

Molecular Epidemiology and Risk Factors Analysis of Major Pathogens Causing Neonatal Calf Diarrhea in Muğla, Türkiye

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Abstract

Neonatal calf diarrhea is a widespread and multifactorial condition that poses a significant challenge in cattle farming. The major etiological pathogens include *Cryptosporidium parvum*, *Escherichia coli*, bovine group A rotavirus (RVA), and bovine coronavirus (BCoV). Accurate identification of these pathogens is essential for the development of targeted prevention and control strategies. This study aimed to obtain comprehensive molecular epidemiological data on neonatal calf diarrhea by determining the prevalence of major pathogens, evaluating the frequency of mixed infections, and analyzing their association with predisposing factors. Between September 2023 and August 2024, 120 fecal samples were collected monthly from diarrheic neonatal calves across 18 different small-scale cattle farms with similar traditional management practices. Samples were initially screened using polymerase chain reaction (PCR), and positive amplicons were confirmed through sequence analysis. A total of 117 (97.5%) calves were found to be infected with at least one pathogen. *Cryptosporidium parvum* was the most

frequently detected agent, identified in 95 samples (79.1%), followed by *E. coli* in 61 (50.8%), RVA in 36 (30%), and BCoV in 27 (22.5%). The *C. parvum* subtype IIaA13G2R1 was identified for the first time in the research area, confirming its dominance as a subtype in calves in Türkiye. Age emerged as the most influential predisposing factor, with *E. coli* infections occurring most frequently during the first week of life, whereas RVA/BCoV and *C. parvum* infections peaked during the second week. Seasonal variation also shaped pathogen patterns, with *C. parvum* occurring at relatively similar rates across seasons, whereas *E. coli* infections were more frequent in autumn and spring, and RVA/BCoV detections peaked in winter. These findings underscore the importance of early-life monitoring and season-aware management strategies to mitigate neonatal calf diarrhea.

Keywords: Bovine coronavirus, bovine group A rotavirus, *Cryptosporidium parvum*, *Escherichia coli*, neonatal calf diarrhea, risk factors

What is already known on this topic?

- Neonatal calf diarrhea is a widespread, multifactorial condition with high morbidity, mortality, and economic losses in cattle farming.
- The main etiological agents include *C. parvum*, *E. coli*, bovine RVA, and BCoV, with mixed infections frequently reported.

Introduction

Neonatal calf (between 24 hours and 28 days of age) diarrhea is a common and multifactorial condition that poses a significant challenge in cattle production systems. It results from interactions between infectious agents and management-related factors such as housing, nutrition, and hygiene, thereby necessitating an integrated management approach for effective control (Astiz et al., 2017; Probo & Veronesi, 2022; Sedky et al., 2025; Torreggiani et al., 2025). The incidence and impact of neonatal calf diarrhea are affected by various factors, including geographical location, production system type, seasonal variations, and management practices. Studies have reported that its incidence ranges from 24% to 30% in Europe and the United States (Donovan et al., 1998; Probo & Veronesi, 2022; Svensson et al., 2003; Virtala et al., 1996). Additionally, the mortality rate of diarrheic calves varies between 15% and 30% in Europe (Heinrichs & Radostits, 2001), while exceeding 50% in the United States (NAHMS, 2008; Probo & Veronesi, 2022). The detrimental effects of neonatal diarrhea extend beyond direct outcomes such as mortality and treatment costs, as it can impair herd productivity and economic viability through reduced weight gain, lower first-lactation milk production, and an increased predisposition

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- Age, season, and management practices are recognized factors influencing the incidence and severity of infections.

What this study adds on this topic?

- Provides the first comprehensive molecular epidemiological assessment of neonatal calf diarrhea in Muğla province of Türkiye, detailing the prevalence and distribution of *C. parvum*, *E. coli*, bovine RVA, and BCoV.
- Identifies the *C. parvum* subtype IlaA13G2R1 for the first time in calves in the study area, confirming its dominance subtype in calves in Türkiye.
- Highlights age and season as significant epidemiological determinants influencing the spread and severity of infections, underscoring the importance of ongoing monitoring and offering baseline data for prevention and control strategies beneficial to veterinarians, animal health professionals, and farmers.

to secondary diseases, particularly respiratory infections. Neonatal calf diarrhea is also associated with sickness behaviors such as increased lying time and reduced milk consumption (Abuelo et al., 2021; Goharshahi et al., 2021; Probo & Veronesi, 2022; Sutherland et al., 2018; Waltner-Toews et al., 1986; Wilson et al., 2023; Virtala et al., 1996). Calf losses have significant economic implications at the national level. In Türkiye, the estimated economic loss per deceased calf has been calculated to be approximately 1.293 USD (Demir et al., 2019).

Neonatal calf diarrhea results from both non-infectious and infectious factors, with various enteric pathogens, including viruses, bacteria, and protozoa, contributing to its onset (Cho & Yoon, 2014; Sedky et al., 2025). While a single primary pathogen may occasionally be responsible, mixed infections are commonly detected in diarrheic calves. Despite considerable advancements in cattle production, including enhancements in herd management, housing conditions, animal health care, nutrition, and the strategic use of biopharmaceuticals, calf diarrhea remains a significant concern due to its complex and multifactorial nature. A thorough understanding of its underlying mechanisms, including the interaction of multiple pathogens, mixed infections, environmental influences, and calving-period management and feeding practices, is essential for the development of effective prevention and control measures before outbreaks occur (Cho & Yoon, 2014).

The major etiological pathogens of neonatal calf diarrhea under one month of age include *Cryptosporidium parvum*, *Escherichia coli*, bovine group A rotavirus (RVA), and bovine coronavirus (BCoV) (Badaracco et al., 2012; Berber et al., 2021; Bok et al., 2015; Garro et al., 2016; López-Novo et al., 2025; Torreggiani et al., 2025; Vega et al., 2020; Yildirim et al., 2020).

Accurate identification of these pathogens is essential for the development of targeted prevention and control strategies against neonatal calf diarrhea (Sedky et al., 2025). Therefore, this study aimed to provide the first comprehensive molecular epidemiological assessment of neonatal calf diarrhea from Muğla province of Türkiye. For this purpose, we aimed to (1) determine the molecular prevalence of major enteric pathogens, including *C. parvum*, *E. coli*, bovine RVA, and BCoV in diarrheic neonatal calves; (2) identify and quantify the frequency of mixed infections; (3) assess the association between pathogen occurrence and predisposing factors such as season and age; and (4) establish baseline data to support the development of future control and prevention strategies for neonatal calf diarrhea.

Materials and Methods

Field Study and Sample Collection

The study was conducted between September 2023 and August 2024 in the Muğla province of Türkiye. Neonatal calves with diarrhea were targeted for sampling throughout this period, and the minimum required sample size was determined through a G-Power analysis assuming a large effect size (0.4). Fecal samples were collected monthly from diarrheic neonatal calves across 18 small-scale farms. These farms typically housed approximately 5–50 calves and shared similar environmental and management characteristics, including traditional barn structures and limited mechanization. Farm enrolment and sample collection were based solely on the presence of neonatal calves with diarrhea during the study period. Fecal samples from each calf were collected once, directly from the rectum, to prevent cross-contamination, using disposable gloves, and placed individually into disposable sterile plastic containers. The fecal specimens were then transferred to the parasitology, bacteriology, and virology laboratories of Muğla Sıtkı Koçman University Faculty of Milas Veterinary Medicine via transport containers with ice and were immediately stored at 4°C for further processing. Each fecal sample was initially portioned for parasitic, bacterial, and viral analyses, while the remaining part was stored at –20°C for possible future use.

Genomic DNA and Viral RNA Extraction

For *Cryptosporidium* spp., genomic DNA (gDNA) from each fecal sample portioned for parasitic analyses was individually isolated using the commercial DNA purification kit (Magen HiPure Stool DNA Kit) according to the manufacturer's instructions. The extracted gDNA samples were stored at –20°C until molecular analysis.

For RVA and BCoV, RNA from each fecal sample portioned for viral analyses was individually isolated using the commercial RNA purification kit (Magen HiPure Stool RNA Kit) according to the manufacturer's instructions. The extracted RNA samples were stored at –20°C for a short period until complementary DNA (cDNA) synthesis was performed.

Isolation of *E. coli*: Fecal samples portioned for bacterial analyses were inoculated onto MacConkey agar, followed by incubation at 37°C for 24 hours. Lactose-positive colonies were selected, purified, and

Table 1.
Polymerase Chain Reaction Primers Used for the Detection and Molecular Characterization of Major Pathogens Associated with Neonatal Calf Diarrhea

Target Species	Primer Name	Target Gene	Oligonucleotide Sequences (5'-3')	Annealing (°C)	Product Size	Reference
Cryptosporidium spp.	CryXF1	SSU rRNA	TTCTAGAGCTAATACATGCG	54	~825 bp	(Xiao et al., 1999)
	CryXR1		CCCTAATCCTTCGAACAGGA			
	CryXF2	SSU rRNA	GGAAGGTTGTATTATTAGATAAAG	60		
	CryXR2		AAGGAGTAAGGAACAACCTCCA			
C. parvum subtyping	AL3531	gp60	ATAGTCTCCGCTGTATTTC	50	~850 bp	(Alves et al., 2003)
	AL3535		GGAAGGAACGATGTATCT			
	AL3532	gp60	TCCGCTGTATTCTCAGCC	55		
	AL3534		GCAGAGGAACCGCATC			
RVA	157 /	VP6	GTTTTCGAAGAGTDATHAHTCAGC	55	~380 bp	(Iturriza Gomara et al., 2002)
	VP6-F		GACGGVGCRACCTACATGGT			
BCoV	BCoVF/BCoVR	N	GCAATCCAGTAGTAGAGCGT	52	~730 bp	(Cho et al., 2001)
			CTTAGTGGCATCCTTGCCAA			
E. coli	ECPF/ECPR	PhoA	GGTAACGTTTCTACCGCAGAGTTG	55/57	~468 bp	(Shome et al., 2011)
			CAGGGTTGGTACACTGTCATTACG			
Target E. coli strains						
EPEC		eaeA	CAGGGTTGGTACACTGTCATTACG	57	~229 bp	(Hegde et al., 2012)
			CTGAACCAAGATCGTAACGGC			
		bfpA	CACCGTTACCGCAGGTGTGA	57	~450 bp	(Hegde et al., 2012)
ETEC			GTTGCCGCTTCAGCAGGAGT			
		elt	CTCTATGTGCACACGGAGC	55	~322 bp	(Hegde et al., 2012)
			CCATACTGATTGCCGCAAT			
EIEC		StIIa	TCTTTCCCTCTTTTAGTCAGTC	55	~170 bp	(Hegde et al., 2012)
			CCGCACAGGCAGGATTAC			
		ial	CTGGTAGTGTGGTGAGG	57	~320 bp	(Hegde et al., 2012)
EAEC			CCAGGCCAACAAATTAATTC			
		CVD432	CTGGCGAAGACTGTATCAT	55	~630 bp	(Hegde et al., 2012)
			CAATGTATAGAAATCCGCTGTT			
EHEC		hlyA	GCATCATCAAGGTACGTTCC	55	~534 bp	(Hegde et al., 2012)
			AATGAGCCAAAGCTGGTTAAGCT			

BCoV, bovine coronavirus; C. parvum, Cryptosporidium parvum; E. coli, Escherichia coli; RVA, bovine group A rotavirus.

re-inoculated onto MacConkey agar (Gibbons et al., 2014). The colonies were then cultured in TSB (Tryptic Soy Broth) at 37°C for 16–18 hours. Subsequently, approximately 1 mL of the TSB culture was transferred to a tube and centrifuged to obtain a pellet. Genomic DNA extraction for *E. coli* was performed according to the protocol described by Güler et al. (2008).

Polymerase Chain Reaction/Reverse Transcription Polymerase Chain Reaction Analyses

Polymerase chain reaction primers used for the detection and molecular characterization of major pathogens associated with neonatal calf diarrhea are presented in Table 1.

For cDNA synthesis from the RNA samples, a commercial kit (Grisp, Xpert cDNA Synthesis Kit, Cat No. GK80.0100) was used. The reaction was carried out in a 20 µL final volume, comprising 4 µL 5X reaction buffer, 1 µL dNTP mix, 1 µL hexaprimer, 1 µL Xpert Rtase, and 11 µL dH₂O. The single-cycle synthesis step was carried out under the following conditions: 10 minutes at 25°C, 50 minutes at 55°C, 5 minutes at 85°C, followed by 2 minutes on ice.

For *Cryptosporidium*, RVA, and BCoV: The PCR reaction was carried out in a 25 µL final volume, comprising 12.5 µL of Dream Taq PCR Master Mix (Thermo Scientific, Waltham, MA, USA), 1 µM of each primer, and 10–30 ng of gDNA/cDNA for the amplification of the target gene region. The thermal cycling conditions were as follows: an initial denaturation step at 95°C for 2 minutes, followed by 35 cycles of 30 seconds at 95°C, 30 seconds at (annealing temperature for each primer pair is given in Table 1), and 60 seconds at 72°C, with a final extension step at 72°C for 10 minutes. Amplicons (5 µL) were analyzed in 1.5% agarose gel, stained with SafeView™ (Applied Biological Materials, Richmond, BC, Canada), and visualized by UV illumination.

For *E. coli*, multiplex PCR analysis was performed using a total reaction mixture of 25 µL, including 0.1 µL of each primer, 5 µL of gDNA, and 18.6 µL of distilled water, prepared as two distinct PCR mixes. To ensure molecular confirmation of culture-positive strains and to maintain internal control in the reactions, the *phoA* gene was included in both PCR mixes, allowing molecular verification of the strains as *E. coli*. The thermal profile for PCR mix 1 consisted of an initial cycle at 95°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 55°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. For PCR mix 2, the conditions included an initial cycle at 95°C for 1 minute, followed by 35 cycles of denaturation at 94°C for 1 minute, annealing at 57°C for 1 minute, extension at 72°C for 1 minute, and a final extension at 72°C for 5 minutes. After the reaction, 10 µL of the amplicon was subjected to electrophoresis on a 2% agarose gel stained with Safeview. The virulence properties of the isolates were further evaluated by examining the specific band sizes.

Sequencing Analyses of *Cryptosporidium parvum*, Bovine Coronavirus, and Bovine Group A Rotavirus Isolates

Polymerase chain reaction products representing study areas were purified and sequenced for further analysis in both directions (MacroGen, Amsterdam, The Netherlands) using the AL3532/AL3534 nested PCR primers for subtyping of *C. parvum* isolates.

For the molecular characterization of RVA and BCoV isolates, PCR products from each were purified and sequenced in both directions

(MacroGen, Amsterdam, The Netherlands) using the 157/VP6-F and BCoV/R primers, respectively.

All sequences obtained were assembled and edited using the Geneious Prime 2024.0.5 software (<https://www.geneious.com>). Sequences were individually assessed by evaluating the phred scores and chromatogram quality, and a consensus sequence was generated. To identify species, final nucleotide sequences were analyzed using the BLASTn plugin in Geneious Prime.

The subtyping of *C. parvum* isolates was performed in accordance with the relevant nomenclature established by Xiao and Feng (2017). The nucleotide sequence of *C. parvum* isolate has been submitted to GenBank with the following isolate name (2MCpar) and accession number PV555434.

Statistical Analysis

The minimum required sample size was calculated using G-Power 3.1.9.4 (Heinrich-Heine-Universität Düsseldorf; Düsseldorf, Germany) (Company; City, Country) for a chi-square goodness-of-fit test, assuming a large effect size ($w=0.40$), $\alpha=0.05$, power=0.95, and 4 degrees of freedom. The analysis showed that a sample size close to the one used in the study would provide sufficient statistical power. Therefore, 120 diarrheic neonatal calves were included to ensure adequate power and representativeness for preliminary epidemiological assessment in the study area.

The data were subjected to descriptive statistics, and the frequency distributions of the pathogens according to the factors studied were summarized as frequencies (n) and percentages (%). The factors that were potentially effective for bacterial, viral, and parasitic pathogens were evaluated. Pearson's chi-squared or Fisher–Freeman–Halton tests were utilized to assess the frequency distributions of the factors according to different factors as a univariate approach, taking into account the proportions of the expected values in the cells. Logistic regression analysis was used to examine the multivariate effect of factors on the presence of bacterial, viral, and parasitic pathogens. In determining the variables to be included in the model, the univariate approach and the potential clinically significant effect were taken into account (Hosmer et al., 2013), and the backward stepwise elimination method was used to build the final model. The Hosmer–Lemeshow test statistic was then employed to assess the model's goodness of fit. The level of statistical significance was determined using a *p*-value cut-off of .05. All statistical analyses were conducted using the SPSS 30 software package (IBM SPSS Corp.; Armonk, NY, USA).

This study was approved by the Local Ethics Committee for Animal Experiments of Muğla Sıtkı Koçman University (2023/03-14/23; Date: March 23, 2023). Written informed consent was obtained from animal owners for the participation of their animals in this study.

Results

Molecular Prevalence and Genetic Characterization of Major Pathogens (*Cryptosporidium parvum*, *Escherichia coli*, Bovine Group A Rotavirus, and Bovine Coronavirus) in Neonatal Calves

Cryptosporidium spp. positivity was detected in 95 out of 120 gDNA of fecal samples by nested PCR analysis of the SSU rRNA gene region, with an overall prevalence of 79.1%. All positive isolates detected in the SSU rRNA gene region analyses were successfully

amplified using PCR analyses targeting the *gp60* gene region, which is employed for subtyping *C. parvum*. Purified representative PCR products of *C. parvum* isolates were successfully sequenced, and all sequences obtained from all isolates were wholly identical. BLASTn analyses revealed that the *gp60* sequence of the isolates characterized in this study showed 100% identity with a single subtype, IlaA13G2R1. Also, the subtyping of *C. parvum* isolates was confirmed in accordance with the relevant nomenclature established by Xiao and Feng (2017). These findings demonstrate for the first time that IlaA13G2R1 is the predominant genotype in the study area. Also, the isolate characterized in this study exhibited ranging from 99.7% to 100% similarity with isolates (accession numbers: KX443780, MN998541, MN815775, KF008182, and JX826602) reported from different regions of Türkiye (Kabir et al., 2020; Taylan-Ozkan et al., 2016; Yildirim et al., 2020, 2021).

Escherichia coli positivity was detected in 61 out of 120 gDNA of fecal samples in the PCR analysis based on the *phoA* gene region, with an overall prevalence of 50.8%. Molecular analyses targeting virulence genes in the isolated *E. coli* strains revealed that no virulence genes were detected.

Group A rotavirus positivity was detected in 36 out of 120 gDNA of fecal samples by PCR analysis of the *VP6* gene region, with an overall prevalence of 30%. Bovine coronavirus positivity was detected in 27 out of 120 gDNA of fecal samples by PCR analysis of the *N* gene region, with an overall prevalence of 22.5%. The purified representative PCR products of the *VP6* gene region of RVA isolates and the *N* gene region of BCoV isolates were successfully sequenced. The RVA isolates identified in this study exhibited 100% similarity among themselves. The characterized RVA isolate showed 94.9%–99% similarity to other isolates recorded in GenBank from different provinces of Türkiye (accession numbers: OP432877–78, MZ848179–81, OQ082573, OQ215305, KX212867,

Table 2.*Sociodemographic Characteristics of the Study Population*

Sex	Male	59 (49.2%)
	Female	61 (50.8%)
Breed	Holstein	92 (76.7%)
	Simmental	28 (23.3%)
Age	1–7 days	37 (30.8%)
	8–14 days	52 (43.3%)
	15–28 days	31 (25.8%)

JQ993400, JQ993399). Similarly, all sequences obtained from BCoV isolates detected in this study were wholly identical. The characterized BCoV isolate demonstrated 99.7%–99.9% similarity to other isolates recorded in GenBank from different provinces of Türkiye (OQ161705; MK787436–37).

Epidemiology and Risk Factors of Major Pathogens Causing Neonatal Calf Diarrhea

Among the 120 calves included in the study, 117 (97.5%) were found to be infected with one or more pathogens. The age, sex, and breed distributions of calves are presented in Table 2. *Cryptosporidium parvum* (25%) was the most frequently detected pathogen as a single infection, followed by dual infections of *C. parvum* + *E. coli* (24.2%) and *E. coli* (10%) single infection. Multi infections (*C. parvum* + *E. coli* + RVA + BCoV) were rare, observed in only 5% of cases. In calves aged 1–7 days, *E. coli* (18.9%) single and *C. parvum* + *E. coli* (18.9%) dual infections were predominant, suggesting that bacterial infections were more prevalent in the early stages of life of calves. In calves aged 8–14 days, *C. parvum* + *E. coli* (30.8%) dual infection was identified as the most common mixed infection. In calves aged 15–28 days,

Table 3.*Epidemiology and Risk Factors of Major Pathogens Causing Neonatal Calf Diarrhea*

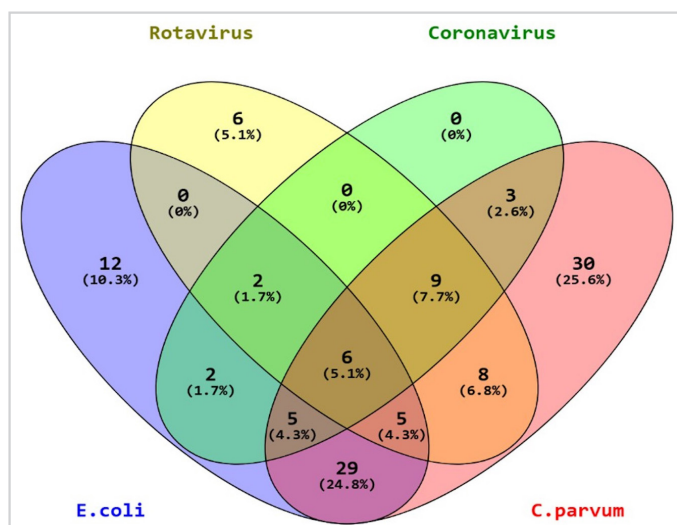
Pathogen(s)	Sex, n (%)		Breed, n (%)		Age, n (%)			Total, n (%)
	Male	Female	Holstein	Simmental	1–7 days	8–14 days	15–28 days	
Absence	2 (3.4)	1 (1.6)	2 (2.2)	1 (3.6)	0 (0)	1 (1.9)	2 (6.5)	3 (2.5)
<i>E. coli</i> (Single)	4 (6.8)	8 (13.1)	6 (6.5)	6 (21.4)	7 (18.9)	1 (1.9)	4 (12.9)	12 (10)
RVA (Single)	2 (3.4)	4 (6.6)	3 (3.3)	3 (10.7)	3 (8.1)	2 (3.8)	1 (3.2)	6 (5)
<i>C. parvum</i> (Single)	15 (25.4)	15 (24.6)	25 (27.2)	5 (17.9)	4 (10.8)	9 (17.3)	17 (54.8)	30 (25)
<i>E. coli</i> + BCoV	0 (0)	2 (3.3)	0 (0)	2 (7.1)	2 (5.4)	0 (0)	0 (0)	2 (1.7)
<i>E. coli</i> + <i>C. parvum</i>	14 (23.7)	15 (24.6)	23 (25)	6 (21.4)	7 (18.9)	16 (30.8)	6 (19.4)	29 (24.2)
RVA + <i>C. parvum</i>	5 (8.5)	3 (4.9)	7 (7.6)	1 (3.6)	1 (2.7)	7 (13.5)	0 (0)	8 (6.7)
BCoV + <i>C. parvum</i>	1 (1.7)	2 (3.3)	2 (2.2)	1 (3.6)	1 (2.7)	2 (3.8)	0 (0)	3 (2.5)
<i>E. coli</i> + RVA + BCoV	1 (1.7)	1 (1.6)	1 (1.1)	1 (3.6)	2 (5.4)	0 (0)	0 (0)	2 (1.7)
<i>E. coli</i> + RVA + <i>C. parvum</i>	4 (6.8)	1 (1.6)	4 (4.3)	1 (3.6)	3 (8.1)	2 (3.8)	0 (0)	5 (4.2)
<i>E. coli</i> + BCoV + <i>C. parvum</i>	1 (1.7)	4 (6.6)	5 (5.4)	0 (0)	1 (2.7)	3 (5.8)	1 (3.2)	5 (4.2)
RVA + BCoV + <i>C. parvum</i>	8 (13.6)	1 (1.6)	8 (8.7)	1 (3.6)	2 (5.4)	7 (13.5)	0 (0)	9 (7.5)
<i>E. coli</i> + RVA + BCoV + <i>C. parvum</i>	2 (3.4)	4 (6.6)	6 (6.5)	0 (0)	4 (10.8)	2 (3.8)	0 (0)	6 (5)
Total	59 (100)	61 (100)	92 (100)	28 (100)	37 (100)	52 (100)	31 (100)	120 (100)

BCoV, bovine coronavirus; *C. parvum*, *Cryptosporidium parvum*; *E. coli*, *Escherichia coli*; RVA, bovine group A rotavirus.

Table 4.
Seasonal Distribution of Major Pathogens Causing Neonatal Calf Diarrhea

Pathogen(s)	Winter n (%)			Spring n (%)			Summer n (%)			Autumn n (%)		
	December	January	February	March	April	May	June	July	August	September	October	November
Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)	Total N (%)
Absence	0 (0)	1 (10)	0 (0)	1 (3.3)	0 (0)	1 (10)	1 (3.3)	0 (0)	1 (10)	1 (3.3)	0 (0)	0 (0)
<i>E. coli</i> (Single)	1 (10)	1 (10)	0 (0)	2 (6.7)	0 (0)	1 (10)	1 (3.3)	1 (10)	1 (10)	3 (10)	3 (30)	0 (0)
RVA (Single)	0 (0)	1 (10)	1 (10)	2 (6.7)	0 (0)	1 (10)	1 (3.3)	0 (0)	1 (10)	1 (3.3)	0 (0)	2 (20)
<i>C. parvum</i> (Single)	0 (0)	2 (20)	0 (0)	2 (6.7)	2 (20)	1 (10)	5 (16.7)	8 (80)	5 (50)	18 (60)	4 (40)	1 (10)
<i>E. coli</i> + BCoV	1 (10)	0 (0)	0 (0)	1 (3.3)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	0 (0)
<i>E. coli</i> + <i>C. parvum</i>	4 (40)	0 (0)	4 (40)	8 (26.7)	4 (40)	3 (30)	11 (36.7)	0 (0)	2 (20)	1 (10)	4 (40)	2 (20)
RVA + <i>C. parvum</i>	1 (10)	0 (0)	1 (10)	2 (6.7)	2 (20)	1 (10)	4 (13.3)	0 (0)	0 (0)	0 (0)	0 (0)	2 (20)
BCoV + <i>C. parvum</i>	0 (0)	1 (10)	1 (10)	2 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (3.3)	0 (0)	0 (0)
<i>E. coli</i> + RVA + BCoV	1 (10)	1 (10)	0 (0)	2 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)	0 (0)
<i>E. coli</i> + RVA + <i>C. parvum</i>	1 (10)	0 (0)	0 (0)	1 (3.3)	0 (0)	1 (10)	1 (3.3)	0 (0)	0 (0)	1 (10)	1 (10)	1 (10)
<i>E. coli</i> + BCoV + <i>C. parvum</i>	0 (0)	0 (0)	0 (0)	0 (0)	1 (10)	1 (10)	2 (6.7)	0 (0)	0 (0)	0 (0)	0 (0)	3 (30)
RVA + BCoV + <i>C. parvum</i>	0 (0)	3 (30)	2 (20)	5 (16.7)	0 (0)	1 (10)	2 (6.7)	1 (10)	1 (10)	2 (6.7)	0 (0)	0 (0)
<i>E. coli</i> + RVA + BCoV + <i>C. parvum</i>	1 (10)	0 (0)	1 (10)	2 (6.7)	1 (10)	0 (0)	2 (6.7)	0 (0)	1 (10)	1 (3.3)	0 (0)	0 (0)
Total	10 (100)	10 (100)	10 (100)	30 (100)	10 (100)	10 (100)	30 (100)	10 (100)	10 (100)	30 (100)	10 (100)	10 (100)

BCoV, bovine coronavirus; *C. parvum*, *Cryptosporidium parvum*; *E. coli*, *Escherichia coli*; RVA, bovine group A rotavirus.

**Figure 1.**

A Venn Diagram Represents the Distribution of Single and Mixed Infections Attributed to Major Pathogens Detected in Diarrheic Neonatal Calves.

C. parvum (54.8%) single infection emerged as the dominant agent, suggesting that parasitic infections tend to become more noticeable after the second week of age (Table 3).

When the seasonal distribution of the identified pathogens was evaluated, *C. parvum* single infection exhibited a notably high prevalence of 60% during the summer. The most common dual infection in winter was *C. parvum*+*E. coli* (26.7%), followed by *C. parvum*+RVA+BCoV (16.7%) multiple infection. During spring, *C. parvum* (16.7%) single infection and *C. parvum*+*E. coli* (36.7%) dual infection were observed at high rates, followed by *C. parvum*+RVA dual infection (13.3%). In autumn, *C. parvum*+*E. coli* (23.3%) was the most frequently detected dual infection, followed by *E. coli* single infection (20%).

These findings indicate that *C. parvum* is a particularly dominant pathogen during the summer period, and *C. parvum*+*E. coli* dual infection commonly poses a significant risk (Table 4).

The Venn diagram illustrates the distribution of single and mixed infections caused by major pathogens detected in diarrheic neonatal calves (Figure 1).

The occurrence of *C. parvum* was significantly influenced by both breed and age. Holstein calves exhibited a significantly higher prevalence of *C. parvum* compared to Simmental calves (84.2% vs. 15.8%, $p < .001$). The highest infection rate was observed in calves aged 8–14 days (50.5%), followed by those aged 15–28 days (25.3%) and 1–7 days (24.2%) ($p = .002$), suggesting a greater risk in the second week of life. Neither sex ($p = .179$) nor season ($p = .204$) showed a significant association with *C. parvum* infection. The univariate analysis for *E. coli* indicated that age and season were significantly associated with the presence of the pathogen. Calves aged 1–7 days had a significantly higher prevalence of *E. coli* infection compared to older age groups ($p = .011$), aligning with the heightened susceptibility observed in the earliest days of life. Seasonally, infections were

Table 5.

Statistical Analysis of the Distribution of Major Pathogens Causing Neonatal Calf Diarrhea

		E. coli				RVA/BCoV				C. parvum			
		Absence		Presence		Absence		Presence		Absence		Presence	
		n	%	n	%	n	%	n	%	n	%	n	%
Breed	Holstein	47	79.70	45	73.80	56	75.70	36	78.30	12	48.00	80	84.20
	Simmental	12	20.30	16	26.20	18	24.30	10	21.70	13	52.00	15	15.80
p-value													
					.52								<.001
Age	1-7 days	11	18.60	26	42.60	18	24.30	19	41.30	14	56.00	23	24.20
	8-14 days	28	47.50	24	39.30	27	36.50	25	54.30	4	16.00	48	50.50
	15-28 days	20	33.90	11	18.00	29	39.20	2	4.30	7	28.00	24	25.30
					.011								.002
Sex	Male	33	55.90	26	42.60	35	47.30	24	52.20	9	36.00	50	52.60
	Female	26	44.10	35	57.40	39	52.70	22	47.80	16	64.00	45	47.40
p-value													
					.201								.179
Season	Spring	13	22.00	17	27.90	18	24.30	12	26.10	3	12.00	27	28.40
	Summer	23	39.00	7	11.50	25	33.80	5	10.90	5	20.00	25	26.30
	Autumn	9	15.30	21	34.40	18	24.30	12	26.10	9	36.00	21	22.10
	Winter	14	23.70	16	26.20	13	17.60	17	37.00	8	32.00	22	23.20
p-value													
					.003								.204

BCoV, bovine coronavirus; C. parvum, *Cryptosporidium parvum*; E. coli, *Escherichia coli*; RVA, bovine group A rotavirus.

Table 6.

Evaluation of Factors Influencing the Likelihood of Cryptosporidium parvum Occurrence—Final Logistic Regression Model

	Beta (B)	SE	Wald	df	p	Odds Ratio	95% CI	
							Lower	Upper
Age			5.812	2	.055			
15–28 days (Reference)					–			
1–7 days	–0.376	0.601	0.391	1	.532	0.687	0.211	2.23
8–14 days	1.23	0.704	3.049	1	.081	3.42	0.86	13.592
Constant term	0.017	0.685	0.001	1	.98	1.017		

Nagelkerke $R^2 = 0.270$, $-2LL = 100.062$, SE: Standart Error, CI: Confidence Interval, df: degree of freedom

most frequent in autumn (34.4%) and spring (27.9%), with the lowest prevalence in summer (11.5%), demonstrating a significant seasonal effect ($p = .003$). No significant differences were detected in *E. coli* prevalence by breed ($p = .52$) or sex ($p = .201$). For RVA/BCoV, age and season were also significant determinants. The highest prevalence occurred in the early ages of calves (1–7 days: 41.3%, 8–14 days: 54.3%), whereas the oldest group (15–28 days) exhibited the lowest prevalence (4.3%) ($p < .001$), suggesting a peak susceptibility during the early ages. Additionally, seasonal differences were observed ($p = .017$), with winter showing the highest infection rate (37%) and summer the lowest (10.9%). Neither breed ($p = .826$) nor sex ($p = .708$) significantly influenced RVA/BCoV occurrence. These findings collectively highlight the critical role of age in pathogen susceptibility and the potential influence of breed and season, underlining the importance of targeted management practices during specific risk periods (Table 5).

These factors were further evaluated with multivariate logistic regression to reveal possible effects. The final models for each pathogen were presented in Tables 6–8.

When factors influencing the likelihood of *C. parvum* occurrence were modeled, age was included in the final model. Although the

effect of age was not statistically significant in the model, calves aged 1–7 days were identified as the lowest-risk group, whereas calves aged 8–14 days constituted the highest-risk group (Table 6).

The final logistic regression model evaluating factors influencing the likelihood of *E. coli* occurrence revealed that age was a statistically significant predictor (Wald = 6.829, $df = 2$, $p = .033$), while season approached but did not reach statistical significance (Wald = 6.935, $df = 3$, $p = .074$) unlike the univariate approach. Compared to calves aged 15–28 days (reference group), those aged 1–7 days were significantly more likely to exhibit *E. coli* infection, with an odds ratio (OR) of 3.84 (95% CI: 1.205–12.235, $p = .023$), indicating a markedly elevated risk in the youngest age group. In contrast, calves aged 8–14 days did not differ significantly from the reference group (OR = 1.218, 95% CI: 0.425–3.49, $p = .714$). Regarding seasonal effects, no statistically significant associations were observed when compared to winter; however, the odds of *E. coli* occurrence were not significantly higher in spring (OR = 1.772, 95% CI: 0.588–5.343, $p = .310$) and autumn (OR = 2.21, 95% CI: 0.698–6.998, $p = .177$), while a non-significant potential protective effect was noted in summer (OR = 0.338, 95% CI: 0.1–1.148, $p = .082$). Although the seasonal associations were not statistically significant, the trends suggest possible seasonal influences on *E. coli* occurrence that warrant further investigation. The overall

Table 7.

Evaluation of Factors Influencing the Likelihood of Escherichia coli Occurrence—Final Logistic Regression Model

	Beta (B)	SE	Wald	df	p	Odds Ratio	95% CI	
							Lower	Upper
Age			6.829	2	.033			
15–28 days (Reference)					–			
1–7 days	1.346	0.591	5.18	1	.023	3.84	1.205	12.235
8–14 days	0.197	0.537	0.134	1	.714	1.218	0.425	3.49
Season			6.935	3	.074			
Winter (Reference)					–			
Spring	0.572	0.563	1.032	1	.31	1.772	0.588	5.343
Summer	–1.085	0.624	3.025	1	.082	0.338	0.1	1.148
Autumn	0.793	0.588	1.82	1	.177	2.21	0.698	6.998
Constant term	–0.546	0.63	0.75	1	.387	0.579		

Nagelkerke $R^2 = 0.221$, $-2LL = 144.54$, SE: Standart Error, CI: Confidence Interval, df: degree of freedom

Table 8.

Evaluation of Factors Influencing the Likelihood of Bovine Coronavirus and/or Bovine Group A Rotavirus Occurrence—Final Logistic Regression Model

								95% CI	
		Beta (B)	SE	Wald	df	p	Odds Ratio	Lower	Upper
Age				9.31	2	.01			
	15–28 days (Reference)					–			
	1–7 days	2.479	0.831	8.905	1	.003	11.931	2.342	60.789
	8–14 days	2.294	0.806	8.099	1	.004	9.918	2.043	48.158
Season				3.294	3	.348			
	Winter (Reference)					–			
	Spring	–0.168	0.566	0.088	1	.767	0.845	0.279	2.565
	Summer	–1.143	0.65	3.089	1	.079	0.319	0.089	1.141
	Autumn	–0.25	0.56	0.2	1	.655	0.778	0.26	2.333
Constant term		–2.106	0.866	5.915	1	.015	0.122		

Nagelkerke $R^2 = 0.258$, $-2LL = 134.55$, SE: Standart Error, CI: Confidence Interval, df: degree of freedom.

model demonstrated moderate explanatory power (Nagelkerke R^2 of 0.221), supporting the relevance of age, particularly in the first week of life, as a critical factor in the risk of *E. coli* infection in calves (Table 7).

The final model evaluating the likelihood of BCoV and/or RVA occurrence identified age as a statistically significant predictor, while season did not exert a significant influence (Table 8). Calves aged 1–7 days had a markedly increased risk of infection compared to the reference group (15–28 days), with an odds ratio (OR) of 11.931 (95% CI: 2.342–60.789, $p = .003$), indicating an almost 12-fold higher likelihood of infection in this youngest age group. Similarly, calves aged 8–14 days exhibited a significantly elevated risk (OR = 9.918, 95% CI: 2.043–48.158, $p = .004$), underscoring a peak vulnerability to BCoV and/or RVA infections within the first two weeks of life. In contrast, seasonal variation did not significantly affect infection risk. Although the spring season approached marginal significance ($p = .079$), its odds ratio (0.319, 95% CI: 0.089–1.141) suggested a potential trend toward reduced risk compared to winter, the reference season. Other seasons (summer and autumn) also did not show statistically significant associations ($p = .655$ and $p = .767$, respectively). These results reinforce the critical influence of calf age, particularly during the first 2 weeks postpartum, in determining susceptibility to RVA and/or BCoV, while seasonal factors appeared to play a less definitive role in infection dynamics.

Discussion

The study focused on identifying and analyzing the major pathogens associated with neonatal calf diarrhea in cattle farms in Muğla province, Türkiye. The study also explored the seasonal dynamics of these infections and noted variations in pathogen prevalence throughout the year. Among the 120 calves included in the study, 117 (97.5%) were found to be infected with one or more major pathogens, including *C. parvum*, *E. coli*, RVA, and BCoV. These findings highlight the significant risk these pathogens pose to the livestock industry in the study region. Also, the high prevalence of infections indicates a critical need for improved management and preventive measures on farms to mitigate the impact of these pathogens.

Among enteropathogens, *C. parvum* is recognized as one of the most common causative agents responsible for neonatal calf diarrhea worldwide (Gharieb et al., 2019; Meganck et al., 2015). Numerous studies in different countries have identified *C. parvum* as the predominant species affecting pre-weaned calves, including Portugal (Alves et al., 2003), Spain (Quílez et al., 2008), Slovenia (Soba & Logar, 2008), Poland (Kaupke & Rzeżutka, 2015), and Austria (Lichtmannsperger et al., 2019). For example, a study in Spain reported that 57.8% of 287 diarrheic calves under 21 days of age tested positive for *C. parvum* (Quílez et al., 2008). In Poland, prevalence was found to be 22.5% in calves up to 14 days of age (Kaupke & Rzeżutka, 2015). In Türkiye, the prevalence of cryptosporidiosis in calves has been reported to vary between 3.9% and 63.92% across different provinces, depending on the diagnostic methods used (Berber et al., 2021; Gündüz & Arslan, 2017; Güven et al., 2013; Keleş et al., 2022; Yildirim et al., 2020). It is important to understand that these rates may vary according to the sensitivity and specificity of the diagnostic methods used, as emphasized by Brunauer et al. (2021). In this study, consistent with previous studies, *C. parvum* was detected in 79.1% of diarrheic neonatal calves in the present study. These findings further confirm that *C. parvum* is a primary etiological parasitic agent of neonatal calf diarrhea and poses a significant risk to the livestock industry in Türkiye.

Limited molecular studies of *C. parvum* conducted in Türkiye have identified several subtypes, including IlaA11G2R1, IlaA12G3R1, IlaA13G2R1, IlaA14G1R1, IlaA15G2R1, IldA16G1, IldA18G1, IldA22G1, and IldA20G1b (Kabir et al., 2020; Taylan-Ozkan et al., 2016; Yildirim et al., 2020, 2021). Notably, all three studies highlighted IlaA13G2R1 as the predominant subtype. In line with these findings, the IlaA13G2R1 subtype was also identified in this study, which suggests that this subtype is the predominant *C. parvum* circulating on cattle farms throughout Türkiye. This subtype has been detected not only in calves but also in goat kids, lambs, ponies, and even humans in various countries, including Belgium, the Netherlands, Tunisia, the United States, and Algeria (Baroudi et al., 2018; Benhouda et al., 2017; Geurden et al., 2007; Iqbal et al., 2012; Rahmouni et al., 2014; Wagnerová et al., 2016; Wielinga et al., 2008). The identification of this subtype in humans highlights its zoonotic importance.

In this study, *C. parvum* single infection was identified as the causative agent of neonatal calf diarrhea in 25% of cases. This finding is consistent with previous studies reporting prevalence rates of 21.9%–23.1% in the Central Anatolia Region (Berber et al., 2021; Keleş et al., 2022) and 21.8% in the Eastern and Southeastern Anatolia Regions of Türkiye (Içen et al., 2013).

In the present study, calves were divided into three age groups (1–7, 8–14, 15–28 days). The highest infection rate (50.5%) was observed in calves aged 8–14 days, suggesting a greater risk in the second week of life, similar to *Cryptosporidium* spp. prevalence of 43.8% reported by Berber et al. (2021). Also, a strong age dependence was observed by Lichtmannsperger et al. (2019), and cryptosporidiosis was reported mostly in the first two weeks of age of calves. Indeed, Santin et al. (2004) reported that *C. parvum* prevalence peaked in the calves aged two weeks. *Cryptosporidium parvum* infection is known to cause the most severe diarrhea primarily during the first 2 weeks of life. However, in the present study, *C. parvum* was also detected in diarrheic calves aged 15–28 days. Since all samples were collected from clinically diarrheic animals, the positivity observed in this older age group may still be relevant to the clinical picture; at the same time, it should be kept in mind that oocyst shedding can continue beyond the early neonatal period. For this reason, the presence of *C. parvum* in older calves should be interpreted with care, considering both the possibility of a pathogenic role and the likelihood of ongoing oocyst excretion.

Regarding seasonal distribution, this study revealed that infection rates of *C. parvum* remained consistent across all seasons. However, previous studies by Berber et al. (2021) and Keleş et al. (2022) indicated a higher prevalence of diarrhea caused by cryptosporidiosis during winter months. This seasonal increase may be related to the increased number of sick animals being admitted to veterinary hospitals during this period. In addition, Hamnes et al. (2006) stated that intensive calving leads to a higher stocking density, which in turn leads to a higher number of susceptible young animals being kept together. Overpopulation may prevent optimal hygiene practices, increase the burden of pathogens, and facilitate direct transmission between animals. In addition, the prolonged survival of *Cryptosporidium* oocysts at moderate temperatures may contribute to the increased prevalence observed in winter. Gulliksen et al. (2009) also reported that cows that calve in winter produce a lower quality of colostrum than cows that calve at other seasons, which may further increase the risk of diarrhea in neonatal calves. In summary, the findings of the present study underscore the need for targeted management strategies, particularly for calves aged 8–14 days, and highlight the importance of addressing seasonal factors that may contribute to the increased prevalence of *C. parvum* infections. Implementing effective biosecurity measures and ensuring optimal sanitation practices, especially during peak calving seasons, will be critical in mitigating the impact of these infections on neonatal calf health.

Escherichia coli is widely recognized as the most significant bacterial pathogen causing neonatal calf diarrhea. The presence of specific virulence factors in pathogenic strains of *E. coli* can increase the susceptibility of calves to diarrhea (Cengiz & Adıgüzel, 2020). In this study, molecular analyses targeting virulence genes in the isolated *E. coli* strains revealed that no virulence genes were detected. Similarly, Cengiz and Adıgüzel (2020) reported that no virulence-associated features could be identified in 100 out of 133 *E. coli* strains isolated from diarrheic calves. It has been well documented since

2004 (Constable, 2004) that, regardless of the inciting cause of diarrhea, intestinal overgrowth of *E. coli* strains other than ETEC occurs in about 30% of diarrheic calves. These strains cause secondary infections, increasing mortality rates. Accordingly, these bacterial isolates may act synergistically with viral or parasitic agents in the pathogenesis of calf diarrhea, potentially contributing to increased calf mortality. Additionally, understanding the distribution of *E. coli* infections across different age groups is crucial for effective management and prevention strategies. We found that calves aged 1–7 days had a significantly higher prevalence (42.6%) of *E. coli* infection compared to older age groups. This finding is most likely attributable to age-related physiological and immunological immaturity during the early neonatal period rather than to pathogen-specific virulence factors, given that no virulence genes were detected in the isolates. These findings align with the observations of Berber et al. (2021), who reported an alarmingly high *E. coli* infection rate of 91.7% in similar age groups. In contrast, Güreli and Kara (2023) documented a significantly lower infection rate of 13.75%, while Bartels et al. (2010) reported only 4.6%. Additionally, we identified that the prevalence of *E. coli* single infections in calves aged 1–7 days was 18.9%. This finding is consistent with the 16.6% infection rate reported by Içen et al. (2013) for similar age groups. However, the rates reported by Berber et al. (2021) and Keleş et al. (2022), which were 85.7% and 33.6%, respectively, were notably higher than those observed in the present study results. Such variations in infection rates can be attributed to several factors, including the diagnostic techniques used, the types of farms sampled, housing conditions, hygiene standards, and herd management practices (Cho & Yoon, 2014; Keleş et al. 2022). Moreover, Keleş et al. (2022) noted a higher prevalence of *E. coli* in calves born to unvaccinated dams, emphasizing the role of maternal immunity and vaccination status in influencing calf health. This suggests that improving vaccination protocols for pregnant cows may be an effective strategy for reducing *E. coli* infections in neonatal calves. It is important to note that vaccination status could not be evaluated in the present study, as most of the small-scale farms lacked reliable herd-recording systems, and information provided by owners regarding dam vaccination was often incomplete or unverifiable. This limitation made it impossible to assess whether vaccination practices influenced pathogen prevalence, particularly for *E. coli*. Therefore, although vaccination data could not be analyzed in the present study, the potential role of maternal immunization should still be considered when interpreting *E. coli* infection patterns in neonatal calves. Indeed, the study by Bartels et al. (2010) identified a low *E. coli* prevalence, which was suggested to be associated with regularly scheduled vaccination programs. In another study, Keleş et al. (2022) reported that outbreaks of *E. coli*-associated diarrhea were more frequently observed on farms with low vaccination coverage against enteropathogens. These findings underline the critical role of vaccination programs in controlling *E. coli* infection and show that a lack of vaccination is a major factor in increasing the risk of infection in calves.

In this study, the rate of RVA infection among neonatal calves was found to be 30%, positioning RVA as the third most common cause of diarrhea in the study area. This finding aligns with several reports from different regions of Türkiye, where infection rates varied from 30% to 37.27% (Aksoy et al., 2021; Güreli & Kara, 2023; Keleş et al., 2022; Öztrürk et al., 2024). In contrast, Berber et al. (2021) reported a significantly higher RVA infection rate of 56.4% in the Central Anatolia Region of Türkiye, which is comparable to other international reports, including 55% in Israel (Brenner et al., 1993), 42.7% in

Spain (García et al., 2000), and 42% in the United Kingdom (Reynolds et al., 1986). BCV was found in 22.5% of the cases in this study. This is consistent with other reports from different regions of Türkiye, where infection rates varied from 16.67% to 42.25%. Aksoy et al., 2021; Güreli & Kara, 2023; Keleş et al., 2022; Öztrürk et al., 2024). Berber et al. (2021) attributed the regional variability in the distribution of related pathogens and in the incidence of infections to differences in herd management, calf husbandry, and the sensitivity of diagnostic techniques. Also, their study, conducted in an intensive care unit of an animal hospital, likely included calves with more severe conditions, which could explain the higher infection rates observed. In the present study, seasonal differences were observed in the distribution of viral infections, with winter showing the highest infection rate (37%) and summer the lowest (10.9%). Berber et al. (2021) also reported a higher prevalence of viral infections (both RVA and BCoV) during winter. This suggests that the winter season may pose an increased risk for these viral infections, highlighting the need for preventive veterinary measures tailored to this seasonal risk in the study area. The highest prevalence of viral infections occurred in the early ages of calves (1–7 days and 8–14 days), whereas the oldest group (15–28 days) exhibited the lowest prevalence, suggesting a peak susceptibility during early life. These rates are consistent with findings from other studies across Türkiye (Berber et al., 2021; Güreli & Kara, 2023; Keleş et al., 2022). Overall, the observed age-related patterns indicate that the first two weeks of life represent a critical period of increased susceptibility to RVA and/or BCoV infections in neonatal calves. Considering these results, targeted preventive measures are essential for mitigating the impact of these viral infections and improving calf health outcomes in the region.

Moreover, investigations into diarrheic calves in Türkiye indicate that dual infections involving *Cryptosporidium* spp. and rotavirus (RVA) are particularly common. For instance, Içen et al. (2013) found that *C. parvum* + *E. coli* dual infections accounted for 5.2% of cases, while *C. parvum* + RVA dual infections were observed in 15.62% of cases. Similarly, Berber et al. (2021) detected *Cryptosporidium* spp. + *E. coli* dual infections in 1.3% of cases, *Cryptosporidium* spp. + RVA dual infections in 23.1% of cases, and *Cryptosporidium* spp. + BCoV dual infections in 6.4% of cases. Furthermore, Keleş et al. (2022) reported *Cryptosporidium* spp. + *E. coli* dual infections in 0.4% of cases, *Cryptosporidium* spp. + RVA dual infections in 9.3% of cases, and *Cryptosporidium* spp. + BCoV dual infections in 1.1% of cases. In contrast to these previous studies, the present study revealed that the prevalence of dual infections caused by *C. parvum* + *E. coli* (24.2%) was higher. This was followed by dual infections associated with *C. parvum* + RVA (6.7%) and *C. parvum* + BCoV (2.5%).

This study has certain limitations that should be considered when interpreting the findings. Detailed farm-level information, including vaccination status, standardized management practices, and biosecurity measures, could not be systematically evaluated due to the limited availability and consistency of farm records across the included small-scale farms. Moreover, all included farms were small-scale holdings operating under broadly similar management and environmental conditions. Therefore, the risk factor analysis was intentionally focused on calf-level and seasonal variables that were reliably and uniformly recorded. Furthermore, although RVA was molecularly detected and confirmed through sequencing of the VP6 gene, a highly conserved and antigenic inner capsid protein widely used for group-level identification of rotaviruses, further

genotyping based on the VP4 (P type) and VP7 (G type) genes was not performed. This was primarily related to the scope of the study, which aimed to characterize the molecular prevalence and epidemiological patterns of major enteric pathogens rather than to conduct detailed genotype-level analyses. Despite these limitations, the study provides robust molecular and epidemiological baseline data on neonatal calf diarrhea in the region and identifies critical age and season-related risk patterns. These findings may serve as a foundation for future studies incorporating comprehensive farm-level data and advanced molecular characterization, including RVA VP4 and VP7 genotyping.

Unlike routine surveillance systems that typically provide descriptive diagnostic summaries, this study offers an integrative molecular and epidemiological assessment of neonatal calf diarrhea in the region. By combining molecular confirmation of major enteric pathogens with an analysis of co-infection patterns and temporally structured risk factors, the present investigation provides a level of epidemiological resolution that extends beyond routine monitoring approaches. Consequently, the findings generate scientifically interpretable baseline data that can directly inform evidence-based prevention and control strategies tailored to regional conditions.

Conclusion

This study revealed the major pathogens (*C. parvum*, *E. coli*, RVA, and BCoV) and predisposing factors involved in the etiology of neonatal calf diarrhea. The distribution of these pathogens across different parameters was analyzed. Results suggest that age and season are significant epidemiological determinants that influence the spread and severity of infections, highlighting the importance of monitoring these pathogens over time. Additionally, the IIA13G2R1 subtype of *C. parvum* was identified for the first time in the research area, confirming its dominance subtype in calves in Türkiye. This finding is significant as it contributes to the current understanding of *C. parvum* dynamics in this area. The results of this study are expected to serve as a valuable resource for veterinarians, animal health professionals, relevant non-governmental organizations, and farmers in the study area. Further comprehensive studies are required to elucidate the genotypes, subtypes, and serotypes diversity of pathogens associated with the etiology of neonatal calf diarrhea across the country, thereby facilitating the development and implementation of effective prevention and control strategies.

Data Availability Statement: The data that support the findings of this study are available on request from the corresponding author.

Ethics Committee Approval: This study was approved by the Local Ethics Committee for Animal Experiments of Muğla Sıtkı Koçman University (2023/03-14/23; Date: March 23, 2023).

Informed Consent: Written informed consent was obtained from animal owners for the participation of their animals in this study.

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Data Collection and/or Processing – N.S.Ş., I.Ç., S.C., N.A.Ç., N.Ç., E.Ş.; Analysis and/or Interpretation – N.S.Ş., I.Ç., D.Ö., S.C., N.A.Ç., N.Ç., E.Ş.; Literature – Review – N.S.Ş., I.Ç., D.Ö., S.C., N.A.Ç., N.Ç., E.Ş.; Writing – N.S.Ş., I.Ç., D.Ö., S.C., N.A.Ç., N.Ç., E.Ş.; Critical Review – N.S.Ş., E.Ş.

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