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REGULAR ARTICLES

Molecular epidemiology of group A rotavirus (RVA) and bovine coronavirus (BCoV) associated with neonatal calf diarrhea in Türkiye

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Abstract

Neonatal calf diarrhea is mainly associated with group A rotavirus (RVA) and bovine coronavirus (BCoV), continuing to pose a major issue in livestock due to its high prevalence and economic impact. This study aimed to investigate the prevalence, molecular features, and genotype diversity of RVA and BCoV in fecal samples from 213 diarrheic calves under 28 days old, collected across various regions of Türkiye. Using antigen ELISA, RVA was detected in 32.3% ($n=69$), BCoV in 7.9% ($n=17$), and coinfection in 1.8% ($n=4$) of samples. Out of 69 samples that tested positive for RVA, 53 were confirmed using the RT-PCR method, targeting the VP6 gene. The predominant genotype combinations were G10P[11] and G6P[5], whereas G8P[5], G6P[11], and G6P[1] were detected at lower frequencies. The presence of the rarely observed G8P[5] and G6P[1] genotypes in Türkiye indicates the circulation of uncommon strains and underscores the necessity for continued genotyping surveillance. SDS-PAGE analysis showed a typical 4/2/3/2 electrophoretic pattern for Group A rotaviruses. For BCoV, nested RT-PCR targeting the N gene confirmed infection in 13 of 17 ELISA-positive samples. Phylogenetic analysis of three selected samples from three provinces demonstrated that the analyzed BCoV sequences clustered within the genus *Betacoronavirus* and showed genetic similarity to classical BCoV strains. The findings suggest genetically diverse RVA strains and more conserved BCoV strains are co-circulating in Türkiye. Despite vaccination and biosecurity measures, the ongoing presence of RVA and BCoV in neonatal diarrhea cases highlights the need to reassess control strategies and align vaccine formulations with the molecular profiles of circulating strains.

Keywords Diarrhea · Calf · ELISA · PCR · Genotyping · Phylogenetic analyses

Introduction

Neonatal calf diarrhea (NCD), predominantly driven by Group A rotavirus (RVA) and Bovine coronavirus (BCoV), inflicts severe financial burdens on dairy and beef producers due to high morbidity, mortality, treatment, and long-term growth retardation costs (Berge and Vertenten 2024; Huang et al. 2025). RVA chiefly affects calves in the first two weeks, while BCoV often strikes between 5 and 20 days of age, frequently co-infecting with pathogens like enterotoxigenic E.coli (ETEC) and Cryptosporidium (Brunauer et al. 2021). These viruses damage intestinal enterocytes, causing malabsorption, diarrhea, and reduced weight gain (Brunauer et al. 2021; Kostanić et al. 2024; Huang et al. 2025). Global prevalence studies report an expected NCD case fatality rate over 5%, with RVA–BCoV co-infections widespread, leading to significant operational losses. Additional risk factors include inadequate colostrum intake, poor hygiene, and environmental stressors, which predispose calves to infection (Brunauer et al. 2021; Berge and Vertenten 2024; Huang et al. 2025).

Rotavirus alphagastroenteritidis, formerly known as Rotavirus Group A (RVA), is a fecal-orally transmitted virus that was first identified in 1974 using electron microscopy and is classified within the genus *Rotavirus* of the family *Sedoreoviridae* (Vita et al. 2025). These viruses are non-enveloped and possess a double-stranded RNA genome with positive polarity, divided into 11 segments. The genome encodes a total of 12 proteins, comprising six structural proteins (VP1–VP4, VP6, and VP7) and six nonstructural

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proteins (NSP1–NSP6). The RVA virion is a triple-layered icosahedral particle, and the main antigenic components of the virion are VP4, VP6, and VP7 (Gomez and Weese 2017; Vita et al. 2025). In addition, one of the important non-structural proteins, NSP4, called viral enterotoxin, causes secretory diarrhea by disrupting calcium mobilization (Kostanić et al. 2024). Rotaviruses infecting different host species are classified into ten distinct species based on the analysis of the VP6 gene, which encodes an important structural protein. (Vita et al. 2025). While group A rotaviruses are the predominant cause of diarrhea in calves, groups B and C are also associated with natural infections in cattle (Rahman et al. 2005; Gomez and Weese 2017). Traditionally, rotavirus strains have been typed based on the outer capsid proteins VP7 (a glycoprotein, designated G) and VP4 (a protease-sensitive protein, designated P). So far, 42 G genotypes (G1–G42) and 58 P genotypes (P[1]–P[58]) have been identified (Park et al. 2022). Field studies have shown that the most common bovine rotavirus genotypes are G6 and G10, with G8 being less frequently detected; these are typically associated with P[5] and P[11], and less commonly with P[1] (Badaracco et al. 2013; Ates and Yesilbag 2023). Genetic reassortment and interspecies transmission, particularly involving human, bovine, and porcine strains, are possible, contributing to viral evolution and zoonotic potential (Redda et al. 2025).

Bovine coronavirus (BCoV) is a globally distributed, enveloped, positive-sense single-stranded RNA virus classified within the *Betacoronavirus* genus, subgenus *Embecovirus* (Liu et al. 2025; Zhao et al. 2025). BCoV infects both the gastrointestinal and respiratory systems of cattle, leading to diseases such as neonatal calf diarrhea, winter dysentery, and bovine respiratory disease complex (BRDC) (Shah et al. 2025; Zhao et al. 2025). An approximately 31 kb genome is organized into several open reading frames (ORFs), including ORF1a/1b encoding non-structural replicase proteins and a 3′ region encoding the five structural proteins: spike (S), hemagglutinin-esterase (HE), membrane (M), envelope (E), and nucleocapsid (N). BCoV continues to evolve through point mutations and recombination events due to its error-prone RNA polymerase. Genetic characterization of circulating strains has revealed substantial diversity, particularly in the S, HE, and N genes, which may influence virulence, immune evasion, and diagnostic detectability (Shah et al. 2025; Zhao et al. 2025). The spike (S) protein plays a key role in viral entry through receptor binding (S1 subunit) and membrane fusion (S2 subunit), and is a major determinant of host specificity and pathogenesis (Shah et al. 2025). The HE protein facilitates viral attachment and release, and recent studies have identified insertion/deletion mutations within its receptor-binding domain (RBD) that may influence viral binding affinity and tissue tropism, potentially enhancing zoonotic potential (Zhao et al. 2025). The nucleocapsid protein, encoded by the N gene, is a highly conserved, phosphorylated structural protein that binds to the viral RNA genome, ensuring its packaging and stability. It contains two major functional domains: the N-terminal domain (NTD) for RNA binding and the C-terminal domain (CTD) for dimerization and genome packaging (Shah et al. 2025). Despite the availability of vaccines, BCoV infections remain widespread, necessitating ongoing molecular surveillance and refinement of control strategies. Notably, BCoV has demonstrated zoonotic potential, as evidenced by its close evolutionary relationship with human coronavirus OC43, thought to have emerged from a BCoV spillover (Zhang et al. 1994; Saif and Jung 2020).

Current findings regarding neonatal calf diarrhea indicate that reducing the biological and economic losses at the herd level requires the integrated implementation of pathogen control strategies, including enhanced colostrum management, targeted nutritional programs, and vaccination practices aligned with biosecurity principles. Given the global significance of neonatal diarrhea, identifying the genotypes responsible for herd-level infections is critical for effective disease control, appropriate vaccine selection, and comprehensive herd health management. Notably, cases of rotavirus- and coronavirus-induced diarrhea have been reported even in calves that received adequate colostrum or were born to vaccinated dams, highlighting the persistence and complexity of these infections. Therefore, this study aimed to assess the current status of RVA and BCoV associated with calf diarrhea in Türkiye, and to characterize the genetic diversity of these viruses through molecular typing techniques.

Material and methods

Samples

Between 2018 and 2023, a total of 213 fecal samples were collected from diarrheic calves (breed information is unavailable). The calves were all under 28 days of age. These samples originated from both intensive and small-scale dairy farms located in various provinces of Türkiye (Table 1). Sample collection was carried out using sterile stool containers, and all specimens were transported to the laboratory under cold chain conditions to preserve sample integrity. Prior to sample collection, informed consent was obtained from farm owners in each respective province for inclusion in the study. Upon receipt, fecal samples were diluted at a ratio of 1:10 in phosphate-buffered saline (PBS) supplemented with 100 IU/mL penicillin, 100 µg/mL streptomycin, and 250 µL/mL amphotericin B. The samples were

Table 1 Details for the samples and sampling provinces

Provinces of samples	Number of samples	Sample type	Age range of animals	Farm type
Adıyaman	47	Feces	<28 old days	Intensive
Bursa	42	Feces	<28 old days	Intensive and small
Çanakkale	38	Feces	<28 old days	Intensive
Konya	33	Feces	<28 old days	Intensive
Balıkesir	20	Feces	<28 old days	Intensive
Edirne	16	Feces	<28 old days	Intensive
Kırklareli	8	Feces	<28 old days	Intensive
Bilecik	7	Feces	<28 old days	Small
Ankara	2	Feces	<28 old days	*
Total	213			

* Data not available

then vortexed and centrifuged at 3000 rpm for 10 min at +4 °C. Following centrifugation, the supernatants were carefully separated and stored at −80 °C until further virological analysis.

Detection of viral antigen with ELISA

For the detection of RVA and BCoV viral antigens, all the collected stool samples ($n=213$) were tested with a commercially available RVA antigen ELISA kit (BioX Diagnostic BIO K 343/2) and BCoV antigen ELISA kit (BioX Diagnostic BIO K 392/2). The test protocol was applied as recommended by the manufacturer. The test plate was scanned in ELISA automatic microplate reader (Thermo-Multiskan EX, Finland) at 450 nm wavelength, and the results were evaluated using suggested calculations.

Nucleic acid extraction and cDNA synthesis

Viral RNA was extracted using a commercial kit (NucleoSpin Virus, Macherey-Nagel, Germany) from 82 stool samples ($n=82$) detected as antigen-positive for RVA and BCoV by ELISA. For RT-PCR, the cDNA was synthesized using the iScript cDNA synthesis kit (Bio-Rad, 170–8891). The kit protocols were applied as recommended by the manufacturer.

RVA RT-PCR, G and P genotyping

Stool samples that tested positive for RVA antigen by ELISA were further analyzed by reverse transcription PCR (RT-PCR) to detect the VP6 gene region of rotavirus A. For genotyping, gene-specific primers were used to identify G6, G8, and G10 genotypes targeting the VP7 gene and P[1], P[5], and P[11] genotypes targeting the VP4 gene (Table 2). The PCR protocols were conducted based on methodologies previously described in the literature (Table 3). Amplified PCR products were loaded onto a 2% agarose gel prepared with 1× TAE buffer and subjected to electrophoresis for 30 min.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis (SDS-PAGE)

To determine the rotavirus group based on the migration pattern of its 11 genomic segments, sodium dodecyl sulfate–polyacrylamide gel electrophoresis (SDS-PAGE) was conducted. RNA extraction was performed from RVA antigen-positive stool samples (Burgu et al. 1995). The extracted RNA was loaded onto a commercially prepared gradient gel

Table 2 The oligonucleotide primer pairs and target regions of virus species for RT-PCR and nested-PCR

Virus Species	Target Region	Primers	Primers' sequence (5'-3')	Location (Nucleotide position)	Size of product (bp)	Reference
RVA	VP6	VP6-F	GACGGVGCRACTACATGGT	747–766	379	(Iturriza Gómara et al. 2002)
		VP6-R	GTCCAATTCATNCCTGGTGG	1126−1106		
	VP7	Beg9-F	GGCTTTAAAAGAGAGAATTTCCGTCTGG	1–28	500 274	(Falcone et al. 1999)
		G6-R	CTAGTTCCTGTGTAGAATC	499−481	715	
		G8-R	CGGTTCCGGATTAGACAC	274−256		
		G10-R	TTCAGCCGTTGCGACTTC	715−697		
	VP4	Pgen-F	TTCATTATTGGGACGATTCACA	1064–1085	463	
		P[1]-R	TTAAATTCATCTCTTAGTTCTC	1526−1505	662	
		P[5]-R	GGCCGCATCGGATAAAGAGTCC	1725−1704	335	
		P[11]-R	TGCCTCATAATATTGTTGGTCT	1398−1377		
BCoV	N	F1	GCAATCCAGTAGTAGAGCGT	21–40	730	(Cho et al. 2001)
		R1	CTTAGTGGCATCCTTGCCAA	731–750		
		F2	GCCGATCAGTCCGACCAATG	79–98	407	
		R2	AGAATGTCAGCCGGGGTAG	467–485		

Table 3 Applied RT-PCR and Nested-PCR protocols

Virus Species			PCR Master mix (25 µL)	Thermal-cycle protocol	Reference
RVA			9.5 µl of nuclease-free water, 12.5 µl Maxima Hot Start Dream Taq (2X) PCR Master Mix, 1 µl each of VP6 forward and VP6 reverse primers (10pmol) and 1 µL cDNA	• Pre-denaturation; 4 min at 95 °C • 35 cycles; 1 min at 95 °C, 1 min at 58 °C, 2 min at 72 °C • Final extension; 10 min at 72 °C	(Iturriza Gómara et al. 2002)
RVA	**G Genotyping**	**G6**	9.5 µl of nuclease-free water, 12.5 µl Maxima Hot Start Dream Taq (2X) PCR Master Mix, 1 µl each of Beg9 forward and G6 reverse primers (50 pmol) and 1 µL cDNA	• Pre-denaturation; 5 min at 95 °C • 40 cycles; 1 min at 94 °C, 1 min at 43,5 °C, 2 min at 72 °C • Final extension; 7 min at 72 °C	(Falcone et al. 1999; Ates and Yesilbag 2023)
		G8	9.5 µl of nuclease-free water, 12.5 µl Maxima Hot Start Dream Taq (2X) PCR Master Mix, 1 µl each of Beg9 forward and G8 reverse primers (50 pmol) and 1 µL cDNA	• Pre-denaturation; 5 min at 95 °C • 40 cycles; 1 min at 94 °C, 1 min at 54 °C, 2 min at 72 °C • Final extension; 10 min at 72 °C	
		G10	9.5 µl of nuclease-free water, 12.5 µl Maxima Hot Start Dream Taq (2X) PCR Master Mix, 1 µl each of Beg9 forward and G10 reverse primers (10 pmol) and 1 µL cDNA	• Pre-denaturation; 5 min at 95 °C • 40 cycles; 1 min at 94 °C, 1 min at 55 °C, 2 min at 72 °C • Final extension; 7 min at 72 °C	
RVA	**P Genotyping**	**P[1]**	6,5 µl of nuclease-free water, 12.5 µl Maxima Hot Start Dream Taq (2X) PCR Master Mix, 2,5 µl each of Pgen-F forward and P[1]-R reverse primers (10 pmol) and 1 µL cDNA	• Pre-denaturation; 5 min at 95 °C • 40 cycles; 1 min at 94 °C, 1 min at 45,7 °C, 2 min at 72 °C • Final extension; 7 min at 72 °C	
		P[5]	9,5 µl of nuclease-free water, 12.5 µl Maxima Hot Start Dream Taq (2X) PCR Master Mix, 1 µl each of Pgen-F forward and P[5]-R reverse primers (50 pmol) and 1 µL cDNA	• Pre-denaturation; 5 min at 95 °C • 40 cycles; 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C • Final extension; 7 min at 72 °C	
		P[11]	9,5 µl of nuclease-free water, 12.5 µl Maxima Hot Start Dream Taq (2X) PCR Master Mix, 1 µl each of Pgen-F forward and P[11]-R reverse primers (10 pmol) and 1 µL cDNA	• Pre-denaturation; 5 min at 95 °C • 40 cycles; 1 min at 94 °C, 1 min at 50 °C, 2 min at 72 °C • Final extension; 7 min at 72 °C	
BCoV			**1st Round**: 37,7 µL nuclease free water, 2 µL MgCl2 (25 mM), 5 µL 10x PCR buffer, 1 µL F1 forward primer (25 pmol), 1 µL R1 reverse primer (25 pmol), 1 µL dNTPs (10 mM), 0,3 µL Taq DNA polimerase (500 U/µL) and 2 µL cDNA	• Pre-denaturation; 5 min at 94 °C • 35 cycles; 1 min at 94 °C, 1 min at 58 °C, 2 min at 72 °C • Final extension; 7 min at 72 °C	(Cho et al. 2001)
			2nd Round: 34,7 µL nuclease free water, 2 µL MgCl2 (25 mM), 5 µL 10x PCR buffer, 1 µL F2 forward primer (25 pmol), 1 µL R2 reverse primer (25 pmol), 1 µL dNTPs (10 mM), 0,3 µL Taq DNA polimerase (500 U/µL) and 5 µL DNA of 1st round	• Pre-denaturation; 5 min at 94 °C • 30 cycles; 1 min at 94 °C, 1 min at 58 °C, 2 min at 72 °C • Final extension; 7 min at 72 °C	

system containing 5–14% polyacrylamide (Bio-Rad, Cat. No.: 4561083). Electrophoresis was carried out for 6 h at 30 mA and 100 V. Following electrophoresis, silver nitrate staining was applied according to standard protocols. The banding patterns were subsequently evaluated by macroscopic examination.

BCoV nested PCR and sequencing

Stool samples that tested positive for BCoV antigen using commercial ELISA kits were further analyzed for the presence of BCoV-specific nucleic acid by nested PCR (Table 2). A nested PCR protocol targeting the N gene region of BCoV was applied based on previously published methods (Table 3). Following the second round of amplification, PCR products were visualised as described above. Among the BCoV nested PCR positive samples, three samples obtained from different provinces and farms were selected for Sanger sequencing (BM Lab, Türkiye). The obtained nucleotide sequences were aligned using the ClustalW multiple alignment tool implemented in BioEdit software. Phylogenetic relationships were inferred using the maximum likelihood method with 1000 bootstrap replicates in MEGA11. Reference sequences for phylogenetic tree construction were retrieved from the GenBank database.

Result

Antigen ELISA results

All 213 stool samples were analyzed using commercially available ELISA kits for the detection of both RVA and BCoV antigens. When evaluated individually, RVA was detected in 69 samples (32.39%) and BCoV in 17 samples (7.98%). At least one of the two viral agents (RVA and/or

BCoV) was identified in 82 samples (38.5%). Specifically, RVA alone was detected in 65 samples (30.52%), BCoV alone in 13 samples (6.10%), and co-infection with both RVA and BCoV was identified in 4 samples (1.88%).

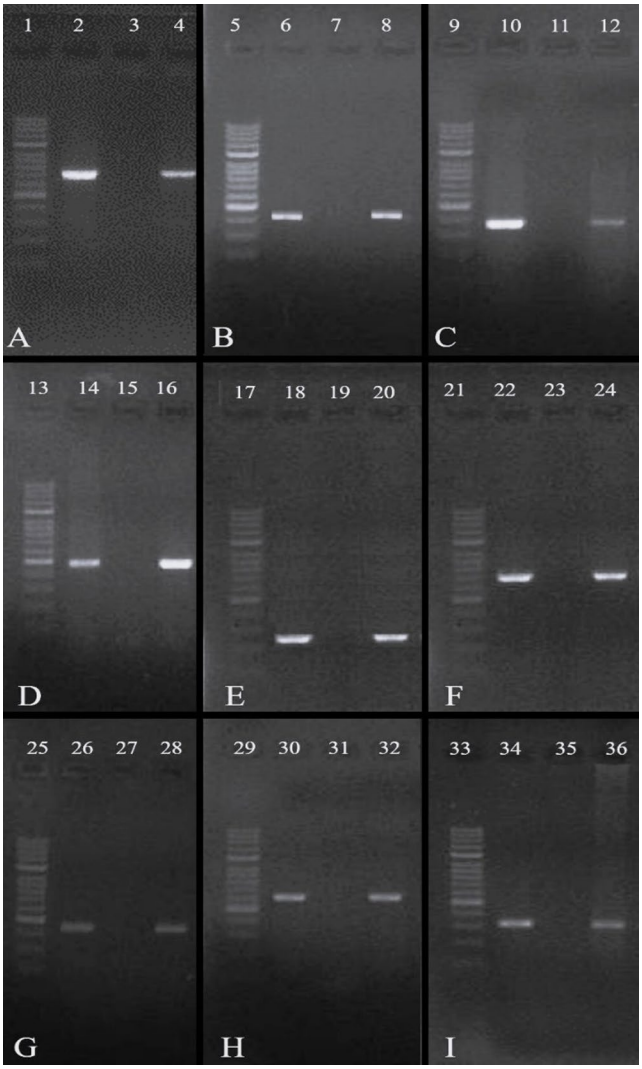

Fig. 1 Amplification profiles of RT-PCR and nested PCR products from reference viruses and field strains of rotavirus and coronavirus in fecal samples. **A**: BCoV Nested PCR – First Round (amplicon size: 730 bp), **B**: BCoV Nested PCR – Second Round (amplicon size: 407 bp), **C**: RVA RT PCR (amplicon size: 379 bp), **D**: RVA G6 RT-PCR (amplicon size: 500 bp), **E**: RVA G8 RT-PCR (amplicon size: 274 bp), **F**: RVA G10 RT-PCR (amplicon size: 715 bp), **G**: RVA P[1] RT-PCR (amplicon size: 463 bp), **H**: RVA P[5] RT-PCR (amplicon size: 662 bp), **I**: RVA P[11] RT-PCR (amplicon size: 335 bp); (**Lines 1**,**5**,**9**,**13**,**17**,**21**,**25**,**29**,**33**: DNA Ladder 100 bp (GeneAll, GA-010); **Lines 2**,**6**: Positive control (BCoV mebus strain); **Lines 10**,**14**,**26**: Positive control (RVA G6P[1] strain); **Line 18**: Positive control (RVA G8 strain), **Lines 22**,**34**: Positive control (RVA G10P[11] strain); **Line 30**: Positive control (RVA P[5] strain); **Lines 3**,**7**,**11**,**15**,**19**,**23**,**27**,**31**,**35**: Negative control; **Lines 4**,**8**: BCoV field strains, **Lines 12**,**16**,**20**,**24**,**28**,**32**,**36**: RVA field strains)

RVA RT-PCR, G and P genotyping results

The amplification profiles of RT-PCR products obtained from fecal samples of RVA reference strains and selected field strains are shown in Fig. 1. Out of the 69 stool samples that tested positive for RVA antigen using the ELISA kit, 53 were confirmed as RVA-positive by RT-PCR. Genotyping analysis revealed that the G6 genotype was identified in 32 samples, G8 in 8 samples, and G10 in 28 samples (The sum exceeds the number of PCR-positive samples (53) due to the detection of mixed G genotypes in some samples ($n=5$) and because some samples could not be genotyped for G types ($n=11$)). However, in 11 of the 69 ELISA-positive samples, none of the G6, G8, or G10 genotypes could be detected by RT-PCR. Regarding P genotypes, P[1] was identified in 1 sample, P[5] in 23 samples, and P[11] in 25 samples. Notably, P[1], P[5], and P[11] genotypes could not be determined in 20 of the RVA-positive samples by RT-PCR. Furthermore, P genotypes could not be identified in 20 of the samples where a G genotype was successfully determined, while G genotypes could not be identified in 2 of the samples where P genotypes were detected, despite using species-specific primers in RT-PCR assays. Based on the genotyping results, a total of 45 samples were successfully genotyped. The following G/P genotype combinations were identified: G10P[11] in 18 samples, G6P[5] in 16 samples, G10P[5] in 3 samples, G6P[1] in 1 sample, G6P[11] in 1 sample, and G8P[5] in 1 sample. In addition, mixed G genotypes were detected in five samples: G8 + G10P[11] in two samples, G6 + G10P[11] in one sample, and G6 + G8P[5] in two samples.

SDS-PAGE results

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis was performed on 69 stool samples that had previously tested positive for RVA antigen by ELISA, as a supplementary molecular diagnostic approach to detect RVA viral nucleic acid. The characteristic 11-segmented RNA genome of RVA was identified in all tested samples. Based on the SDS-PAGE results, the electrophoretic migration patterns of the RNA segments on polyacrylamide gel exhibited a typical 4/2/3/2 distribution, consistent with the banding profile of Group A bovine rotaviruses (Fig. 2).

BCoV Nested PCR results and phylogenetic analyses results

The amplification profiles of nested-PCR products obtained from fecal samples of BCoV reference strains and selected field strains are shown in Fig. 1. Of the 17 stool samples that tested positive for BCoV antigen by ELISA, 13 were

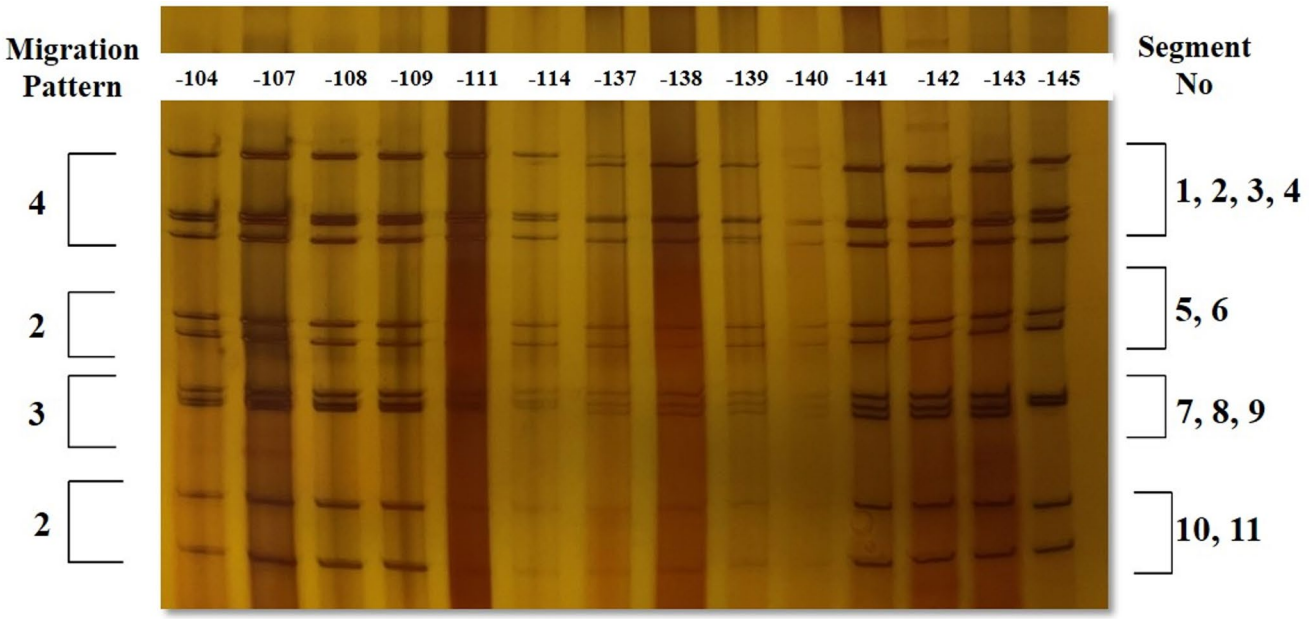

Fig. 2 SDS-PAGE analysis showing the 11-segment band profiles of some RVA-positive samples on a polyacrylamide gel (Sample IDs are indicated on the figure)

confirmed to be positive for BCoV by nested reverse transcription PCR (nested RT-PCR). Following the first round of PCR, 9 samples yielded positive results, whereas all 13 were confirmed positive after the second round of amplification. Among these 13 samples in which BCoV-specific nucleic acid was detected using a primer pair targeting the N gene region, three samples, RV-51 (BCoV/TR-Adiyaman/Bovine/RV-51/2020; GenBank accession no.: OQ927576), RV-195 (BCoV/TR-Bursa/Bovine/RV-195/2021; GenBank accession no.: OQ927577), and RV-205 (BCoV/TR-Edirne/Bovine/RV-205/2021; GenBank accession no.: OQ927578) originating from different provinces, were selected for nucleotide sequencing. Phylogenetic analysis revealed that the BCoV strains identified in these three samples clustered within the Betacoronavirus genus (Fig. 3).

Discussion

This study aimed to investigate the prevalence and molecular characterization of RVA and BCoV infections associated with diarrhea in newborn calves. Despite herd-level vaccination and biological safety measures, RVA and BCoV remain the major viral agents of neonatal diarrhea (Berge and Vertenten 2024; Huang et al. 2025). Early and accurate diagnosis is crucial due to the high morbidity and mortality associated with these pathogens. Antigenic differences between vaccine strains and circulating genotypes can negatively impact the effectiveness of immunoprophylaxis by reducing maternal antibody response (Gomez and Weese 2017). Therefore, updating vaccination strategies based on circulating strains and appropriate colostrum management plays a critical role in controlling neonatal viral diarrhea.

Prevalence rates of RVA and BCoV, as determined by ELISA, have shown considerable variation worldwide. Reported RVA prevalence varies between 16 and 57%, BCoV prevalence between 1.4 and 38.9%, and co-infection rates between 0.7 and 6.4% worldwide (Abraham et al. 1992; Al-Yousif et al. 2001; Lanz Uhde et al. 2008; Barua et al. 2021). Studies conducted in Turkey have also reported similar variability in RVA (8.92–71.1%), BCoV (1.0–37.1%), and co-infection (3.6–13.4%) rates (Alkan 1998; Erdoğan et al. 2003; Hasoksuz et al. 2005; Ok et al. 2009; Yılmaz 2016). These variations may be attributed to differences in sampling strategies, farm management practices, hygiene conditions, calf immunity status, and seasonal factors. The prevalences of RVA (32.3%), BCoV (7.9%), and co-infection (1.8%) found in the current study are consistent with the literature and support the active circulation of these pathogens in Turkey and the need for effective biosecurity measures at the herd level.

This study evaluated the diagnostic concordance between antigen ELISA and PCR methods used to detect RVA and BCoV, important causative agents of neonatal calf diarrhea. In PCR analyses, the VP6 gene region was targeted for RVA and the N gene region for BCoV (Cho et al. 2001; Iturriza Gómara et al. 2002). ELISA is reported to offer a practical screening method in acute infections, while RT-PCR provides higher sensitivity in early or low viral load infections (Kostanić et al. 2024). In this study, only 53 of the 69

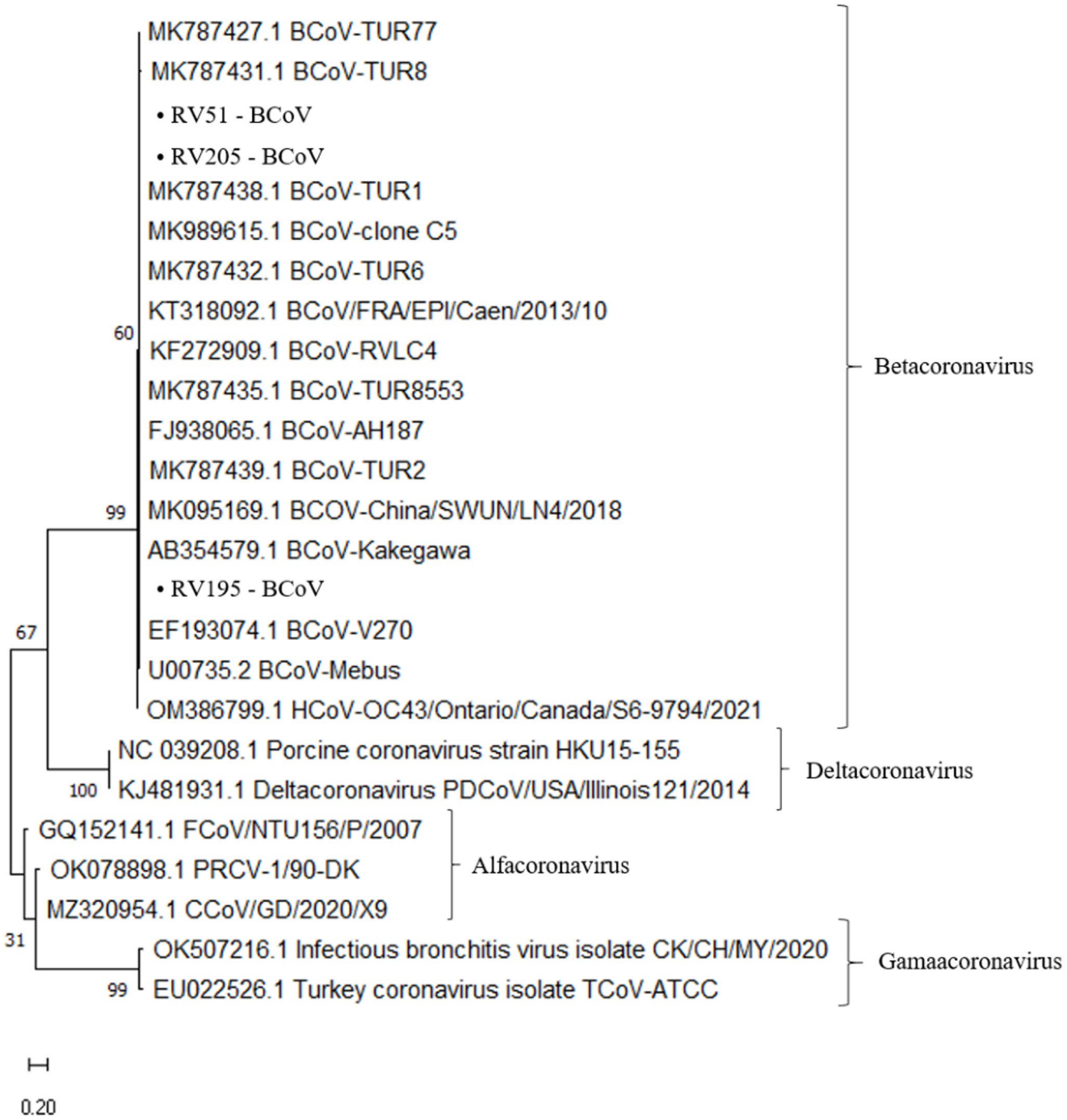

Fig. 3 Phylogenetic tree based on the N gene sequences of BCoV-positive samples coded RV-51 (BCoV/TR-Adiyaman/Bovine/RV-51/2020; GenBank accession no.: OQ927576), RV-195 (BCoV/TR-Bursa/Bovine/RV-195/2021; GenBank accession no.: OQ927577), and RV-205 (BCoV/TR-Edirne/Bovine/RV-205/2021; GenBank accession no.: OQ927578). Sequences marked with a dot (•) were obtained in this study. Reference sequences were retrieved from the GenBank database, and their accession numbers are indicated in the tree

samples that tested positive for RVA by ELISA and only 13 of the 17 samples that tested positive for BCoV were confirmed by PCR. These differences are thought to be related to the stage of infection and sampling time. PCR inhibitors and technical variables should also be considered (Schrader et al. 2012). Previous studies have reported differences in sensitivity between antigen-based and molecular methods, particularly showing that nested RT-PCR provides higher accuracy than ELISA in BCoV detection (Burgu et al. 1995; Cho et al. 2001; Izzo et al. 2012; Mohamed A. Soltan et al. 2016; Barua et al. 2021). However, studies directly comparing the diagnostic concordance of these two methods are

limited, and the presented data contribute to the literature. It is considered that the complementary use of antigen and molecular methods will provide a more reliable diagnosis in field conditions.

Globally, the most common VP7 genotypes in bovine RVA strains have been reported as G6 and G10, while the dominant VP4 genotypes are P[5] and P[11]; the circulation of other genotypes (G1–G3, G5, G8, G11, G15, G17, G21, G24, P[1], P[3], P[6], P[7], P[14], P[17], P[21], P[29] and P[38]) is more limited (Timurkan and Alkan 2012; Papp et al. 2013; Karayel et al. 2017; Karayel-Hacioglu et al. 2022; Ates and Yesilbag 2023). In the present study, consistent with the literature, the genotypes G6, G10, P[5], and P[11] were detected. However, the molecular detection of G8 and P[1] genotypes in field samples is noteworthy as it reveals the circulation, albeit at a low level, of these genotypes, which are rarely reported in Turkey (Timurkan and Alkan 2012; Karayel et al. 2017). These findings highlight that RVA genotype diversity may change over time through reassortment and emphasize the need for continuous epidemiological surveillance. In addition, animal movement between regions and differences in herd management may contribute to the low but persistent circulation of uncommon RVA genotypes, although these factors were not specifically evaluated in the present study. In Türkiye, the spatial proximity of large-scale industrial dairy enterprises and small-scale traditional livestock farming in certain regions reflects a heterogeneous production structure that may facilitate transmission between herds.

Among the RVA genotype combinations reported in cattle worldwide, G6P[5], G6P[11], G10P[5], G10P[11] and G8P[1] stand out. Previous studies in Turkey have also reported that G6P[5], G6P[11], and G10P[11] are the dominant combinations, while rare genotypes such as G6P[1], G8P[5], and G15P[21] have been reported to a more limited extent (Alkan et al. 2010; Timurkan and Alkan 2012; Karayel et al. 2017; Aksoy et al. 2021; Karayel-Hacioglu et al. 2022; Ghonaim et al. 2025a, b).The genotypes identified in the present study are consistent with these findings and show that the endemic RVA strain profile in Turkey is largely preserved. However, the first-ever detection of mixed genotypes such as G8+G10P[11] and G6+G8P[5] in field samples constitutes a noteworthy finding of the study. Furthermore, the repeated detection of rare genotypes such as G6P[1] and G8P[5] provides important epidemiological findings regarding the RVA genotype diversity in the country. The use of alternative RT-PCR primers or whole genome sequencing (WGS) in samples where the genotype could not be determined may contribute to the identification of new or recombinant strains and strengthen surveillance data.

In order to prevent neonatal calf diarrhea, inactivated combination vaccines administered during the last three months of gestation generally contain the classic genotypes of RVA (G6P[1], G10P[11], G6P[5]), BCoV (Mebus strain), and *Escherichia coli* K99. Studies have reported that cross-protection between heterologous G and P genotypes may be limited and that a significant decrease in immune efficacy may be observed, especially in P genotype incompatibility (Zhichang Xu et al., 1993; Lu et al. 1994; Hasan et al. 2023; Ghonaim et al. 2025a, b). In previous field studies, the detection of different genotypes in vaccinated and unvaccinated calves than those included in the vaccine formulation indicated that antigenic incompatibility between vaccine components and circulating strains may limit protection (Rocha et al. 2017). While most of the genotypes identified in this study were compatible with existing vaccine components, the identification of combinations such as G8P[5] and G10P[5], which are not fully represented in the formulations, shows that field strain diversity can extend beyond the vaccine content. This highlights the need for large-scale studies to detect cross-immunity between genotypes. The detection of the G6P[1] genotype, which is present in the vaccine content, in a calf with diarrhea in this study supports the idea that genotype compatibility alone may not guarantee clinical protection and the need to re-evaluate protective efficacy. In addition, host-related factors such as passive transfer status, colostrum quality, maternal antibody levels, and potential antigenic variability within the same genotype may also influence vaccine effectiveness and clinical outcome. Continuous monitoring of circulating strains and considering the obtained genotype data together with the vaccine content is important for explaining the diarrhea cases in the field. In particular, the inclusion of RVA genotypes such as G8 in future vaccine formulations or the continuous optimization of existing vaccines to increase cross-protection against such genotypes should be evaluated in more detailed studies with scientific evidence.

SDS-PAGE is considered one of the gold standard methods for the detection of RVA from field samples and culture fluids. In this method, rotaviruses are classified into Groups A, B, and C based on the electrophoretic migration patterns of their genome segments in polyacrylamide gel. Specifically, Group A rotaviruses exhibit a migration pattern of 4/2/3/2, Group B shows 4/2/2/3, and Group C displays 4/3/2/2 (Rahman et al. 2005). Numerous studies have successfully utilized the SDS-PAGE technique for group determination of rotaviruses (Özkul et al. 2002; Ates and Yesilbag 2023). In this study, samples that tested positive for RVA Ag ELISA ($n=69$) were analyzed by SDS-PAGE, and the characteristic 4/2/3/2 genome migration pattern specific to group A rotaviruses was detected in all tested samples (Fig. 2). Minor morphological differences observed in the sequencing of

genome segments did not affect the overall migration profile or genotype identification. Notably, positive SDS-PAGE profiles were obtained in some samples despite negative RT-PCR results; this highlights the complementary diagnostic value of SDS-PAGE in detecting genomic RNA, especially in conditions where PCR sensitivity may be limited.

In molecular studies targeting coronaviruses, the N gene region is a commonly used genetic target due to its relatively conserved structure (Cho et al. 2001; Hasoksuz et al. 2005). In this study, partial N gene sequence analysis performed on BCoV-positive samples selected from different provinces (Adıyaman, Bursa, and Edirne) revealed that all samples belonged to the Betacoronavirus genus and that no atypical variants were found (Fig. 3). This suggests that BCoV strains circulating in different regions of Turkey exhibit a largely homogeneous phylogenetic structure. Similarly, high nucleotide similarity and limited phylogenetic divergence have been reported in N gene-based analyses of BCoV isolates obtained from different countries and different sample types (Frucchi et al. 2022; Furlan et al. 2025). However, considering the limited variation capacity of the N gene, it is accepted that phylogenetic analyses based on this gene region are meaningful in terms of basic classification and evaluation of genetic stability rather than revealing fine-scale genetic differences between strains. Therefore, the findings of our study show that the BCoV strains circulating in Turkey cluster within classical enteric (Mebus-like) BCoV lineages at the N gene level, rather than with strains associated with respiratory disease or winter dysentery. This phylogenetic positioning supports their enteric origin and reinforces their etiological relevance in neonatal calf diarrhea. However, they indicate that more detailed phylogenetic distinctions require the analysis of gene regions with higher variability.

This study clearly demonstrates that RVA and BCoV continue to be important etiological agents in neonatal calf diarrhea and that current preventive measures may remain insufficient to suppress these viral threats in the field. Genotyping of RVA revealed G10P[11] and G6P[5] as the most common G/P combinations, while less frequent genotypes such as G8P[5], G6P[11], and G6P[1] were also detected. The presence of the rarely reported G8P[5] and G6P[1] genotypes in Türkiye indicates that viral evolution and genotype diversity are more dynamic and regionally variable than previously anticipated. These findings underscore the need for flexible, data-driven vaccination strategies in response to RVA's genetic variability, and for a sustained, long-term monitoring approach for BCoV control. From a practical perspective, veterinarians should consider genotype diversity when designing vaccination programs and emphasize integrated control measures, including appropriate immunization schedules, effective colostrum management, and strict biosecurity practices to reduce the burden of neonatal calf diarrhea.

Author contributions **OA**: Writing – original draft, Methodology, Investigation, Data curation. **KY**: Writing – review & editing, Supervision, Methodology, Funding acquisition, Conceptualization.

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Data availability All data supporting the findings of this study are included in the article. Additional information can be obtained from the corresponding author on request.

Declarations

Ethical approval This study was approved by the Bursa Uludag University Experimental Animals Local Ethics Committee in accordance with ethical guidelines for the Care and Use of Laboratory Animals (approval number: HADYEK; 2020–05/14).

Competing interests The authors declare that they have no known competing financial interests or personal relationships that could have appeared to influence the work reported in this paper.

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