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Research Article / Araştırma Makalesi

Investigation of Bovine Coronavirus and Bovine Rotavirus in Calves with Neonatal Diarrhea in Kırıkkale and Surrounding Provinces

Kırıkkale ve Çevre İllerindeki Neonatal İshalli Buzağılarda Sığır Koronavirüs ve Sığır Rotavirüs Varlığının Araştırılması

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Abstract: Bovine coronavirus (BCoV) and bovine rotavirus (BRV) infections are very common in neonatal diarrhoea of calves which are of the important problems in cattle breeding. In the present study 110 calves with neonatal diarrhoea from Kirikkale and surrounding provinces were investigated for BCoV and BRV presence by RT-PCR in stool samples and positive BRV samples were genotyped by PCR based on VP4 and VP7 genes. In total, 41 samples were BCoV positive (37.27%) and 41 samples were BRV positive (37.27%), whereas 20 samples were positive for both BCoV and BRV (18.18%). According to the results of the study, BCoV and BRV are the neonatal calf diarrhoea agents in calves reared in Kirikkale, Kirsehir, Ankara, Cankiri, Corum, and Yozgat provinces. Genotyping results of positive BRV samples indicated that G6P[5], G10P[5], G10P[11], and G6P[11] genotypes, commonly seen in Turkey, are circulating among these provinces. Detection of these genotypes indicated importance of vaccination against neonatal diarrhoea and selection of vaccine strain.

Keywords: Bovine coronavirus, Bovine rotavirus, Kırıkkale, RT-PCR.

Öz: Sığır koronavirüs (BCoV) ve sığır rotavirüs (BRV) enfeksiyonları, sığır yetiştiriciliğindeki önemli sorunlardan biri olan neonatal buzağı ishallerinin en sık rastlanan viral etkenleri arasında yer almaktadır. Bu çalışmada, Kırıkkale ve çevre illerindeki neonatal ishalli 110 buzağıda BCoV ve BRV etkenleri RT-PCR ile araştırıldı ve pozitif BRV örnekleri PCR ile VP4 ve VP7 genleri temelli olarak genotiplendirildi. RT-PCR sonuçlarına göre toplamda 41 örnek BCoV pozitif (%37,27), 41 örnek BRV pozitif (%37,27) ve 20 örnek hem BCoV hem de BRV pozitif (%18,18) olarak belirlendi. Bu sonuçlara göre Kırıkkale, Kırşehir, Çankırı, Çorum, Yozgat ve Ankara illerinde yetiştirilen buzağılarda BCoV ve BRV, neonatal buzağı ishallerinin etkenleri olarak tespit edildi. BRV genotiplendirme sonuçlarına göre ise bu illerde ülkemizde yaygın olarak görülen genotipler olan G6P[5], G10P[5], G10P[11] ve G6P[11] genotiplerinin sirküle olduğu belirlendi. Bu genotiplerin buzağılarda saptanması, neonatal ishallere karşı aşılamanın ve aşılarda kullanılacak olan suş seçiminin önemini göstermektedir.

Anahtar Kelimeler: Sığır koronavirüs, Sığır rotavirüs, Kırıkkale, RT-PCR.

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Introduction

Neonatal calf diarrhoea is one of the most challenging global problems for dairy and beef cattle industry which is caused by viruses, bacteria and protozoa. Common infectious causes of neonatal calf diarrhoea are Enterotoxic *Escherichia coli, Cryptosporidium parvum*, bovine rotavirus (BRV) and bovine coronavirus (BCoV) (Foster and Smith, 2009; Lorenz and ark., 2011; Azkur and Aksoy, 2018). In a study investigating the diarrhoea of beef and dairy calves, 36.2% of

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enteric pathogens were found to be viral agents (Cho et al., 2013). Among these viral agents, BRV and BCoV are detected in diarrheic calves as 7-80% and 3-79%, respectively (Gomez and Weese, 2017). BCoV and BRV infections, usually affect calves less than 3 weeks old, are found to be responsible for economical losses in cattle breeding including significant effects on the body weight gain of calves (Torres-Medina et al., 1985; Foster and Smith, 2009; Renaud et al., 2020). The most common cause of calf mortality was reported as diarrhoea in Kars province of Turkey (Erdoğan et al., 2009).

BRV is classified in *Rotavirus A* species, *Rotavirus* genus, *Sedoreovirinae* subfamily of *Reoviridae* family, *Reovirales* order, *Riboviria* realm (ICTV, 2020). BRV has 11 segments of double-stranded RNA which encodes 6 structural (VP1, VP2, VP3, VP4, VP6, VP7) and 5 non-structural (NSP1-NSP5/6) proteins. The classifcation system commonly used for Rotaviruses is based on the sequences of the VP7 (G genotype) and VP4 (P genotype) genes (Matthijnssesns et al., 2011). To date 28 G genotypes and 39 P genotypes are determined in Rotavirus A species (Desselberger, 2017), among them G6, G8, G10 and P[1], P[5], P[11] are regarded as the common bovine genotypes worldwide. In Turkey, presence of G6, G8, G10, P[5], P[11] genotypes of BRV were hitherto reported in cattle (Alkan et al., 2010; Karayel et al., 2017; Aydın and Timurkan, 2018).

BCoV is a member of *Betacoronavirus* genus, *Coronaviridae* family of *Nidovirales* order in *Riboviria* realm (ICTV, 2020). BCoV is enveloped, positive-sense single-stranded RNA virus which is associated with respiratory disease in cattle and severe diarrhoea in neonatal calves (Azkur and Aksoy, 2018; Franzo et al., 2020). BCoV infection was reported in Turkey in both enteric and respiratory diseases of cattle as well as calves (Hasoksuz et al., 2005; Pestil et al., 2016; Aydın and Timurkan, 2018).

The aim of the present study was to investigate prevalence of BCoV and BRV in neonatal diarrhoea of calves which are reared in Kirikkale, Kirsehir, Ankara, Cankiri, Corum, and Yozgat provinces and to determine which BRV genotypes are circulating in these provinces.

Materials and Methods

Sampling

The study includes 110 neonatal calves (0-30 days old) which were already patients of Animal Hospital of Kirikkale University that were reared in Kirikkale, Ankara, Kirsehir, Corum, Cankiri, and Yozgat provinces and have acute diarrhoea. Following clinical examination of calves, stool samples were taken in specimen containers from calves by rectal provocation for defecation for routine clinical diagnosis by parasitological examination and rapid diagnostic kits. The stool samples that were taken for routine clinical diagnosis and stored at -20°C were used in the present study.

RNA isolation from stool samples

One gram of each stool samples was taken in sterile centrifuge tubes, diluted 1:5 in phosphate buffered saline (PBS) and homogenized by vortexing. Samples were centrifuged at 2000 rpm for 20 minutes at 4°C (Allegra X100, Beckman Coulter, USA). After centrifugation, the supernatants were transferred into sterile microcentrifuge tubes and stored at -20°C. RNA isolation from supernatants was performed with commercial kit (740956.50, Macherey-Nagel) by following the instructions of the manufacturer. RNA samples were stored at -80°C until use (DF590, Nüve, Turkey).

Reverse Transcription-Polymerase Chain Reaction (RT-PCR)

The cDNAs from the RNA samples were synthesized within 3 steps. In first step, 4 µl RNA and 2.8 µl dimethylsulphoxide (DMSO) was incubated in 95°C for 5 minutes. In second step, 6 µl sterile distilled water and 1 µl random hexamer primer (PM-301S, Jena Bioscience) were added to the mixture and incubated in 70°C for 5 minutes. In the last step, the tubes were put into cracked ice and 2 µl dNTPs (DN001-0250, GeneDirex), 4 µl

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RT buffer and 1 µl reverse transcriptase (M0253, New England Biolabs) were added to the mixture, and incubated in 25°C for 10 minutes, 37°C for 1 hour and 70°C for 5 minutes.

The cDNA samples were used as template in PCR that is carried out with primers specific for bovine coronavirus (Nucleocapsid protein gene), bovine rotavirus (VP6 gene), and bovine glyceraldehyde 3-phosphate dehydrogenase (GAPDH) genes (Table 1). PCR mixture was containing 3 µl template, 1U Taq DNA polymerase (MB101-0500, GeneDirex), 1.25 mM dNTPs (DN001-0250, GeneDirex), 25 mM MgCl2, 10× PCR buffer, 10 pmol forward primer, 10 pmol reverse primer in 50 µl total volume. PCR was carried out in 35 cycles as following conditon: 94°C for 3 minutes, 94°C for 50 second, 55°C for 50 second, 72°C for 1 minute and 72°C for 10 minutes for final extention. PCR products were visualized under UV transluminator after ethidium bromide stained gel electrophoresis.

Genotyping of Bovine Rotavirus

Rotavirus PCR-positive samples were tested for VP4 ([P] genotyping) and VP7 (G genotyping) genes to determine genotypes.

Rotavirus VP4 ([P] genotyping) genotyping were carried out in 2 steps. For first round for VP4 genotyping, 3 µl cDNA, 1U Taq DNA polymerase, 1.25 mM dNTPs, 25 mM MgCl2, 10× PCR buffer, 10 pmol Con3 forward primer, 10 pmol Con2 reverse primer were mixed in 50 µl total volume. According to electrophoresis results, the samples have 877 bp product were determined as positive whereas the samples do not have product were determined as negative. In the second round of PCR, 1 µl (from VP4 positive samples) or 5 µl (from VP4 negative samples) first round PCR product, 1U Taq DNA polymerase, 1.25 mM dNTPs, 25 mM MgCl2, 10× PCR buffer, 10 pmol P1-P5-P11 forward primer mixture, and 10 pmol Con2 reverse primer were used. All optimized PCR conditions were shown in Table 2. Samples were genotyped as P[1], P[5] and P[11] if 624 bp, 552 bp, and 314 bp products were seen, respectively.

Rotavirus VP7 (G genotyping) genotyping were carried out in 2 steps. For the first round of VP7 genotyping, 3 µl cDNA, 1U Taq DNA polymerase, 1.25 mM dNTPs, 25 mM MgCl2, 10× PCR buffer, 10 pmol INI forward primer, and 10 pmol FIN reverse primer were used in first round PCR. According to electrophoresis results, the samples have 1062 bp product were determined as positive whereas the samples do not have product were determined as negative. In the second round PCR, 1 µl (from VP7 positive samples) or 5 µl (from VP7 negative samples) first round PCR product, 1U Taq DNA polymerase, 1.25 mM dNTPs, 25 mM MgCl2, 10× PCR buffer, 10 pmol DT6 primer, 10 pmol HT8 primer, 10 pmol ET10 primer, and 10 pmol INI primer were mixed. All optimized PCR conditions were shown in Table 2. Samples were genotyped as G6, G8 and G10 if 499 bp, 273 bp, and 714 bp products were seen, respectively.

Table 1. Primers used for detection of bovine rotavirus, bovine coronavirus and bovine GAPDH.

Target	Primer	Sequence (5'→3')	Product
Bovine rotavirus	BRV - F	GTTTTCCAAGAGTDATHAHYTCAGC	214 bp
	BRV - R	ACCGCTGGTGTCATGTTTGG	
Bovine coronavirus	BCoV - F	CGATCAGTCCGACCAATCTA	597 bp
	BCoV - R	GAGGTAGGGGTTCTGTTGCC	
Bovine GAPDH	GAPDH - F	GGTCACCAGGGCTGCTTTTA	222 bp
	GAPDH - R	CCAGCATCACCCCACTTGAT	

F: Forward primer, R: Reverse primer, bp: base pair.

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Table 2. Primers used for genotyping of bovine rotavirus and PCR conditions.

Gene	Primer	Sequence (5'→3')	Product	PCR round and number of cycles	PCR conditions
VP4	Con3 - F	TGGCTTCGCTCATTTATAGACA	877 bp	1st 35 cycles	94°C 3 min 94°C 1 min 54°C 1 min 72°C 1 min 72°C 10 min
	Con2 - R	ATTTCGGACCATTTATAACC			
	P1- F	ACCAACGAACGCGGGGGTG	624 bp	2nd 30 cycles	94°C 3 min 94°C 30 sec 47°C 30 sec 72°C 45 sec 72°C 10 min
	P5 - F	RCCAGGTGTCRCATCAGAG	552 bp		
	P11 - F	GGAACGTATTCTAATCCGGTG	314 bp		
VP7	INI - F	GGCTTTAAAAGMGAGAAWTT	1062 bp	1st 35 cycles	94°C 3 min 94°C 1 min 47°C 1 min 72°C 2 min 72°C 10 min
	FIN - R	GGTCWCATCATACAAYTCT			
	DT6 - R	CTAGTTCCTGTGTAGAATC	499 bp	2nd 30 cycles	94°C 3 min 94°C 30 sec 42°C 30 sec 72°C 45 sec 72°C 10 min
	HT8 - R	CGGTTCCGGATTAGACAC	273 bp		
	ET10 - R	TTCAGCCGTTGCGACTTC	714 bp		

F: Forward primer, R: Reverse primer, bp: base pair, min: minutes, sec: seconds.

Table 3. Sampling provinces and cattle breeds included in the study.

		Kirikkale	Kirsehir	Cankiri	Corum	Yozgat	Ankara	Total
Breed	**Simmental**	43	13	4	9	1	8	78
	Montofon	5	5	0	1	1	0	12
	Holstein	3	0	1	0	0	1	5
	Crossbred	4	4	4	0	2	1	15
	Total	**55**	**22**	**9**	**10**	**4**	**10**	**110**

Results

A total of 110 stool samples were included in the present study from calves with neonatal diarrhea which were brought from Kirikkale, Kirsehir, Ankara, Cankiri, Corum, and Yozgat provinces to the Kirikkale University Veterinary Faculty Animal Hospital (Table 3).

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RT-PCR was carried out for screening of BCoV (nucleocapsid protein gene) and BRV (VP6 gene) from 110 stool samples. Samples have 597 bp and 214 bp products were evaluated as BCoV- and BRV-positive, respectively (Figure 1). Forty-one of 110 samples were determined as BCoV positive (37.27%) and 41 samples were BRV positive (37.27%). Distribution of BCoV and BRV positive samples among provinces were given in Table 4. Total of 20 samples were detected as BCoV and BRV double positive (18.18%).

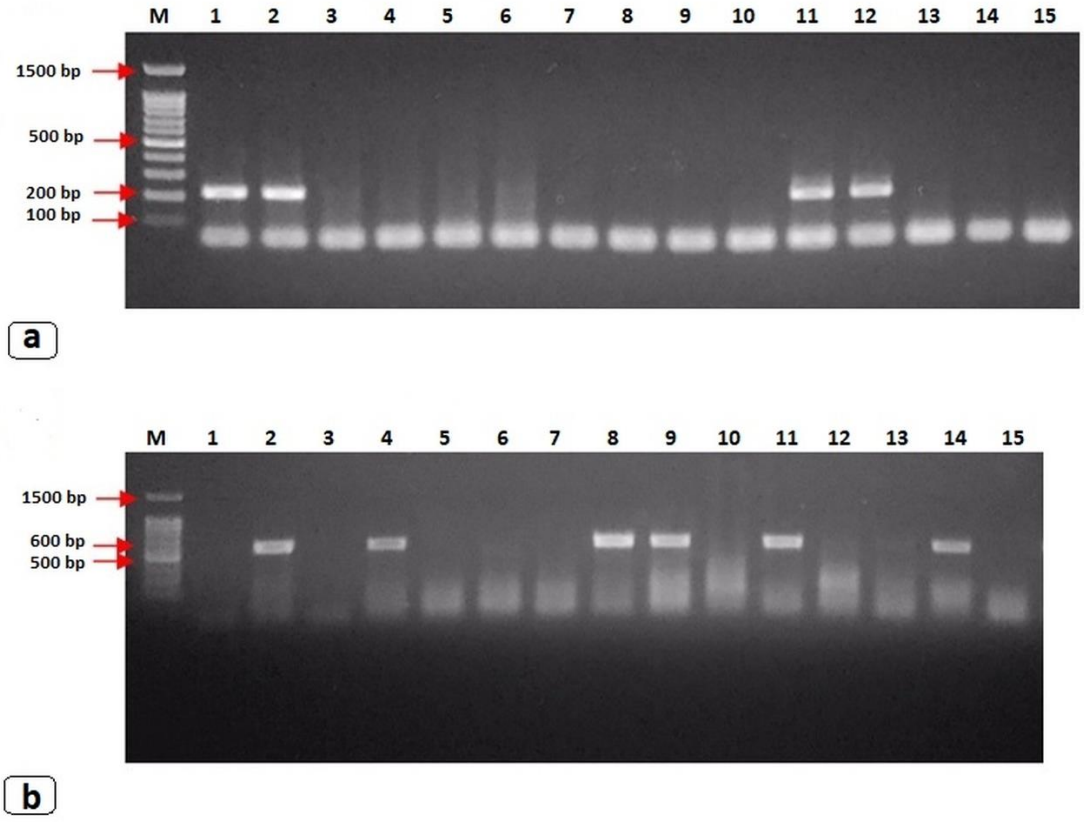

Figure 1. RT-PCR results of **a:** bovine rotavirus (214 bp) and **b:** bovine coronavirus (597 bp). M: DNA marker, 1-15: samples.

Table 4. Number of positive samples for bovine rotavirus (BRV) and bovine coronavirus (BCoV) among provinces.

	Kirikkale	Kirsehir	Cankiri	Corum	Yozgat	Ankara	Total
BRV	19	6	4	5	2	5	41
BCoV	23	8	1	3	1	5	41

Following screening of samples for presence of BCoV and BRV by RT-PCR, BRV-positive samples were analysed for genotyping based on VP4 and VP7 genes. Firstly P genotyping based on VP4 gene was carried out and whole VP4 gene of BRV was amplified (877 bp). The samples have 624 bp, 552 bp, and 314 bp products were characterised as P[1], P[5], and P[11], respectively. According to results of VP4 genotyping, 31 samples were P[5], 7 samples were P[11], 1 sample was P[5]+P[11], and 2 samples were suspected out

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of 41 BRV-positive samples (**Figure 2**). None of the samples were genotype P[1].

VP7 gene based PCR to determine G genotypes of BRV-positive samples were implemented and whole length VP7 gene (1062 bp) was amplified. Following amplification of VP7 gene, the samples have 499 bp, 273 bp, and 714 bp products were determined as G6, G8, and G10, respectively. According to results of VP7 genotyping, 13 samples were G6, 6 samples were G10, 21 samples were G6+G10, 1 sample was suspected out of 41 BRV-positive samples (Figure 3). G8 genotype was not determined in any samples.

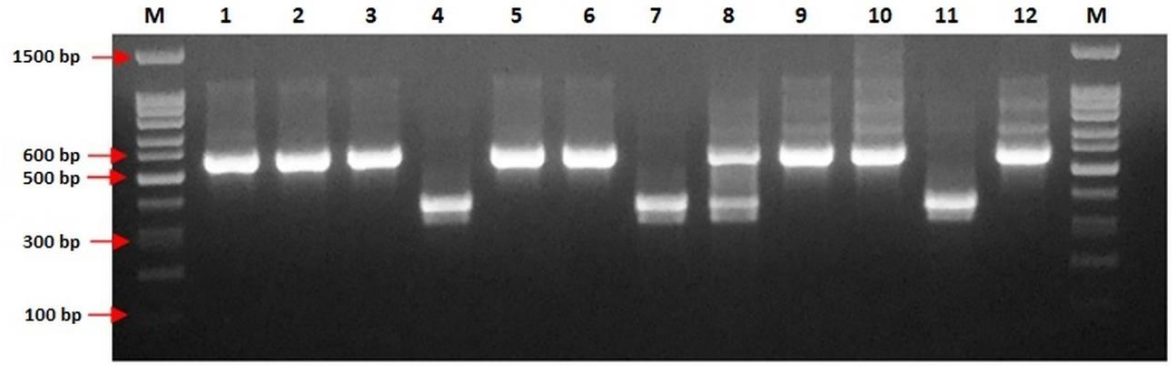

Figure 2. Bovine rotavirus VP4 genotyping results. The samples have 552 bp and 314 bp products were determined as P[5] and P[11], respectively. M: DNA marker, 1-12: samples.

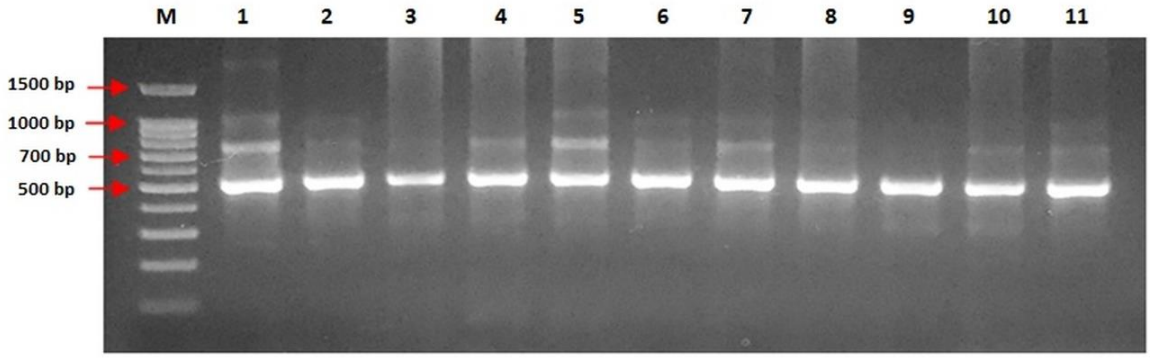

Figure 3. Bovine rotavirus VP7 genotyping results. The samples have 499 bp and 714 bp products were determined as G6 and G10, respectively. M: DNA marker, 1-11: samples.

Genotyping results of BRV-positive samples indicated that 11 samples were G6P[5] and 6 samples were G10P[11]. According to results some samples have two genotypes (19 samples were G6P[5]+G10P[5], 1 sample was G6P[11]+G10P[11]) and one sample has four genotypes (G6P[5] + G10P[5] + G6P[11] + G10P[11]). Three samples were determined as suspected by PCR, 2 out of 3 suspected samples were only genotyped as G6, and 1 sample was only genotyped as P[5].

As a result of the study, it was determined that G6P[5], G6P[11], G10P[5] and G10P[11] genotypes were circulated in diarrhea cases of neonatal calves reared in Kirikkale, Kirsehir, Corum, Cankiri, Yozgat and Ankara provinces (Table 5). In total BRV genotype G6P[5], G6P[11], G10P[5], and G10P[11] were detected in 31, 2, 20, and 8 samples in consideration of double- and multi-positive samples.

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Table 5. Distribution of bovine rotavirus (BRV) genotypes among provinces.

		Kirikkale	Kirsehir	Cankiri	Corum	Yozgat	Ankara	Total
BRV genotypes	**G6P[5]**	17	4	3	4	1	2	31
	G10P[5]	10	4	2	3	1	-	20
	G10P[11]	2	2	1	1	1	1	8
	G6P[11]	1	1	-	-	-	-	2
	G6P[?]*	-	1	-	-	-	1	2
	G?P[5]**	-	-	-	-	-	1	1

**VP4 genotyping can not be determined. **VP7 genotyping can not be determined.

Discussion

Bovine rotavirus (BRV) and bovine coronavirus (BCoV) infections are the most important viral agents of neonatal calf diarrhoea which is economically significant disease for cattle industry because of mortality, cost of medication, labour needed to treat sick calves, delayed growth of calves, etc (Azkur and Aksoy 2018). Due to importance of BRV and BCoV in neonatal calf diarrhoea, in the present study, BRV and BCoV presence was investigated by RT-PCR in stool samples of neonatal diarrhoeic calves reared in Kirikkale, Kirsehir, Ankara, Cankiri, Corum, and Yozgat and circulating genotypes of BRV were determined.

BRV and BCoV presence in Turkey have been reported in many researches, determining prevalence of these infections by serological and molecular methods in numerous provinces. Some researchers used ELISA to detect BRV and/or BCoV in diarrhoea of neonatal calves (Hasoksuz et al., 2005; Çabalar et al., 2007), whereas rapid diagnostic kits are another tool for antibody detection against BRV and BCoV (Al and Balıkçı, 2012; Altuğ et al., 2013). RT-PCR and sequencing are molecular methods for both detection and phylogenetic studies of BRV and BCoV, and many studies had been submitted local strains of BRV and BCoV to Genbank (Alkan et al., 2010; Karayel et al., 2017; Aydın and Timurkan, 2018). In this study, RT-PCR were carried out to determine BRV and BCoV in stool samples of calves and which genotypes of BRV were circulating in the study area, however positive BRV and BCoV samples were not sequenced due to financial reasons.

Studies for BRV and BCoV infection in Turkey showed wide range of prevalence rates. In a study conducted in Erzurum, BRV positivity was found to be 6.1% and BCoV positivity was 12.1% (Aydın and Timurkan, 2018). BCoV positivity in many provinces of Marmara region was reported as 2% (Pestil et al., 2016). BCoV infection in diarrhoeic calves was determined as 10.8% in a study involving many provinces of Turkey including Ankara which is also studied in the present study (Alkan et al., 2011). In the present study, both BCoV positivity and BRV positivity was determined as 37.27% and 18.18% of samples were detected as positive for both BCoV and BRV. The higher rate of positivity could be affected by many factors, such as sampling region, season, sample size, vaccination status of cows, housing of the calves, etc.

The previous studies showed that main genotypes of BRV in many provinces of Turkey are G6P[5], G6P[11], G10P[5], G10P[11] (Alkan et al., 2010; Aydın and Timurkan, 2018), and distinctively G8P[5] genotype of BRV was reported in Amasya (Karayel et al., 2017). In the present study, genotyping of BRV indicated that G6P[5], G6P[11], G10P[5], and G10P[11] genotypes are circulating among Kirikkale, Kirsehir, Ankara, Cankiri, Corum, and Yozgat provinces, consistent

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with the results of the previous studies (Alkan et al., 2010; Aydın and Timurkan, 2018).

Although natural infection of BRV and BCoV in field are very common and most of cows are found to be seropositive, studies showed that antibody titers in milk decrease to non-protective levels. Thus, maternal antibody level in colostrum can be increased by vaccination of cows against BRoV and BCoV in order to protect newborn calves. BRV and BCoV vaccination, usually in combination with *E. coli*, is administrated to pregnant cows in order to induce immunity and maternal antibody production in dams which is then transferred to calves with colostrum. Protection level of the vaccine depends on identity of the vaccine strain and the field strain, suggesting that if the vaccines do not contain field strains, vaccination may not be protective against circulating strains (Gomez and Weese 2017). Vaccines used for BRV and BCoV in Turkey contain G6, G10, P[1], P[5] genotypes of BRV however circulating BRV genotypes in Turkey are G6, G8, G10, P[5], and P[11], which means vaccines in practice could not cover the circulating genotypes and may not be protective for natural field infection.

In the conclusion, BRV and BCoV infections are widely distributed in Kirikkale, Kirsehir, Ankara, Cankiri, Corum, and Yozgat provinces and seem very important agents of neonatal calf diarrhoea in this region. Circulating BRV genotypes are G6P[5], G6P[11], G10P[5], and G10P[11] in these provinces, and this data showed that field strains are different from vaccine strains. Considering the strains available in the BRV and BCoV vaccines used against calf diarrhea in Turkey, this study shows that the vaccine strains are not compatible with the circulating strains in the field and therefore the calves cannot be protected against neonatal calf diarrhea even if they take colostrum. In this context, the fact that the vaccines used in Turkey should contain up-to-date field strains which will prevent calf diarrhea and accompanying economic losses.

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