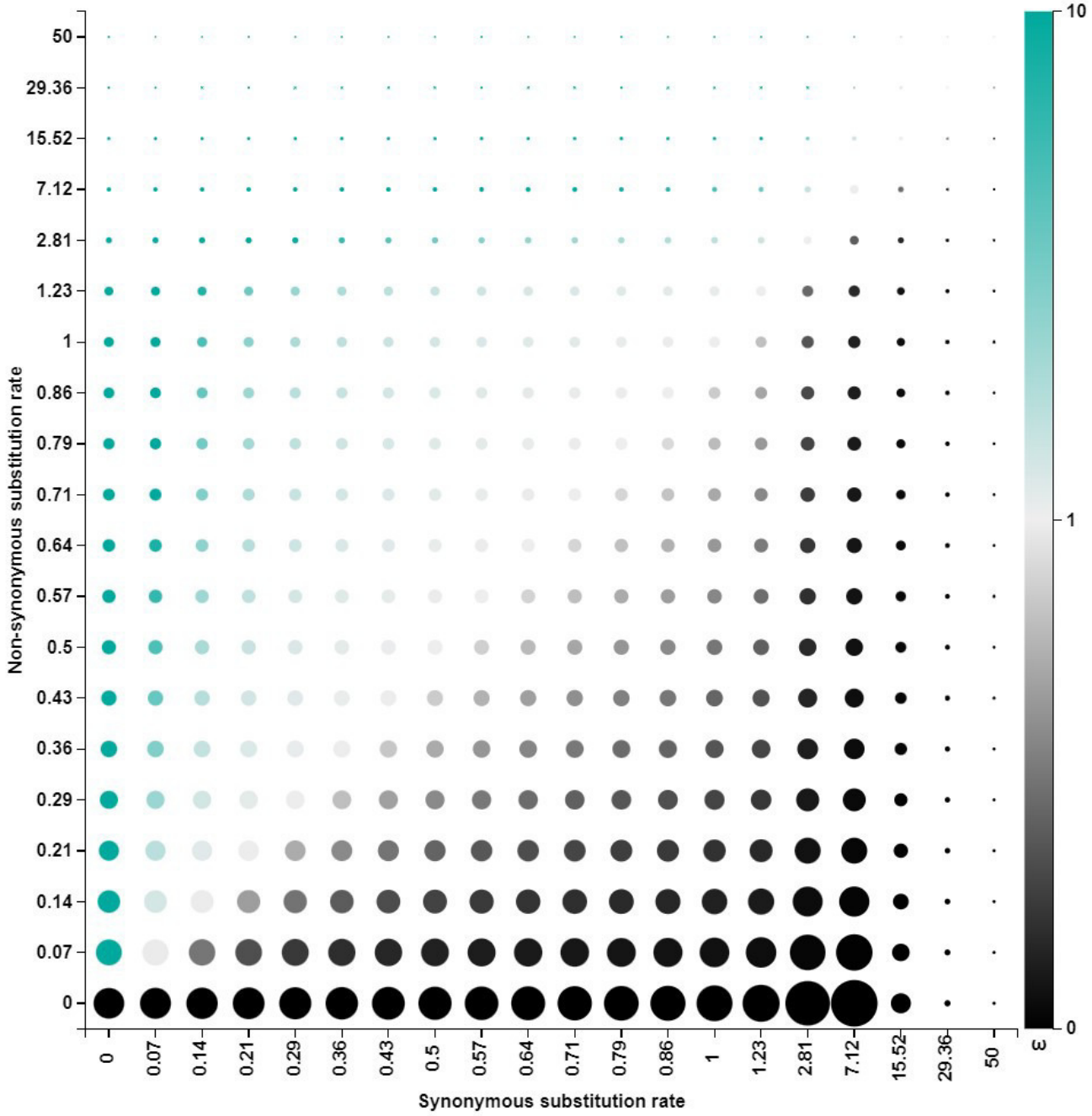

Figure 4 The posterior rate distribution of FUBAR analysis. The dot's size is directly related to the assigned posterior weight for that specific gridpoint, while the color represents the intensity of selection.

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Codon	AA	Fraction	Frequency	Number
GCA	Ala	0.313	14.306	148
GCC	Ala	0.197	8.990	93
GCG	Ala	0.097	4.447	46
GCU	Ala	0.393	17.980	186
UGC	Cys	0.345	23.780	246
UGU	Cys	0.655	45.143	467
GAC	Asp	0.263	8.507	88
GAU	Asp	0.737	23.780	246
GAA	Glu	0.630	16.433	170
GAG	Glu	0.370	9.667	100
UUC	Phe	0.253	16.143	167
UUU	Phe	0.747	47.559	492
GGA	Gly	0.290	14.403	149
GGC	Gly	0.222	11.020	114
GGG	Gly	0.117	5.800	60
GGU	Gly	0.372	18.463	191
CAC	His	0.433	8.797	91
CAU	His	0.567	11.503	119
AUA	Ile	0.282	14.113	146
AUC	Ile	0.172	8.603	89
AUU	Ile	0.546	27.356	283
AAA	Lys	0.679	38.279	396
AAG	Lys	0.321	18.076	187
CUA	Leu	0.074	6.187	64
CUC	Leu	0.070	5.897	61
CUG	Leu	0.081	6.767	70
CUU	Leu	0.174	14.596	151
UUA	Leu	0.312	26.196	271
UUG	Leu	0.290	24.360	252
AUG	Met	1.000	18.173	188
AAC	Asn	0.286	11.213	116
AAU	Asn	0.714	27.936	289
CCA	Pro	0.417	10.633	110
CCC	Pro	0.182	4.640	48
CCG	Pro	0.095	2.417	25
CCU	Pro	0.307	7.830	81
CAA	Gln	0.624	15.563	161
CAG	Gln	0.376	9.377	97
AGA	Arg	0.416	19.913	206
AGG	Arg	0.220	10.536	109
CGA	Arg	0.073	3.480	36
CGC	Arg	0.093	4.447	46
CGG	Arg	0.051	2.417	25
CGU	Arg	0.147	7.057	73
AGC	Ser	0.163	11.600	120
AGU	Ser	0.276	19.720	204
UCA	Ser	0.201	14.306	148
UCC	Ser	0.111	7.927	82
UCG	Ser	0.046	3.287	34
UCU	Ser	0.203	14.500	150
ACA	Thr	0.351	16.336	169
ACC	Thr	0.218	10.150	105
ACG	Thr	0.112	5.220	54
ACU	Thr	0.320	14.886	154
GUA	Val	0.202	12.373	128
GUC	Val	0.134	8.217	85
GUG	Val	0.191	11.696	121
GUU	Val	0.472	28.903	299
UGG	Trp	1.000	24.940	258
UAC	Tyr	0.377	23.200	240
UAU	Tyr	0.623	38.279	396
UAA	TERM	0.486	39.729	411
UAG	TERM	0.181	14.790	153
UGA	TERM	0.333	27.163	281

Table V The codon usage preference of the bocine coronavirus strain Kirikkale. AA: amino acid.

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Table VI The amino acid (aa) identity and similarity of structural and non-structural proteins of bovine rotavirus Kirikkale strain with other rotaviruses. The identity and similarity were determined by SIAS (SIAS, 2023).

VP1 (OQ215302)			
GenBank Accession Numbers*	Rotavirus A strains	AA Identity	AA Similarity
MZ848168.1 (UYH99844)	RVA/Cow-wt/TUR/K060/2008/G10P[11]	98.42%	98.42%
KX212876.1 (APZ86776)	RVA/Cow-wt/TUR/Amasya-2/2015/G8P[5]	97.9%	98.01%
KF729635.1 (AHF49967)	RVA RF clone TOPORF1#2 /UK	97.9%	98.01%
HQ846854.1 (AEK32867)	Rotavirus A strain Rotashield DxRRV /USA	98.11%	98.21%
EU636924.1 (ACC94312)	RVA/Simian-tc/USA/RRV/1975/G3P[3]	98.01%	98.21%
MW924298.1 (UVW67528)	RVA/Oyster-wt/ARG/Crassostrea_gigas-BsAs/2015/G8P[1]	98.21%	98.42%
OL988973.1 (UZY23610)	RVA/Bovine/Northern Ireland/R1WTA16/2013/G6P[5]	97.8%	98.21%
GU565041.1 (ADK27004)	RVA/Vaccine/USA/RotaTeq-WI79-4/1992/G6P1A[8]	97.8%	98.11%
GU565085.1 (ADK27008)	RVA/Vaccine/USA/RotaTeq-BrB-9/1996/G4P7[5]	97.69%	98.01%
GU565052.1 (ADK27005)	RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]	97.69%	98.01%
GU565063.1 (ADK27006)	RVA/Vaccine/USA/RotaTeq-SC2-9/1992/G2P7[5]	97.69%	98.01%
KU708259.1 (AMR44584)	RVA/roe deer-wt/SLO/D38-14/2014/G6P[15]	98.01%	98.21%
KU962128.1 (AOV81271)	RVA/Cervidae/United States/14-02218-2/2014	98.01%	98.32%
FJ495126.1 (ACL27787)	RVA/Antelope-wt/ZAF/RC-18-08/G6P[14]	97.8%	98.11%
JX567760.1 (AFV91311)	RVA/Human-wt/AUS/V585/2011/G10P[14]	98.11%	98.21%
OK651078.1 (UFK32626)	RVA/Horse-wt/IND/ERV2/2015/G6P[1]	97.9%	98.11%
LC133558.1 (BAV35169)	RVA/Cow-tc/JPN/KK3/1983/G10P[11]	98.21%	98.32%
VP2 (OQ215304)			
GenBank Accession Numbers*	Rotavirus A strains	AA Identity	AA Similarity
MN626424.1 (QHG11383)	Rabbit rotavirus A isolate RRVA/L232/France	97.84%	97.95%
OL988914.1 (UZY23615)	RVA/Bovine/Northern Ireland/R1WTA05/2013/G6P[5]	97.95%	98.07%
OL988950.1 (UZY23618)	RVA/Bovine/Northern Ireland/R1WTA12/2013/G6P[5]	97.84%	98.18%
GQ428142.1 (ADB28000)	Rotavirus A giraffe/UCD/IRL/2007	96.36%	96.36%
MN067456.1 (QHU80083)	RVA/Calf-wt/MAR/S18/2012/G6P[14]	97.72%	97.95%
LC055548.1 (BAR88358)	RVA/Human-wt/THA/SKT-27/2012/G6P[14]	97.84%	97.95%
MZ848171.1 (UYH99847)	RVA/Cow-wt/TUR/K063/2008/G10P[11]	97.95%	98.18%
MZ848172.1 (UYH99848)	RVA/Cow-wt/TUR/K060/2008/G10P[11]	97.95%	98.18%
KC488886.1 (AGQ48108)	RVA/Rabbit-tc/NLD/K1130027/2011/G6P[11]	98.18%	98.29%
MW771177.1 (UQQ66507)	RVA/Cow-wt/ZAF/MRC-DPRU456/2009/G10P[11]	98.07%	98.18%
MW771122.1 (UQQ66502)	RVA/Cow-wt/ZAF/Bov1/2009/G6P[5]	97.72%	98.07%
KJ751923.1 (AHZ32701)	RVA/Cow-wt/ZAF/MRC-DPRU3005/2009/G6P[11]	97.84%	98.18%
JN831221.1 (AER51155)	RVA/Cow-wt/ZAF/1604/2007/G8P[1]	97.84%	97.95%
KY055428.1 (ASM56663)	RVA/Goat-wt/UGA/BUW-14-085/2014/G6P[1]	97.04%	97.72%
EU708913.1 (ACH97418)	Canine rotavirus CU-1 /USA/G3P[3]	96.25%	97.72%
LC477477.1 (BBJ70244)	RVA/Human-wt/JPN/Tokyo17-10/2017/G2P[4]	95.68%	97.38%
KX171525.1 (APT71323)	Human rotavirus A strain CAU11-04 /South Korea / G2P[4]	95.8%	97.5%
EU708924.1 (ACH97429)	Canine rotavirus K9 /USA /G3P[3]	96.48%	97.72%
EU708891.1 (ACH97396)	Human rotavirus Ro1845 / Israel / G3P[3]	96.7%	97.72%
LC477479.1 (BBJ70246)	RVA/Human-wt/JPN/Tokyo17-15/2017/G2P[4]	95.68%	97.38%
EU708957.1 (ACH97462)	Feline rotavirus Cat2/Australia/ G3P[9]	96.7%	97.72%
KJ940064.1 (AIS93076)	RVA/Human-wt/BRA/SC19868/2011/G2P[4]	95.91%	97.5%
HQ661135.1 (AEH95559)	RVA/Dog-tc/ITA/RV198-95/1995/G3P[3]	96.48%	97.61%
KX363215.1 (ANN83175)	RVA/Human-wt/VNM/12067_87/	95.68%	97.38%
LC169985.1 (BAV57941)	RVA/Human-wt/THA/LS-L7/2014/G2P[4]	95.57%	97.38%
MT232577.1 (QNG71094)	RVA/Human-wt/USA/2009727045/2009/G8P[4]	95.45%	97.27%
VP6 (OQ215305)			
GenBank Accession Numbers*	Rotavirus A strains	AA Identity	AA Similarity
MZ848179.1 (UYH99855)	RVA/Cow-wt/TUR/K063/2008/G10P[11]	99.5%	98.5%
MZ848180.1 (UYH99856)	RVA/Cow-wt/TUR/K060/2008/G10P[11]	99.25%	98.25%
KX212867.1 (APZ86767)	RVA/Cow-wt/TUR/Amasya-1/2015/G8P[5]	99.5%	98.5%
MW771142.1 (UQQ66486)	RVA/Cow-wt/ZAF/Bov7/2003/G10P[11]	99.25%	98.5%
MW771197.1 (UQQ66491)	RVA/Cow-wt/ZAF/MRC-DPRU3005/2009/G6P[11]	99.5%	98.5%
MW771175.1 (UQQ66489)	RVA/Cow-wt/ZAF/MRC-DPRU456/2009/G10P[11]	99.5%	98.5%
MW771119.1 (UQQ66484)	RVA/Cow-wt/ZAF/Bov1/2009/G6P[5]	99.25%	98.25%
LC055551.1 (BAR88361)	RVA/Human-wt/THA/SKT-27/2012/G6P[14]	99.25%	98.5%
KP753064.1 (AKA40770)	RVA/Pig-wt/ZAF/MRC-DPRU3878/2008/G5P[X]	99.25%	98.25%
MN067459.1 (QHU80089)	RVA/Calf-wt/MAR/S18/2012/G6P[14]	99.5%	98.5%
GU565078.1 (ADK26987)	RVA/Vaccine/USA/RotaTeq-WI78-8/1992/G3P7[5]	99.5%	98.5%
LC133562.1 (BAV35173)	RVA/Cow-tc/JPN/KK3/1983/G10P[11]	99.25%	98.5%
MW924302.1 (UVW67532)	RVA/Oyster-wt/ARG/Crassostrea_gigas-BsAs/2015/G8P[1]	99.5%	98.5%
OK651101.1 (UFK32649)	RVA/Horse-wt/IND/ERV2/2015/G6P[1]	99%	98.25%
KC815662.1 (AGS43821)	RVA/Horse-tc/JPN/OH-4/1982/G6P[5]	99.5%	98.5%
MN067448.1 (QHU80088)	RVA/Goat-wt/MAR/S19/2012/G10P[14]	99.25%	98.5%
KJ940164.1 (AIM40192)	RVA/dog-wt/GER/88977/2013/G8P1	99.25%	98.5%
MT234355.1 (QST88821)	RVA/Buffalo-wt/ZAF/1442/2007/G10P[11]	99%	98.5%
MN626422.1 (QHG11381)	Rabbit rotavirus A isolate RRVA/L232/France	98.25%	98.5%
GU384194.1 (ADC42128)	Bovine rotavirus A isolate DQ-75/China/	98.5%	98.25%
KU128896.1 (ANS11443)	RVA/Human-wt/Bel/BEF06018/2014/G29P41	98.75%	98.5%
MW771153.1 (UQQ66487)	RVA/Cow-wt/MOZ/MPT-93/2016/G10P[11]	99%	98.5%
NSP4 (OQ215306)			
GenBank Accession Numbers*	Rotavirus A strains	AA Identity	AA Similarity
KX212879.1 (APZ86779)	RVA/Cow-wt/TUR/Amasya-1/2015/G8P[5]	99.42%	99.42%

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AF166353.1 (AAD46918)	Bovine rotavirus strain CBNU-1/South Korea	99.42%	99.42%
KC815667.1 (AGS43826)	RVA/Horse-tc/JPN/OH-4/1982/G6P[5]	97.71%	98.28%
LC133567.1 (BAV35178)	RVA/Cow-tc/JPN/KK3/1983/G10P[11]	98.28%	98.28%
MW924307.1 (UVW67537)	RVA/Oyster-wt/ARG/Crassostrea_gigas-BsAs/2015/G8P[1]	97.71%	97.71%
KM454490.1 (AIZ08924)	Equine rotavirus A strain FI-14/USA/G3P[12]	97.14%	97.14%
AB908945.1 (BAQ95482)	RVA/Horse-tc/JPN/JE29/1997/G3P[12]	97.71%	97.71%
EU200802.1 (ABY64690)	Human rotavirus A isolate N155/India/G10P[11]	97.71%	97.71%
OK651114.1 (UFK32662)	RVA/Horse-wt/IND/ERV2/2015/G6P[1]	96%	96.57%
KC175212.1 (AGF85911)	RVA/Human-wt/IND/N228/2004/G10P[11]	97.71%	97.71%
LC074704.1 (BAU88417)	RVA/Cat-tc/JPN/FRV537/2004/G6P[5]	97.14%	97.14%
JF327746.1 (AEL87275)	Rotavirus A strain TURV01/Brazil/turkey/	96.57%	96.57%
FJ422140.1 (ACL36062)	RVA/Rhesus-tc/USA/PTRV/1990/G8P[1]	96%	96.57%
DQ838625.2 (ABG75799)	RVA/Simian-tc/ZAF/SA11-H96/1958/G3P5B[2]	94.28%	94.85%
KY972048.1 (AUC63157)	RVA/Llama-wt/PER/L25/2014	93.71%	94.28%
MN067453.1 (QHU80077)	RVA/Calf-wt/MAR/S18/2012/G6P[14]	97.71%	97.71%
K03384.1 (AAA47288)	UK bovine rotavirus/	96%	96.57%
JN248455.1 (AEP96171)	Bovine rotavirus A strain DK11601/Denmark/G6P[5]	97.71%	97.71%
EF554102.1 (ABU49806)	RVA/Human-tc/AUS/MG6/1993/G6P[14]	96%	97.14%
NSP5 (OQ215303)			
GenBank Accession Numbers*	Rotavirus A strains	AA Identity	AA Similarity
KX212877.1 (APZ86777)	RVA/Cow-wt/TUR/Amasya-1/2015/G8P[5]	94.44%	94.44%
LC119114.1 (BAU88580)	RVA/Cow-tc/NGA/NGRBg8/1998/G8P[1]	95.95%	95.95%
EF554125.1 (ABU49815)	RVA/Human-wt/BEL/B10925/1997/G6P[14]	95.45%	95.45%
MF940692.1 (AXF43190)	RVA/Bovine-tc/KOR/KJ19-2/2006/G6P[7]	94.94%	94.94%
OK651118.1 (UFK32666)	RVA/Horse-wt/IND/ERV2/2015/G6P[1]	95.45%	95.45%
GU565062.1 (ADK27010)	RVA/Vaccine/USA/RotaTeq-WI79-9/1992/G1P7[5]	95.45%	95.45%
MW924308.1 (UVW67538)	RVA/Oyster-wt/ARG/Crassostrea_gigas-BsAs/2015/G8P[1]	94.94%	94.94%
MN067454.1 (QHU80079)	RVA/Calf-wt/MAR/S18/2012/G6P[14]	94.94%	95.45%
KU708266.1 (AMR44592)	RVA/roe deer-wt/SLO/D38-14/2014/G6P[15]	95.45%	95.95%
KJ411442.1 (AII82561)	RVA/Human-wt/US/2012841174/2012/G8P[14]	95.95%	95.95%
EU708966.1 (ACH97471)	Feline rotavirus Cat2/Australia/1984/G3P[9]	94.94%	94.94%
MG926766.1 (AYE56610)	RVA/Human-wt/MOZ/0060b/2012/G12P[8]P[14]	95.45%	95.45%
KT281130.1 (ANI85934)	RVA/Human-wt/USA/2012741499/2012/G24P[14]	94.44%	94.94%
KJ748486.1 (AIE90137)	RVA/Human-wt/GHA/GH019-08/2008/G8P[6]	95.45%	95.45%
MW771161.1 (UQQ66563)	RVA/Cow-wt/MOZ/MPT-93/2016/G10P[11]	93.93%	93.93%
HQ834206.1 (AEK20851)	Lamb rotavirus strain CC0812-1/China/2008/G10P[15]	91.91%	93.43%
GU384198.1 (ADC42132)	Bovine rotavirus A isolate DQ-75/China/2008/	93.43%	93.93%
FJ031023.1 (ACN18215)	Lamb rotavirus strain Lamb-NT/China/2007/G10P[15]	91.91%	93.43%
NSP6 (OQ215303)			
GenBank Accession Numbers*	Rotavirus A strains	AA Identity	AA Similarity
KX880446.1 (ATI14969)	RVA/Human-wt/DEU/GER29-14/2014/G6P[9]	93.87%	94.89%
FN665687.1 (CBJ23808)	RVA/Human-wt/HUN/BP1879/2003/G6P[14]	93.87%	94.89%
EU659854.1 (ACH72471)	RVA/Cow-wt/Italy/G6P[5]	92.85%	93.87%
EU659852.1 (ACH72468)	RVA/Human-wt/Italy/Pa5/89/G6P[14]	91.83%	92.85%
KP752470.1 (AKA40125)	RVA/Human-wt/CMR/MRC-DPRU3032/XXXX/G3P[6]	90.81%	91.83%
OL988981.1 (UZY23690)	RVA/Bovine/Northern Ireland/R1WTA16/2013/G6P[5]	91.83%	93.87%
KU708266.1 (AMR44591)	RVA/roe deer-wt/SLO/D38-14/2014/G6P[15]	90.81%	91.83%
MT501462.1 (QOY58099)	Rotavirus A/caprine/K-98/India/G8-P[1]	89.79%	92.85%
KJ752059.1 (AHZ32849)	RVA/Cow-wt/ZAF/MRC-DPRU3010/2009/G6P[5]	89.79%	90.81%
AY622998.1 (AAT40861)	Lamb rotavirus/China/G10 P[12]	89.79%	93.87%
MW771161.1 (UQQ66564)	RVA/Cow-wt/MOZ/MPT-93/2016/G10P[11]	88.77%	91.83%
HQ844026.1 (AEK94039)	Rotavirus A HC91xUK reassortant (UKg9KC-1)/USA/2006/G12PX	85.71%	87.75%

*The GenBank accession numbers have been provided in “nucleotide (protein)” format.

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Phylogenetic Analysis of Bovine Rotavirus strain

Five out of 11 viral genomic segments of the BRV Kirikkale strain were sequenced: VP1, VP2, VP6, NSP4, NSP5/6. The VP1 nt sequence was obtained partially and has high identity ranging between 97.31% and 98.53% with other Turkish bovine RVA strains. According to the phylogeny of the VP1 nt sequence, the bovine RVA Kirikkale strain was close to other bovine RVAs with nt identity up to 97.35%, human RVAs up to 95.83% nt identity, and RVA strains from other species such as simian, oyster, giraffe, rabbit, horse, antelope, and buffalo (Figure 5).

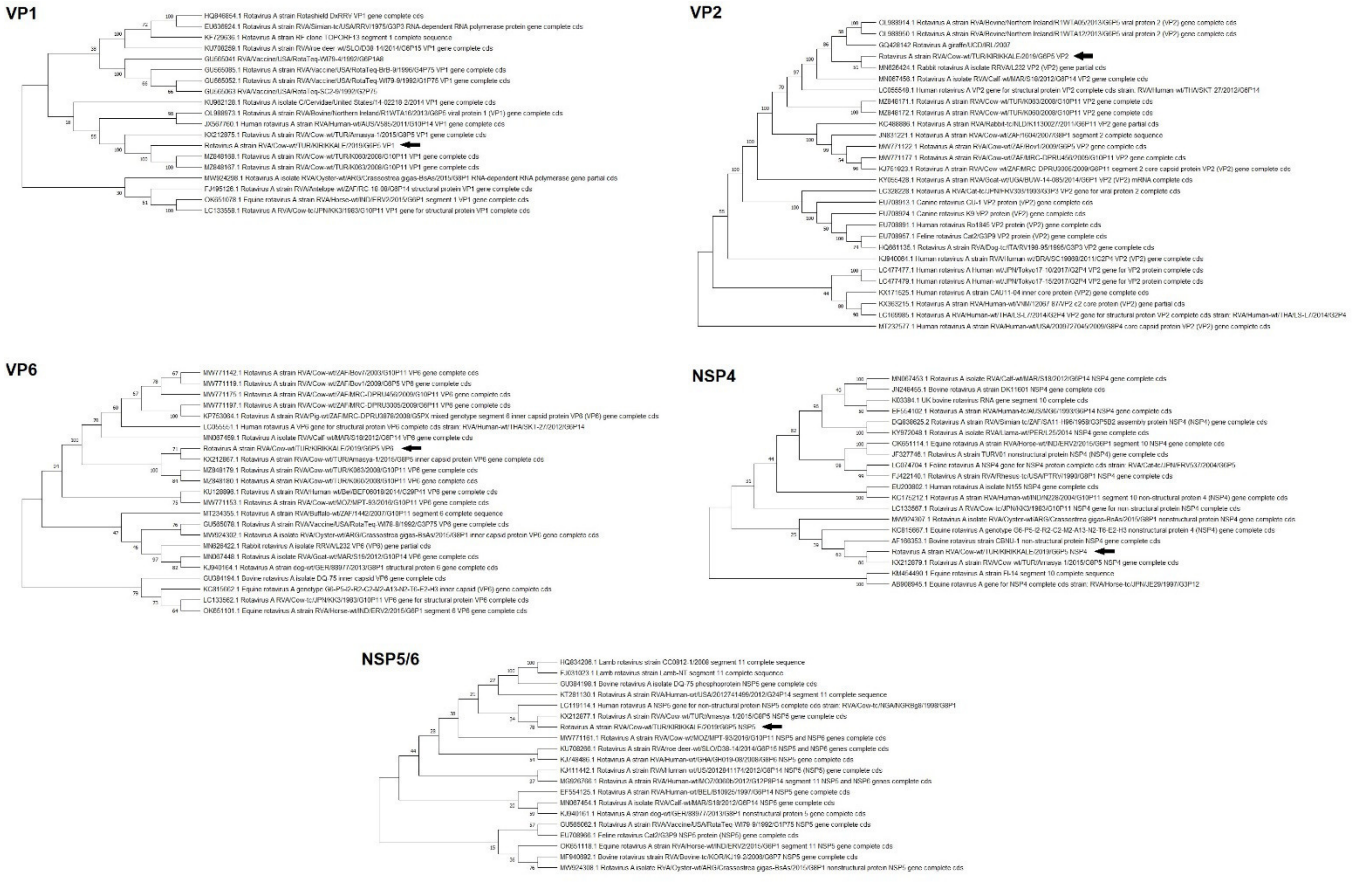

Figure 5 The phylogenetic tree of nucleotide sequences of the structural (VP1, VP2, VP6) and non-structural (NSP4, NSP5/6) genes of the bovine rotavirus Kirikkale strain and other selected rotaviruses from different species.

Interesting findings of the nt sequence of the VP2 gene demonstrated that the highest nt identity was with the giraffe RVA strain from Ireland (97.57%) and the rabbit rotavirus from France (97.35%). The other closer sequences were bovine and pudu RVA strains from Northern Ireland (between 97.08% and 97.35%), a buffalo RVA strain from South Africa (95.80%), human RVAs (up to 95.43%). The present blastn analysis revealed that numerous other RVAs from various animal species, including pig, dog, cat, and goat, exhibited a genetic relationship (Figure 5).

The VP6 gene sequence phylogeny revealed that the present BRV strain was highly close to other Turkish BRV strains and nt identities were between 98.8% and 99.03% amongst Turkish BRVs. Bovine RVAs from South Africa were the other closest strains showing up to 97.45% nt identity (Figure 5). Interestingly, the rabbit rotavirus from France exhibited high nt identity (94.88%) in the VP6 gene, as detected in the VP2 gene phylogeny.

For the NSP4 gene, bovine RVA strains from Türkiye (98.30%) and South Korea (up to 97.45%) were found to be showed the highest nt identity with the present Turkish BRV strain. Notably, a number of equine RVAs from Japan, USA, UK, and India revealed close genetic relationship in nt level (Figure 5). The NSP5/6 gene showed high nt identity up to 96.95% with previous Turkish bovine RVA strains, and according to the Blastn result, the NSP5/6 gene was very close to human (up to 95.99%), equine (95.67%), ovine (up to 95.67%), canine (95.35%) and feline (up to 95.17%) RVAs.

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Analysis of Deduced Amino Acid Sequences of Bovine Rotavirus strain

Three structural proteins of the bovine RVA strain Kirikkale, VP1, VP2, and VP6 proteins, were identified and analysed in order to determine aa sequence identity and similarity amongst other selected RVAs and aa substitutions to reveal mutations. The VP1 protein sequence of the present BRV strain was achieved as partial and consists of 955 aa. There were 37 aa substitutions amongst selected RVA strains (Table S6). Turkish strains' VP1 proteins have up to 98.42% aa identity and similarity. Our present VP1 sequence showed the highest identity and similarity with oyster RVA, RotaShield vaccine strain, and Japanese bovine RVA (Table VI). The VP2 protein (880 residues) showed 69 aa differences when compared to the other sequences (Table S6). An important result was that which is consistent with the nt identity result, the VP2 protein showed the highest aa identity (98.18%) and similarity (98.29%) with rabbit RVA and the lowest 95.45% identity and 97.27% similarity with human RVA (Table VI). When compared to other sequences, the VP6 protein which consists of 397 residues exhibited 11 aa mutations, but not with Turkish bovine RVA strains (Table S6). The VP6 protein showed 98.25-99.5% aa identity and 98.25-98.5 % similarity the selected RVA strains (Table VI).

Three non-structural proteins of the bovine RVA strain Kirikkale (NSP4, NSP5 and NSP6) were sequenced and analysed in aa level for determination of aa identity, similarity, and mutations. A comparison of the NSP4 protein (175 residues) with other sequences revealed the presence of 29 aa substitutions (Table S6). The aa identity and similarity of the NSP4 protein was determined to be 99.42%. The NSP4 nucleotide sequence analysis yielded findings that were compatible with the matching aa sequence, exhibiting strong identity and similarity with equine RVA strains (Figure 5 and Table VI). An analysis of the NSP5 protein (198 residues) demonstrated 22 substitutions in aa composition relative to other sequences (Table S6). The NSP5 aa sequence showed the highest aa identity and similarity with bovine RVA from Nigeria and human RVAs. Interestingly, the aa sequence of NSP5 differs significantly from that of previous Turkish bovine RVA, but closer to feline and equine RVA (Table VI). An analysis of the NSP6 protein, consists of 98 residues, revealed 28 aa substitutions when compared to other sequences. The highest aa identity and similarity of NSP6 were with human RVA strains (Table VI and Table S6).

Discussion

Bovine coronavirus (BCoV) and bovine rotavirus (BRV) are the leading causes of neonatal calf diarrhoea which results in significant economic losses, including high calf mortality from diarrhoea and/or bronchopneumonia, and increased susceptibility to secondary infections (Lorenz *et al.*, 2011; Vlasova and Saif, 2021). BCoV and BRV have attracted attention as zoonotic viral agents causing diarrhoea in newborns and animals, with interspecies transmission (Kim*et al.*, 2018; Tsugawa and Hoshino, 2008; Zhang *et al.*, 1994). The objective of this study was to perform a viral metagenomic analysis in the faeces of a calf with diarrhoea, with a focus on obtaining the sequences of BCoV and BRV.

Oxford Nanopore Technologies (ONT) have been used for obtaining complete or near-complete viral sequences (Choga *et al.*, 2023; Gauthier *et al.*, 2021; Yandle *et al.*, 2023). To our knowledge, for the first time, the near-complete genome of the BCoV was sequenced using the ONT MinION® and this is the first report of a near-complete genome of BCoV from Türkiye (Figure 1, Table I). Despite the presence of partial BCoV sequences from our country, complete or near-complete genome sequence of the Turkish BCoV has never been obtained to date. Therefore, this is the first report of near-complete genome of a Turkish BCoV strain. The near-complete sequence of the BCoV strain Kirikkale, obtained via ONT, is attributed to the presence of ambiguous nucleotides within the acquired sequence. The presence of ambiguous nucleotides in viral sequences has also been reported in other studies conducted using ONT technology (Choga *et al.*, 2023; Gauthier *et al.*, 2021; Yandle *et al.*, 2023).

A recent classification of BCoV strains, based on the full-length genomes of the virus, divides BCoVs into three major genogroups: GI, GII, and GIII (Bahoussi *et al.*, 2022). According to this recent classification, our phylogenetic analysis assigned the BCoV strain Kirikkale to the GII genogroup (Figure 2). Furthermore, our near-complete genome sequence shows a high degree of similarity to French respiratory BCoVs (MG757140.1 and MG757141.1) and enteric BCoV (KX982264), and Irish respiratory BCoVs (ON792957.1, ON792946.1 and ON792963.1) (Figure 2 and Table S1).

Recombination analysis of available complete and near-complete genomes of BCoV strains (n=145) were conducted in this study and our strain was only positive in chimaera method. The major parent was unknown and the minor parent was French enteric BCoV strain (KX982264) (Table S4). In our analysis recombination event was considered if it was positive more than two methods (statistical significance with $p<0.001$), so BCoV strain Kirikkale was not accepted as a recombinant.

The codon usage and G+C content of viruses may reveal significant insights on viral evolution (Butt *et al.*, 2014;

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Castells *et al.*, 2017). We analysed G+C content and codon usage in BCoV strain Kirikkale and closely related strains (French respiratory BCoV, Irish respiratory BCoV, French enteric BCoV, and Mebus strain). G+C contents ranged from 36.31% to 37.09% (Table V and Table S5). The most common codon across all strains was UUU (Phe). The two most frequent codons were identical (UUU, UGU) in Kirikkale and French respiratory/enteric BCoV strains, while the Irish respiratory BCoV and Mebus strain had GUU as the second most frequent codon (Table S5). Kirikkale showed a prevalence of U-ending codons, consistent with a previous study (Castells *et al.*, 2017).

In this study, the BCoV strain Kirikkale was found to have no aa substitution at the S1/S2 cleavage site (Table S2). The S1 subunit is mainly responsible for viral attachment and production of neutralizing antibodies, and has hypervariable region which affects the protein structure, function and attachment pattern. In a previous study by Hasoksuz et al. (2002) (Hasoksuz *et al.*, 2002), it was demonstrated that an aa change from K to N at position 115 resulted in reduced surface probability and hydrophilicity of the S protein. Our analysis revealed that this specific aa substitution (K115N) was identified in both BCoV strain Kirikkale and other BCoV strain sequences belonging to the GII group (Figure 2, Table S2). The results of this study are in agreement with previous report of Bahoussi et al., 2022.

The aa at 113, 409, 501, and 509 positions of the S protein of the Mebus strain are putative RBD (Zhu *et al.*, 2022), and the BCoV Kirikkale strain exhibited I113T, N499S, P501S, and N509T mutations which are also present some other selected coronaviruses. Of these mutations exhibited by the Kirikkale strain, only I113T was detected a respiratory BCoV from Ireland and human enteric coronaviruses, but not in other selected coronaviruses (Table S2). In selective pressure analysis, 113, 499, 501, and 509 codon sites were also found to be pervasive diversifying selection sites in FUBAR (Table IV and Figure 4), indicating agreement with previous studies (Franzo *et al.*, 2020; Zhu *et al.*, 2022).

An insertion at aa position 549 (549K) which is located at the RBD of the S1 subunit was determined in BCoV strain Kirikkale, and this aa insertion is also present in French respiratory BCoV strains, but not detected in the other coronaviruses (Table S2). Due to no protein structure analysis performed in this study, it is unknown whether these aa insertions affect the structure, physicochemical properties and function of the S protein. Future research should consist of more in-depth protein analyses to comprehend the alterations that affect the S protein of the BCoV strain Kirikkale.

Some recent studies from China and USA reported that insertion and/or deletion can occur in the RBD of the HE protein of the BCoV strains (Abi *et al.*, 2020; Workman *et al.*, 2022). However, in the HE protein of the Kirikkale strain, only aa substitutions were detected. Additionally, a report indicated that virulent BCoV strains exhibited proline substitutions in the HE at specific aa positions 5 and 367 (Gélinas AM *et al.*, 2001). These substitutions (L5P and S367P) (Table S2) are also present in the Kirikkale strain that was the causative viral agent of NCD and calf death in our study. Previous studies (Chouljenko *et al.*, 1998; Gélinas AM *et al.*, 2001) reported that G53V substitution in the E protein was present in virulent BCoV strains. The G53V substitution is also exhibited by the Kirikkale strain which was from a calf died due to diarrhoea (Table S2), indicating the consistence with aforementioned previous studies.

The ns4.8 protein has 45 residues in the Mebus and some other coronavirus strains, while the BCoV Kirikkale and French BCoV strains have 43 aa residues for the ns4.8 protein (Table S2). Still the reason of the absent aa residues remains unclear, one may speculate that the non-structural proteins could alter the pathogenicity or tropism of the virus, such as indicated in a previous study that the truncated ns4.9 protein was identified only for respiratory BCoV field isolates (Gélinas AM *et al.*, 2001).

Interspecies transmission is one of the most important characteristics of rotaviruses which is maintained via genetic reassortment and there have been many reports of transmission from bovine-to-other species including human (Ghosh *et al.*, 2013; Komoto *et al.*, 2016; O'Shea *et al.*, 2014; Schoondermark-van de Ven *et al.*, 2013; Tacharoenmuang *et al.*, 2018; Tsoleridis *et al.*, 2019). In the present study, the bovine RVA Kirikkale strain showed high nt and aa identity with previous Turkish bovine RVAs for the VP1, VP6, NSP4, and NSP5/6. However, an interesting result obtain from the VP2 analysis which exhibited the highest nt and aa identity with a giraffe RVA from Ireland and a rabbit RVA from France (Figure 5, Table VI). This result could be evidence for interspecies transmission which occurred international according to the previous studies. It was reported that the French rabbit RVA, having 97.35% nt identity with the Kirikkale strain, has 98.38% nt identity with the Irish giraffe RVA for the VP2 (Tsoleridis *et al.*, 2019). The Irish giraffe RVA which was interpreted as transmitted from a bovine host to the giraffe, was declared to be most closely related to the Slovenian human bovine-like RVA strain, exhibiting 97.1% nt identity for the VP2 (O'Shea *et al.*, 2014). In this study, the bovine RVA Kirikkale strain has 97.57% with the giraffe RVA, 97.35% with the rabbit RVA, and 96.02% with the Slovenian human bovine-like RVA in VP2 nt level. All these results indicated an exciting route of evolution and epidemiology of the rotaviruses, both for interspecies and international transmission.

Although it has an economical importance for cattle industry and zoonotic potential, the genetic and antigenic diversity of BCoV is poorly defined globally and the sequence of the BCoV near-complete genome has never been obtained in

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Türkiye to date. This study provided the first near-complete genome of a Turkish BCoV strain. This dataset has enabled a significant expansion of the previously constrained knowledge pertaining to the genome sequence of BCoV in Türkiye. Moreover, the primary near-complete sequence of the Turkish BCoV strain has been rendered accessible for global dissemination. The phylogenetic analyses at both nt and aa level revealed that the BCoV strain Kirikkale was very close to French and Irish BCoV strains and has insertion in the S protein that affects the host range and tropism. For detailed antigenic and genetic characterization of BCoVs in Türkiye, further investigations are necessary to analyse other Turkish BCoV strains with providing the full genome sequences. The phylogenetic analyses of the bovine RVA Kirikkale strain demonstrated that the VP2 segment could be a genetic ancestor for reassortant rabbit and giraffe RVA strains which are previously described to transmit between different species. This intriguing possibility may have an answer in the future by providing more complete genome sequences of rotaviruses in Türkiye.

Ethical statement

No ethical approval was required for this study.

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