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Diagnosis of bovine group A rotavirus, bovine coronavirus, *Escherichia coli* K99, *Cryptosporidium* spp., *Giardia* spp. in calf diarrhea with one-step duplex RT-PCR: histopathological and immunochromotographic methods

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Abstract

Infections causing diarrhea in cattle breeding pose a substantial threat to calf breeding and health, causing considerable economic losses worldwide, including in Türkiye. The primary causative agents of calf diarrhea include bovine group A rotavirus, bovine coronavirus (BCoV), *Escherichia coli* K99, *Cryptosporidium* spp., and *Giardia* spp. The objective of this study was to diagnose the pathogens present in samples taken from calves exhibiting diarrhea symptoms in the Elaziğ province of Türkiye. The study material consisted of 85 fecal samples from calves up to 30 days of age with diarrhea symptoms and 42 small intestine samples from calves died as a result of diarrhea symptoms after necropsy. Following total RNA isolation from the samples, one-step duplex reverse transcription polymerase chain reaction (RT-PCR) analysis was conducted to identify the bovine rotavirus (BRV) and BCoV. Our results indicated that 24 of the samples were positive for BRV, 13 were positive for BCoV, and 25 were positive for BRV and BCoV in the form of mixed infection. The immunochromatographic analysis revealed that three samples were positive for *E. coli* K99 antigens, 12 for *Cryptosporidium* spp. antigens, and 28 for *Giardia* spp. antigens. Our results indicate that one-step duplex RT-PCR, immunohistochemistry, and immunochromatography methods can be valuable tests to be used in routine diagnostic laboratories for the detection of pathogens associated with calf diarrhea.

Introduction

Neonatal calf diarrhea (NCD) represents a substantial threat to cattle breeding on a global scale. The NCD results in considerable economic losses due to high morbidity, mortality, and treatment costs in calves, and delayed 1st calving in cattle.[1] Calf diarrhea is the primary cause of mortality in dairy calves up to the first 30 days of age after birth, and the mortality rate in this period is around 5.00%.[2,3] A number of enteric pathogens have been identified as a cause of NCD, including viral agents, such as bovine rotavirus (BRV), bovine coronavirus (BCoV), and bovine viral diarrhea virus, parasitic agents, such as *Cryptosporidium parvum* and *Giardia duodenalis*, and bacterial agents, such as *Escherichia coli* K99, *Salmonella* spp., and *Clostridium perfringens.*[1,4-6]

The BRV, BCoV, enterotoxigenic *E. coli* K99, and *C. parvum* are agentscausing diarrhea in calves up to 30 days of age and are frequently diagnosed in calf diarrhea.[7-10] While BRV infections are commonly detected in calves in the 1st to 2nd weeks after birth, BCoV infections are more commonly seen in the age range of five to twenty days.[11] Calves show high susceptibility to *C. parvum* infections between the 1st and 3rd weeks after birth.[12] Mixed infections involving these pathogens are observed more intensively in calves with diarrhea symptoms compared to the healthy ones.[9,13,14] In addition to the infection-causing pathogenic agents, factors, like herd management practices (vaccination, colostrum intake, herd size, biosecurity measures, calf housing, hygiene standards, age-based animal segregation, and nutrition) and environmental conditions, also affect the occurrence of calf diarrhea.[14]

The *C. parvum* and *G. duodenalis* are protozoan parasites infecting both humans and animals,[15,16] and the forms of these parasites found in nature (oocysts and cysts, respectively) are resistant to environmental conditions

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and can be transmitted by fecal-oral route through contaminated food or water sources.[17,18] Currently, 31 *Cryptosporidium* species are recognized in the literature and *Cryptosporidium hominis* and *C. parvum* constitute the majority of human cryptosporidiosis cases.[19,20] The *C. parvum* infects especially unweaned calves and may cause severe symptoms.[21,22]

In this study, it was aimed to investigate the positivity rates of BRV, BCoV, *E. coli* K99, *Cryptosporidium* spp., and *Giardia* spp. in calves up to 30 days of age showing diarrhea symptoms in and around Elazığ province of Türkiye using various diagnostic methods.

Materials and Methods

Fecal and tissue samples collection. In Elazığ and surrounding cattle farms, 85 fecal samples were collected from live calves up to 30 days of age showing diarrhea symptoms and 42 small intestine samples were collected after necropsy from calves of the same age range died with diarrhea symptoms. Necropsy was performed in the necropsy unit at Elazığ Veterinary Control Institute, Elazığ, Türkiye. The samples were immediately transported to the laboratory under cold conditions. A total of 127 fecal and 42 small intestinal samples constituted the study material.

Total RNA isolation. Fecal samples were mixed with phosphate-buffered saline (PBS; pH = 7.40) solution at 10.00% concentration. After incubation at room temperature for at least 30 min, the samples were centrifuged at 3,000 rpm for 10 min and the supernatant was carefully removed and transferred to another tube. For total RNA extraction, a commercial total nucleic acid extraction kit (QIAamp Cador Pathogen Mini Kit (Qiagen, Hilden, Germany) was used and RNA isolation was performed following the manufacturer's instructions. The isolated total RNA was stored at - 80.00 °C until used in the analysis.

Total RNA NanoDrop measurements. The quality and quantity of isolated total RNA were evaluated by measuring the absorbance at 260/280 nm using a NanoDrop device (Denovix ds-11, Wilmington, USA). It was aimed to have a quality value of 2.00 ± 0.10 and a quantitation value of ≥ 50.0 ng μL^{-1}. For samples that did not meet the specified criteria, total RNA isolations and subsequent NanoDrop measurements were repeated.

One-step duplex reverse transcription polymerase chain reaction (RT-PCR). Total RNA isolates were subjected to the one-step duplex RT-PCR analysis for nucleic acid detection of BRV and BCoV. One-step RT-PCR commercial kit (Qiagen, Hilden, Germany) was used the kit protocol was followed, and the analysis was performed. Highly conserved gene regions for both viruses were selected and used in PCR. For this purpose, BCoVF (5'-CGATCATCAGTCGACCAATTCTA3'), and BCoVR (5'-GAGG TAGGGGTTCTTCTGTTGTTGCC-3') primer pair specific for the *N* gene of BCoV, and BRVF (5'-ATGGGTACGATGTGGC TCAA3'), and BRVR (5'-ACCGCTGGTGTCATGTTTTGG-3') primer pair specific for the *VP6* gene of BRV were used. As a result of the PCR reaction, a PCR product of 597 bp for BCoV and 383 bp for BRV was obtained.[23] In one-step duplex RT-PCR analysis, a gradient was applied using positive control samples at 12 different temperature values ranging from 50.00 to 60.00 ˚C for both agents to determine the optimal binding temperature for the selected primers. As a result of the applied gradient, the optimal binding temperature for both pathogen-specific primer pairs was determined to be 55.00 ˚C. The mastermix mixture prepared for the one-step RT-PCR reaction was programmed in the thermal cycler device for the reverse transcription step for 30 min at 50.00 ˚C, initial PCR activation for 15 min at 95.00 ˚C, and PCR steps for 1 min at 94.00 ˚C, for 1 min at 55.00 ˚C, and for 90 sec at 72.00 ˚C (40 cycles), followed by a final extension at 72.00 ˚C for 10 min. The PCR products were subjected to the agarose gel electrophoresis and the results were visualized using the Gel Logic 212 PRO Imaging System (Carestream, Rochester, USA).

Immunohistochemistry (IHC). The BRV and BCoV immunopositivities were determined using streptavidin-biotin complex method using LSAB+ System-HRP commercial kit (Dako, Carpinteria, USA) protocols. Five µm thick small intestinal tissue sections were placed on polylysine slides and subjected to the alcohol and xylene series treatment for endogenous peroxidase inactivation. After washing with PBS solution, the samples were exposed to 3.00% H_2O_2 solution for 10 min. Antigen retrieval was performed by incubating the tissue samples twice at 500 watts for 5 min. The samples were then incubated with rotavirus primary antibody (Anti-BRV mAB; Abnova, Taipei, Taiwan) and coronavirus primary antibody (Anti-BcoV; Biozol, Eching, Germany) for 30 min at 37.00 °C. After incubation, the tissues were washed with PBS solution and incubated with biotinylated antibody and streptavidin-horseradish peroxidase for 15 min each. The 3,3'-diaminobenzidine was used as a chromogen. Sections were then counterstained with Mayer's hematoxylin (Sigma-Aldrich Chemie GmbH, Taufkirchen, Germany) coated with Entellan (Merck, Rahway, USA), and examined under a light microscope (BX53; Olympus, Tokyo, Japan).

Immunofluorescence (IF) and direct IF (DIF). Using a frozen microtome (Leica CM1100, Wetzlar, Germany), 8.00 µm thick sections were taken from 85 fecal and 42 small intestinal samples. The sections were fixed in ethyl alcohol for 15 min and washed with PBS solution. The samples were then incubated with 1/50 diluted fluorescein izotiyosiyanat (FITC)-linked monoclonal rotavirus and coronavirus primary antibodies (FITC Moab a-Bovine Rotavirus BIO 020-BIOX, FITC Moab a-Bovine Coronavirus BIO 023-BIOX) at 37.00 ˚C for 30 min. After incubation, the sections were washed with PBS, treated

with distilled water-glycerol (1/10), cover-slipped, and examined under a fluorescence microscope (Olympus).

Indirect IF (IIF). Using a frozen microtome, 8.00 µm thick sections were taken from 85 fecal and 42 small intestinal samples. After fixation in ethyl alcohol for 15 min and washing with PBS solution, the samples were incubated with 1/50 diluted monoclonal rotavirus and coronavirus primary antibodies (Rotavirus clone-3C10 Abnova, and Coronavirus; Thermo-Fisher, Waltham, USA) at 37.00 ˚C for 30 min. After incubation, the samples were washed with PBS and incubated with fluorescently labeled 1/20 diluted secondary antibody (goat anti-mouse ımmunoglobulin G FITC; Abcam, Cambridge, UK) at 37.00 ˚C for 30 min. After incubation, the samples were washed with distilled water-glycerol solution (1/10), cover-slipped, and examined under the fluorescence microscope (Olympus).

Histopathological examination. Small intestinal tissue sections (5.00 µm thick) were stained with Hematoxylin and Eosin and evaluated for inflammatory, necrotic, and degenerative changes under a light microscope.

Immunochromatographic (ICG) method. In 127 fecal samples, a commercial kit (Anigen Rapid Bovid-5, Bionote, Minnesota, USA) was used for the detection of BRV, BCoV, *Cryptosporidium* spp., *Giardia* spp., and *E. coli* K99 antigens. This test kit is capable of rapid and quantitative detection of BRV, BCoV, *Cryptosporidium* spp., *Giardia* spp., and *E. coli* K99 antigens in calf fecal samples by ICG method. The test protocol of the manufacturer was followed for the detection of antigens of the targeted pathogens in fecal samples.

Statistical analysis. The SPSS Software (version 20.0; IBM Corp., Armonk, USA) for Windows using the Chi-square test was used to evaluate whether there was a statistically substantial difference between one-step duplex RT-PCR, IHC, DIF, IIF, and ICG methods. The results revealed no statistically notable difference among the tests ($p > 0.05$).

Results

One-step duplex RT-PCR. A total of 127 samples, including 42 small intestinal and 85 fecal samples, were analyzed for BRV and BCoV pathogens using the one-step duplex RT-PCR method. In the duplex PCR analysis, bands of 597 bp and 383 bp in size respectively indicated BCoV and BRV positivity, while the presence of both bands in the same sample indicated that the two viral pathogens were seen as a mixed infection (Fig. 1). Duplex RT-PCR analysis revealed that 24 of the samples were positive for BRV, 13 were positive for BcoV, and 25 were positive for both pathogens as a mixed infection. By one-step duplex RT-PCR analysis, viral nucleic acid positivity rates were 18.89% (24/127) for BRV, 10.23% (13/127) for BcoV, and 19.68% (25/127) for mixed infection of both pathogens. It was determined that 48.81% (62/127) of the samples were infected with at least one of the two viral pathogens.

Immunohistochemistry. In 42 small intestinal samples, the presence of BCoV and BRV pathogens was investigated by IHC method. The BRV was positive in 21.42% (9/42), BCoV in 9.52% (4/42), and both pathogens in 14.28% (6/42) as a mixed infection. Viral antigens were predominantly concentrated in intestinal villi and crypts epithelium (Fig. 2).

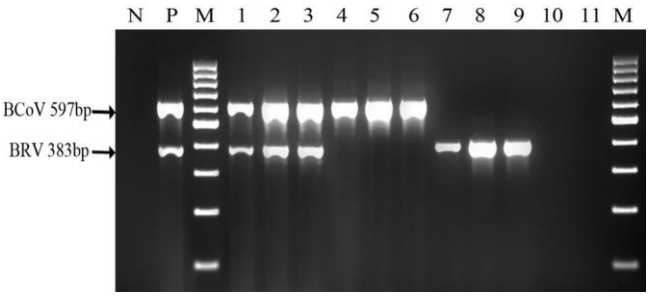

Fig. 1. Bovine rotavirus (BRV) and bovine coronavirus (BCoV) one-step duplex reverse transcription polymerase chain reaction (RT-PCR). Lane N: Negative control; Lane P: Positive control (BRV and BCoV); Lane M: Marker (100 - 1,000 bp); Lanes 1 - 3: BRV and BCoV positive samples; Lanes 4 - 6: BCoV positive samples; Lanes 7 - 9: BRV positive samples; Lanes 10 - 11: BRV and BCoV negative samples.

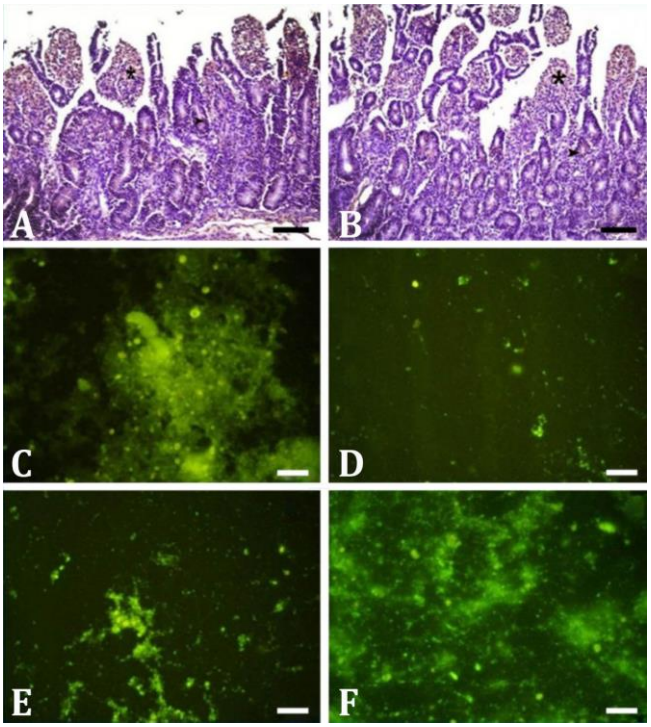

Fig. 2. Immunopositivity and immunofluorescence positivity in intestinal tissue. **A)** Bovine rotavirus (BRV) immunopositivity; **B)** Bovine coronavirus (BCoV) immunopositivity; Asterick: Villi; Arrowheads: crypts epithelium; **C)** BRV direct immuno-fluorescence (DIF) positivity; **D)** BCoV DIF positivity; **E)** BRV indirect immunofluorescence (IIF) positivity; **F)** BCoV IIF positivity; (Bars = 20.00 µm).

Immunofluorescence. A total of 127 samples (42 small intestine and 85 feces) were analyzed for the presence of BCoV and BRV pathogens using DIF and IIF methods. Immunofluorescence positivity was observed as a reflection of fluorescent light of varying sizes and intensities (Fig. 2). The results of the analysis of fecal and small intestinal samples by DIF and IIF are presented in Table 1.

Direct immunofluorescence. A total of 127 samples (42 small intestine and 85 feces) were subjected to the DIF analysis. 26.19% (11/42), 16.66% (7/42), and 23.80% (10/42) of the small intestine samples were positive for BRV, BcoV, and BRV with BCoV, respectively. The BRV was positive in 27.05% (23/85), BCoV in 24.70% (21/85), and mixed infection in 12.94% (11/85) of the fecal samples. Overall, 26.77% (34/127), 22.04% (28/127), and 16.53% (21/127) of a total of 127 samples, including both fecal and small intestinal samples, were positive for BRV, BcoV, and mixed infection, respectively.

Indirect immunofluorescence. A total of 127 samples (42 small intestine and 85 feces) were subjected to the IIF analysis. Of the small intestinal samples, 19.04% (8/42) were positive for BRV, 26.19% (11/42) for BCoV, and 14.28% (6/42) for mixed infection. It was determined that 16.47% (14/85) of the stool samples were infected with BRV, 11.76% (10/85) with BCoV, and 20.00% (17/85) with mixed infection. Overall, 17.32% (22/127), 16.53% (21/127), and 18.11% (23/127) of the 127 samples were positive for BRV, BCoV, and mixed infection, respectively.

Histopathology. Small intestinal specimens being determined positive for BRV and BCoV pathogens by various methods were subjected to the histopathological examination. Histopathological findings indicating necrotic and degenerative changes, villus massing, atrophy, intestinal villi desquamation, inflammatory cell infiltration, and hyperemia in the lamina propria were detected (Fig. 3).

Immunochromatographic method. A total of 127 samples (42 small intestine and 85 feces) were examined by ICG method. It was determined that 17.32% (22/127) of the samples were infected with BRV, 9.44% (12/127) with BCoV, and 18.11% (23/127) with mixed infection. As a result, a total of 127 samples (42 small intestinal and 85 fecal samples) were analyzed for BRV, BCoV, *E. coli* K99, *Cryptosporidium* spp., and *Giardia* spp. by different

Table 1. One-step duplex reverse transcription polymerase chain reaction (RT-PCR), immunohistochemmistry (IHC), direct immunofluorescence (DIF), indirect immunofluorescence (IIF), and immunochromatographic (ICG) analyses results used in the diagnosis of bovine rotavirus (BRV), bovine coronavirus (BCoV), *Escherichia coli* K99, *Cryptosporidium* spp., and *Giardia* spp.

Samples	Pathogens	Number of samples	Diagnostic methods	Positive rate (%)
Small intestine + Stool	**BRV**	127	One-step duplex RT-PCR	24 (18.89)
			DIF	34 (26.77)
			IIF	22 (17.32)
			ICG	22 (17.32)
	BCoV	127	One-step duplex RT-PCR	13 (10.23)
			DIF	28 (22.04)
			IIF	21 (16.53)
			ICG	12 (9.44)
	BRV + BCoV	127	One-step duplex RT-PCR	25 (19.68)
			DIF	21 (16.53)
			IIF	23 (18.11)
			ICG	23 (18.11)
	***E. coli* K99**	127	ICG	3 (2.36)
	***Cryptosporidium* spp.**			12 (9.44)
	***Giardia* spp.**			28 (22.04)
Small intestine	**BRV**	42	One-step duplex RT-PCR	8 (19.04)
			DIF	11 (26.19)
			IIF	8 (19.04)
			ICG	8 (19.04)
			IHC	9 (21.42)
	BCoV	42	One-step duplex RT-PCR	7 (16.66)
			DIF	7 (16.66)
			IIF	11 (26.19)
			ICG	4 (9.52)
			IHC	4 (9.52)
	BRV + BCoV	42	One-step duplex RT-PCR	7 (16.66)
			DIF	10 (23.80)
			IIF	6 (14.28)
			ICG	7 (16.66)
			IHC	6 (14.28)

analytical methods. The BRV agents were detected as 18.89% (24/127), 21.42% (9/42), 26.77% (34/127), 17.32% (22/127), and 17.32% (22/127), using one-step duplex RT-PCR, IHC, DIF, IIF and ICG analyses, respectively. One- step duplex RT-PCR, IHC, DIF, IIF, and ICG analyses were positive for BCoV agents in 10.23% (13/127), 9.52% (4/42), 22.04% (28/127), 16.53% (21/127) of samples, respectively. The frequency of BCoV and BRV pathogens as mixed infections was respectively 19.68% (25/127), 14.28% (6/42), 16.53% (21/127), 18.11% (23/127), 18.11% (23/127), and 18.11% (23/127), by one-step duplex RT-PCR, IHC, DIF, IIF, and ICG analyses. In addition, antigenic positivity rates of *E. coli* K99, *Cryptosporidium* spp., and *Giardia* spp. were 2.36% (3/127), 9.45% (12/127), and 22.04% (28/127), respectively.

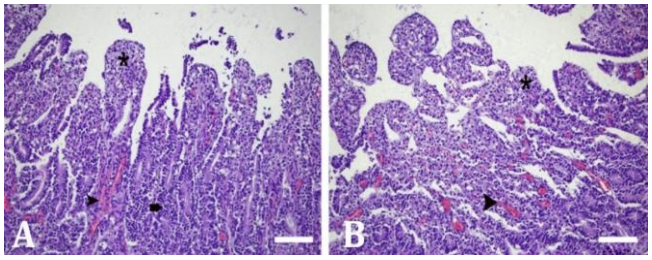

Fig. 3. Histopathological analyses of positive samples of **A)** bovine rotavirus and bovine **B)** coronavirus. Asterick: Villi mass; Arrow: Inflammatory cells infiltration; Arrowhead: Hyperemic vessel; (Hematoxylin and Eosin staining, Bars = 50.00 µm).

Discussion

Neonatal calf diarrhea is a widespread disease worldwide that can cause serious long-term economic losses, such as high morbidity, mortality, growth retardation, treatment costs, and delayed 1st calving.[1] The NCD is the most common cause of death in the 1st 30 days in dairy calves.[2,3] In addition to viral agents, such as BRV, BCoV, and bovine viral diarrhea virüs, enteric pathogens, including parasitic pathogens, such as *C. parvum*, *G. duodenalis*, and *Eimeria* spp., and bacterial pathogens, such as *E. coli* K99, *Salmonella* spp., and *C. Perfringens*, are the infectious agents of NCD.[1,4-6]

The BRV infection can cause substantial economic losses in livestock farms. Techniques, such as electron microscopy, latex agglutination, and enzyme-linked immunosorbent assay (ELISA) are widely used in the diagnosis of the virus. However, it has been suggested that the efficiency of these methods may decrease four to eight days after infection, and it is emphasized that more sensitive diagnostic approaches, such as PCR, which can detect the virus even two to three weeks after infection, are needed.[1,2,24] Although there are limited numbers of studies on BCoV infections in Türkiye, it is widely accepted that it is an important factor in calf diarrhea caused by viral agents worldwide, especially after BRV.[7]

Various diagnostic methods, such as electron microscopy, IF, IHC, hemagglutination, ELISA, cell culture, and PCR, are used for the detection of BRV and BCoV.[4,5,25,26] Among these, PCR is thought to be more sensitive and specific than ELISA, especially in subclinical infections.[25,26]

Epidemiological studies worldwide have reported varying prevalence rates of BRV and BCoV in cattle populations. For example, in Australia, BRV was detected in 79.90% and BCoV in 21.60% of fecal samples.[27] Similar studies conducted in the Netherlands and Sweden reported BRV positivity rates of 17.70% and 47.00%, respectively, and BCoV rates of 7.40% and 8.00%, respectively.[28,29] Differences in prevalence rates may be attributed to differences in practices and environmental factors in cattle breeding facilities.

The prevalence of BRV and BCoV was also investigated in domestic studies. In a study conducted in Elaziğ province, BRV and BCoV were detected in 30.00 and 13.00% of calf fecal samples, respectively, and mixed infection rate was reported as 3.30%.[30] In another study conducted in Kars, BRV and BCoV positivity rates using rapid test methods were reported as 26.90 and 1.00%, respectively.[31] In our study, BRV positivity rates were 18.89 and 17.32%, BCoV positivity rates were 10.23 and 9.44%, and mixed infection rates were 19.68 and 18.11%, using one-step duplex RT-PCR and ICG methods, respectively. Differences in positivity rates may be due to regional differences, animal husbandry practices, and differences in diagnostic methods.

Escherichia coli is one of the most important bacterial agents causing calf diarrhea and various pathotypes are blamed for its etiology.[32,33] *Cryptosporidium* and *Giardia* are also important protozoan agents in calf diarrhea.[11,12] In our study, the positivity rates of *E. coli*, *Giardia* spp., and *Cryptosporidium* spp. by ICG method were 2.36, 22.04, and 9.44%, respectively, and it is thought that the presence of these agents has an important place in calf diarrhea.

In histopathological examination, characteristic changes, such as villous enlargement, lamina propria infiltration, and epithelial cells degeneration associated with BRV and BCoV infections have been reported.[34,35]

There are limited numbers of studies in which immunohistochemical and IF methods were evaluated together in BRV and BCoV infections. Immuno-histochemically, BCoV antigens have been reported to be localized in the lamina propria, villi, and crypts epithelium.[35] In our study, viral antigens were also detected at similar locations using IHC method and positive results were obtained in 83 and 66 samples using DIF and IIF methods, respectively. Compared to the PCR results of our study, it was found that IIF provides similar diagnostic accuracy and can be used safely.

In conclusion, it is thought that it is important to evaluate more than one major pathogenic agent causing diarrhea at the same time for accurate diagnosis in calf

diarrhea. One-step duplex RT-PCR was found to be the most reliable method, while IHC and ICG methods also gave promising results for diagnostic purposes. Although IF method offers ease of application, it can make diagnosis difficult due to the non-specific staining. Therefore, it can be concluded that a comprehensive diagnostic approach combining molecular and immunological methods is the right approach to accurately determine the calf diarrhea etiology.

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Conflict of interest

The authors declare no competing financial interests or personal relationships that could have influenced the work reported in this paper.

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