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Diarrhea in Neonatal Calves - Bacterial and Viral Factors

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ABSTRACT

***Background*:** Neonatal calf diarrhea is one of the most important problems of calf breeding in the world. It causes serious economic losses by causing deaths in calves in the 1st 10 days of their lives. Although many factors cause calf diarrhea, *Escherichia coli* (*E. coli*), *Salmonella* spp., *Campylobacter* spp., viral agents such as Bovine Rotavirus (BRV) and Bovine Coronavirus (BCoV) and parasitic agents such as *Cryptosporidium* spp., *Eimeria* spp. and *Toxocara* spp. frequently plays a role in calf diarrhea. In addition to these infectious factors, the formation of diarrhea has been linked to a number of other factors, including unfavorable barn conditions, mass rearing, inadequate cleaning and disinfection of barn tools, delaying the feeding of colostrum to newborn calves, failing to do so, and failing to disinfect the umbilical cord after delivery. The identification of the microorganisms and virulence factors responsible for diarrhea in newborn calves will direct the development of preventative measures and control strategies. The present study aimed to determine various virulence factors (*Stx1*, *Stx2*, *STa*, *eaeA*, *K99* and *F41*) of *Escherichia coli* isolated from fecal samples and to investigate the prevalence of BRV, BCoV and *E. coli K99* agents which play a role in the etiology of neonatal calf diarrhea in Burdur province.

***Materials, Methods & Results*:** This study was carried out in 75 different cattle farms with diarrhea problem in Burdur province and its districts. Rectal swab samples were taken from 90 calves with diarrhea between 0-4 weeks of age, using 2 sterile swabs from each animal. The swabs are used for the detection of BRV, BCoV and *E. coli* K99 by direct ELISA, and the isolation of *E. coli*. The swabs were cultured on blood agar including 7% sheep blood and MacConkey agar. The isolated bacteria were identified by conventional bacteriological culture methods such as Gram staining, triple tube method, oxidase. The bacteria isolated and identified as *E. coli* were stored at -20ºC by using. Enzyme linked immunobinding assay (ELISA) was positive in 45 of the calf fecal samples. BRV was detected in 28 (31.11%) of samples, BCoV in 15 (16.67%) and *E. coli K99* in 11 (12.22%) samples by ELISA. While BRV and BCoV were detected together in 5.56% of the samples, BRV and *E. coli* K99 were detected in 1.11% of samples, BCoV, *E. coli K99* were detected in 1.11% of samples and all three infections were detected together in 1.11% of samples. The virulence factors of *E. coli* was investigated by polymerase chain reaction (PCR) and *Stx1*, *K99*, *F41* and *eaeA* virulence genes were determined in 2, 5, 3, 4 of samples, respectively. *K99* and *F41* antigens were detected together only in 2 of the *E. coli* isolates. The according to ages, it was determined that the highest BRV was detected between 1-7 days of age and 8-15 days of age. While *E. coli* was detected in 5 of 1-day-old calves, BCoV was also detected in 1 of these calves.

***Discussion*:** In this study, it has been determined that neonatal calf diarrhea is mostly caused by BRV in Burdur province, followed by BCoV and *E. coli*. Even though *E. coli* was recovered from the samples, the inability to extract virulence factors suggests that additional virulence factors might potentially be involved in the infection. It was concluded that the determination of virulence factors of *E. coli* isolates can be a guide in the preparation of protection and control strategies in calf diarrhea.

Keywords: calf diarrhea, bovine coronavirus, *E. coli*, bovine rotavirus, ELISA, PCR.

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INTRODUCTION

Calf deaths are one of the most important causes of calf losses in Türkiye, as well as all over the world. About 45% of calf deaths can be listed as diarrhea, 25% as pneumonia and enteritis, 20% as pneumonia and 10% as other causes [10]. The etiology of calf diarrhea includes viral factors such as BRV and BCoV, bacterial factors such as *E. coli*, *Salmonella* spp. and *Campylobacter* spp., and parasitic factors such as *Cryptosporidium*, *Toxocara* and *Eimeria* [5,21]. Apart from these infectious factors, it has been reported that many factors such as unfavorable barn conditions, mass rearing, lack of cleaning and disinfection of tools used in barns, not giving colostrum to newborn calves at the appropriate time and in sufficient quantities or not at all, and not performing umbilical cord disinfection after birth are effective in the formation of diarrhea [21,23]. The most frequently isolated bacterial agent from newborn calf diarrhea and deaths is *E. coli*. *E. coli* is named as enterotoxigenic *E. coli* (ETEC), enteropathogenic *E. coli* (EPEC), shigatoxigenic *E. coli* (STEC), and necrotoxigenic *E. coli* according to their virulence properties and clinical symptoms in animals [22]. Rotavirus and Coronavirus are the most common viral factors detected in neonatal calf diarrhea. Infections caused by these viruses generally have a subclinical course in adult cattle. This situation leads to the spread of infection within the herd and infections of newborns [25,29]. The aim of this study, it is to determine various virulence factors (*Stx1*, *Stx2*, *STa*, *eaeA*, *K99* and *F41*) of *E. coli* isolates by PCR, and the prevalence of BRV, BCoV and *E. coli K99* agents which play a role in the etiology of neonatal calf diarrhea.

MATERIALS AND METHODS

Animal sampling

This study was conducted in 75 different cattle farms with diarrhea problems in Burdur province and its districts. Rectal swab samples were taken from 90 diarrheal calves between the ages of 0-4 weeks in the farms, using sterile swabs, 2 from each animal. One of the swabs was brought to the Department of Microbiology for bacteriological culture, and the other was brought to the Department of Virology in a cold chain for virological diagnosis.

Isolation and identification

Swab samples were inoculated onto blood agar[1] including 7% sheep blood and MacConkey agar[2]. Petri dishes were incubated in an aerobic environment at 37°C for 18-20 h. Colonies obtained at the end of this period were identified by conventional bacteriological methods (Gram staining, triple tube method, oxidase, etc.) [9]. The bacteria isolated and identified as *E. coli* were stored at -20 ºC until use.

Enzyme linked immunosorbent assay (ELISA)

Swab samples collected from calves were 1st diluted with phosphate buffer solution (PBS) with a pH of 7.2-7.4 at a ratio of 1:2 and vortexed. Fecal samples were collected by pipetting into a clean eppendorf tube. The presence of specific antigens against BRV, BCoV and *E. coli* K99 in fecal samples was investigated with a commercial ELISA kit[3] (IDEXX Rota-Corona–K99 Ag Test). ELISA testing was performed following the kit protocols. The obtained values were calculated by placing them in the formula according to the kit procedure.

Molecular diagnostics

-DNA extraction

DNA was extracted by the boiling method from bacteria isolated from fecal samples and identified as *E. coli* by conventional methods [27]. For this purpose, *E. coli* isolates were inoculated onto MacConkey agar and incubated in an aerobic environment at 37°C for 18-20 h. Colonies were collected into sterile eppendorf tubes and homogenized by adding 100 µL bidistilled water and then boiled at 100°C for 10 min and centrifuged. The upper liquid containing DNA was transferred to sterile eppendorf tubes and stored at -20ºC until use.

-Polymerase chain reaction (PCR)

K99, *F41*, *Sxt1* and *Sxt2*, intimin (*eaeA*), *F41*, *K99* and *STa* virulence genes of *E. coli* were investigated by multiplex PCR. Amplification was performed following an initial denaturation of 1 min at 94°C, followed by 25 cycles of 30 s at 94°C, 45 s at 50°C, 1 min at 70°C, and 10 min at 70°C [14]. The obtained PCR products were run on a gel containing 1.5% agarose and the bands formed according to Table 1 were evaluated as positive.

Table 1. Virulence factors, primer sequence and DNA size of *E. coli*.

Target gene	Primer sequence	DNA size
Stx1	Forward-TTCGCTCTGCAATAGGTA Reverse-TTCCCCAGTTCAATGTAAGAT	555 bp
Stx2	forward-GTGCCTGTTACTGGGTTTTTCTTC reverse-AGGGGTCGATATCTCTGTCC	118 bp
eaeA	forward-ATATCCGTTTTAATGGCTATCT reverse-AATCTTCTGCGTACTGTGTTCA	425 bp
F41	forward-GCATCAGCGGCAGTATCT reverse-GTCCCTAGCTCAGTATTATCACCT	380 bp
K99	forward-TATTATCTTAGGTGGTATGG reverse-GGTATCCTTTAGCAGCAGTATTTC	314 bp
STa	forward-GCTAATGTTGGCAATTTTTATTTCTGTA reverse-AGGATTACAACAAAGTTCACAGCAGTAA	190 bp

RESULTS

In this study, fecal samples were collected from 90 diarrheal calves from 75 farms in Burdur province and surrounding villages. It was learned that pregnant cows were not vaccinated as a preventive measure in the sampled enterprises. In bacteriological culture, 79 *E. coli*, 2 *Klebsiella pneumoniae* and 2 *Citrobacter* spp. were isolated from the samples, while 8 samples were not isolated. *E. coli* and *Citrobacter* spp. were isolated together from 1 of the samples.

In the direct ELISA, which investigated the presence of antigen, positivity was detected in 45 of the fecal samples. The presence of rotavirus was determined mostly in calves with diarrhea. While *E. coli* was isolated from 79 of the samples in the culture of the feces, *E. coli K99* positivity was detected by antigen ELISA in only 11 of the same samples (Table 2). By ELISA, BRV was detected in 28 (31.11%) of the samples, BCoV in 15 (16.67%) and *E. coli K99* in 11 (12.22%). While BRV and BCoV were detected together in 5.56% of the samples, BRV and *E. coli K99* were detected in 1.11%, BCoV and *E. coli K99* were detected in 1.11%, and all three infections were detected together in 1.11%.

When BRV, BCoV and *E. coli K99* positivity was evaluated by ELISA according to ages, it was determined that the highest BRV was detected between 1-7 days of age (n = 11 samples) and 8-15 days of age. While *E. coli* was detected in 5 of 1-day-old calves, BCoV was also detected in 1 of these calves (Table 3).

When the presence of infection was evaluated according to gender by antigen ELISA, 28 female and 26 male animals out of 90 calves were found to be positive for the investigated diseases. Accordingly, the highest BRV was detected in male and female calves (Table 4).

The presence of *Stx1*, *Stx2*, *eaeA*, *F41*, *K99* and *STa* virulence factors in the *E. coli* isolates was investigated by conventional PCR (Figure 1-3). The presence of *Stx1* in 2 of the *E. coli* isolates, *K99* in 5, *F41* in 3, and *eaeA* in 4 were detected. *K99* and *F41* antigens were detected together in 2 of the *E. coli* isolates (Figure 3). *F41* was not found as a single virulence factor in any of the *E. coli* isolates. One of the isolates carrying *F41* also carried *eaeA*, one *K99* and one *K99* + *eaeA* virulence factors. *Stx2* and *STa* could not be detected in any of the *E. coli* isolates (Table 2).

While *K99* antigen and other virulence factors were not detected by PCR in 7 of the 11 fecal samples in which *E. coli K99* was detected by ELISA, *K99* was detected in 4 (Table 2). It was determined that the *E. coli* isolated from 4 samples found to be *K99* positive by ELISA carried the K99 antigen by PCR, and the *E. coli* isolated from a sample determined to be PCR negative by ELISA also had the *K99* antigen (Table 2). F41 and *eaeA* genes were found together in only 1 of the *K99* positive isolates. In the fecal samples detected negative by ELISA, virulence factors were determined at different rates in 4 of the *E. coli* isolates. *eaeA* and *F41* were detected in 1 of these isolates, *eaeA* and *Stx1* in 2, and *F41* and *K99* in 1 (Table 2).

Table 2. Samples and virulence factors found to be *E. coli K99* positive by ELISA.

Sample nº.	Antigen ELISA	Virulence genes					
		Stx1	*Stx2*	*eaeA*	*F41*	*K99*	*STa*
21	+	-	-	-	-	-	-
31	+	-	-	-	-	-	-
39	+	-	-	-	-	-	-
44	+	-	-	-	-	+	-
50	-	-	-	+	+	-	-
63	-	+	-	+	-	-	-
66	+	-	-	-	-	+	-
67	+	-	-	-	-	-	-
69	-	+	-	+	-	-	-
75	+	-	-	-	-	-	-
77	+	-	-	-	-	-	-
87	+	-	-	-	-	-	-
89	+	-	-	+	+	+	-
90	-	-	-	-	+	+	-
96	+	-	-	-	-	+	-

Table 3. Distribution of infection in calves according to age by antigen ELISA.

Antigen ELISA	Age of calves (days)				
	1-7 days	8-15 days	16-21 days	22-28 days	29-40 days
BCoV (n = 15)	9	6	-	-	-
BRV (n = 28)	11	14	-	-	3
E. coli K99 (n = 11)	9	1	-	-	1
Total (n = 54)	28	21	-	-	4

n= Number of samples.

Table 4. Distribution of infection in calves according to gender by antigen ELISA.

Calves	BCoV	BRV	*E. coli K99*
Female (n = 28)	9	15	4
Male (n = 26)	6	13	7
Total (n = 54)	15	28	11

n= Number of samples.

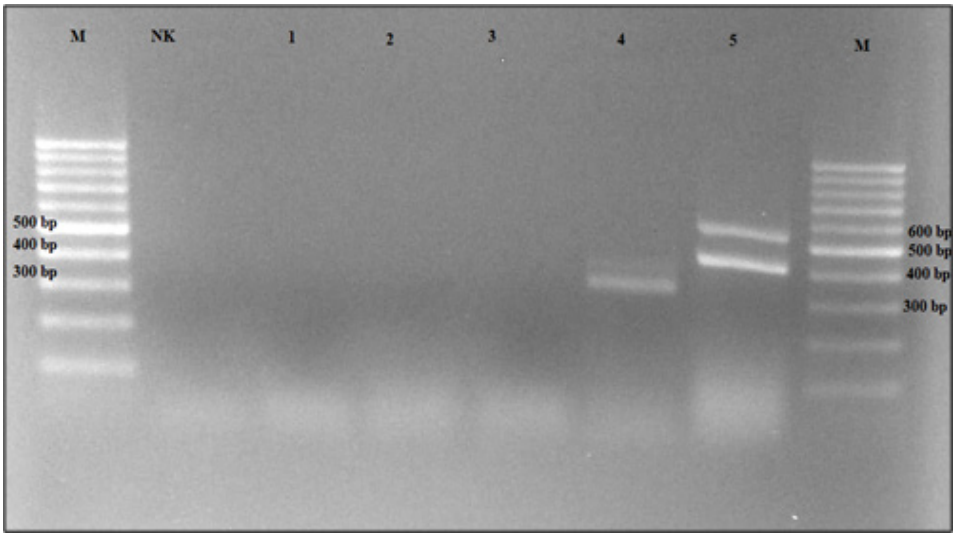

Figure 1. Multiplex PCR results of *E. coli* isolates (*eaeA*-425 bp, *F41*-380 bp, *Stx1*-555 bp). M- Marker, NK-Negative control, 1,2,3-Negative samples, 4-sample number 89 (*eaeA* ve *F41* positive), 5-sample number 63 (*eaeA* and *Stx1* positive).

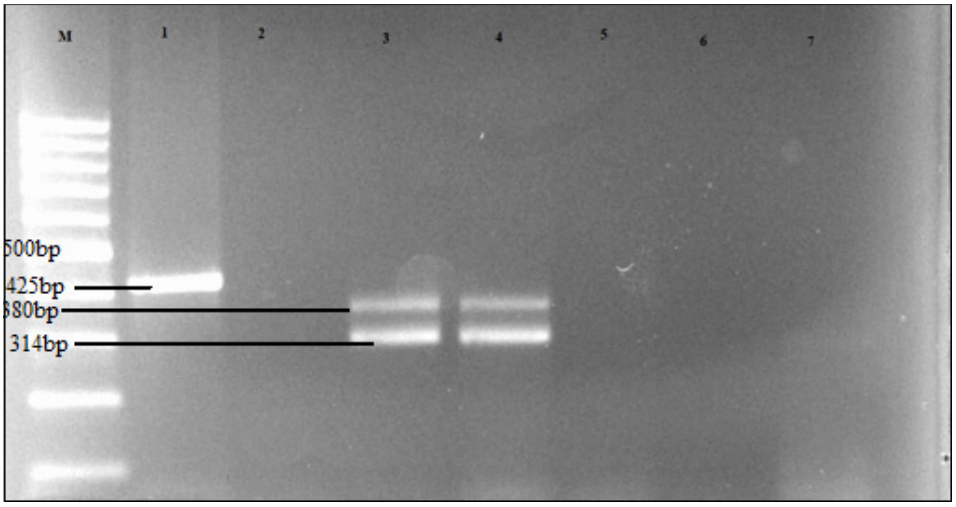

Figure 2. Multiplex PCR results of *E. coli* isolates (*eaeA*-425 bp, *K99*-314 bp, *F41*-380 bp) M-Marker, 1- sample number 50 (*eaeA* positive), 2, 5-7-Negative samples, 3,4- samples number 89 and 90 (*K99* and *F41* positive).

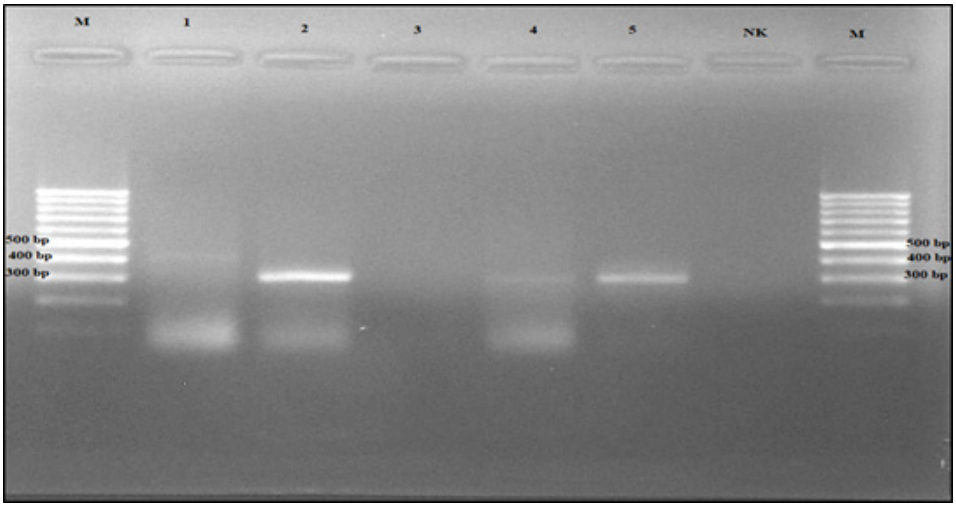

Figure 3. Multiplex PCR results of *E. coli* isolates (*eaeA*-425 bp, *F41*-380 bp *K99*-314 bp) M-Marker, 1- sample number 63 (*eaeA* positive), 2,4,5- sample numbers 66, 44 and 96 (K99 positive) NK-negative sample.

DISCUSSION

Calf diarrhea is a disease symptom that causes serious economic losses by causing high rates of mortality and morbidity in neonatal calves in cattle breeding enterprises. Although many factors cause calf diarrhea, microorganisms are the most important cause. *E. coli* from bacterial agents, BRV and BCoV from viral agents, and *Cryptosporidium* spp. from parasitic agents are frequently detected in calves with diarrhea [8,11,16]. In studies, these microorganisms were reported alone or as mixed infections in calves with diarrhea [2,11,16]. Many researchers found that *E. coli* was mostly isolated from calves with diarrhea, in addition, they stated that other bacterial factors may also cause diarrhea [1,2,8,15]. It was reported the isolation of *E. coli* from 47% of calves with diarrhea, followed by *Salmonella* Typhimurium (1%) and *Pseudomonas aeruginosa* (4%) [1]. In this study, similar to the findings obtained in the previous studies, the highest number of *E. coli* (n = 79) was isolated from calves with diarrhea problems in different enterprises. Additionally, *K. pneumoniae* was isolated from 2 of the calves and *Citrobacter* spp. was isolated from 2 of the calves, but no isolation was made from 8 samples. Similar to other research results, in this study, *E. coli* was detected alone and as a mixed infection in calves with diarrhea. *E. coli* was not isolated from 8 calves with diarrhea. It was thought that the cause of diarrhea in these calves might be other microorganisms. Many researchers have reported that neonatal calf diarrhea may be caused by bacterial, viral and parasitic infections [8,15,29]. As a matter of fact, in this study, the presence of parasitic infections and other viral infections other than BRV and BCoV was not investigated.

Diarrhea progresses within 2-10 days in newborn calves, usually with high mortality and morbidity in the neonatal period. *E. coli* causes diarrhea in calves younger than 7 days of age and mostly in the 1st 12 h of their lives [15]. While BRV generally causes diarrhea in calves aged 2-21 days, BCoV is detected in calves with diarrhea aged 5-30 days [8,28]. In Turkey, it was reported BRV from 16 using PAGE method of the samples collected from 89 diarrheal calves between the ages of 1 and 30 days in Van province between 2002 and 2003, and reported BCoV from 1 sample using indirect ELISA [13]. They did not detect the 2 viruses together in any of the samples. Some researchers detected BRV in 6 calves, BCoV in 3, and *E. coli K99* in 2 calves in the 0-7 day age range, and BRV and BCoV were detected in 1 animal each in the 8-14 age range, BRV was detected in only 1 animal in the age range of 15-21 days, while BRV and *E. coli K99* were detected in 1 animal in the age range of 22-28 days by the rapid diagnostic kit [5]. However, the presence of BRV, 67% was detected in calves aged 0-5 days, 31% was found in calves aged 6-15 days, 6% was found in calves aged 16-21 days, and 13% was detected in calves aged 21-30 days with RT-PCR method [28]. In the same study, while BCoV was not detected in calves aged 0-5 days, they determined the presence of BCoV in 34% of calves aged 6-15 days, 13% in calves aged 16-20 days, and 2% in calves aged 21-30 days. In Şanlıurfa province, BCoV positivity detected at a rate of 20% in calves with diarrhea at the age of 1-7 days and 7% at the age of 22-30 days by indirect ELISA. In this study, the presence of BRV, BCoV in 15, and *E. coli K99* in 11 of the fecal samples was determined by direct ELISA. In this study, BRV was detected mostly in calves aged 1-7 days by ELISA, while BCoV and *E. coli K99* were detected in 9 of the calves. While BRV was detected at a high rate at 8-15 days of age, it was followed by BCoV, and *E. coli K99* was detected in only 1 animal. BRV was detected at a higher rate in animals aged 8-15 days than in animals aged 1-7 days. In this study, *E. coli K99* was detected more frequently in 1-7 days of age compared to other ages. These findings support researchers reporting that *E. coli* causes diarrhea in calves less than 7 days old.

Isolating viruses is a very difficult, time-consuming and expensive method. Similarly, the isolation of *E. coli* and the determination of its virulence factors are time-consuming factors. For this reason, rapid diagnostic kits and antigen ELISA tests are generally used to detect BRV, BCoV and *E. coli*, which are frequently isolated from calf diarrhea [4-6,13,18,20,29]. In the presented study, BRV, BCoV and *E. coli K99* were tried to be detected in fecals with the antigen ELISA kit. Accordingly, the highest presence of BRV was detected in calves, followed by BCoV and *E. coli K99*. Although it has been reported that BRV and *E. coli K99* are generally detected more frequently in studies investigating the etiology of calf diarrhea, the presence of BCoV was detected at a higher rate than *E. coli K99* in this study.

E. coli has been reported to cause diarrhea in calves less than 1 week old. In these calves, *K99* and F41 fimbrial antigens are very important in the colonization of ETEC into intestinal epithelial cells.

Some researchers reported that 36 *E. coli* isolates with K99 were obtained from calves with diarrhea under 1-7 days of age, 17 of them also had F41 fimbriae, 46% of *E. coli* isolates carrying fimbrial genes have shiga toxin genes, however, they reported that the gene encoding enterotoxins (STa) was not found in these isolates [22]. In this study, it was determined that *E. coli* isolates carrying fimbrial genes did not carry enterotoxin (STa) genes and shiga toxin genes. ETEC isolates have *K99*, *F41* and *F17* fimbrial adhesins and are important in the colonization of the intestines of neonatal calves [17,24]. In other studies, it was reported that they isolated *K99* in only 3 of 689 fecal samples obtained from calves with diarrhea, and *F41* was not isolated from only 3 fecal samples and was not isolated alone, but was detected together with the *F17* gene [24]. Similarly, in this study, the *F41* gene was not detected alone. The presence of the *F17* gene was not investigated in this study.

Shigatoxin genes are detected in *E. coli* isolated from neonatal calf diarrhea. The researchers reported presence of *Stx1* in 5 and *Stx2* in 3 of 10 *E. coli* isolated from neonatal calf diarrhea [1]. In a different studies, it was determined *Stx1* in 20 and *Stx2* in 13 of 205 *E. coli* isolated from 139 calves with diarrhea [26]. In other studies, it was identified only *Stx1* in 17 of 388 *E. coli* isolated from calves with diarrhea, *Stx1* and other virulence factors together in 14, and *Stx2* and other virulence factors together in 1 isolate [24]. *Stx2* was not detected alone in any isolate. In these studies, it was observed that *Stx1* could be detected more in *E. coli* isolates isolated from calf diarrhea. Contrary to these studies, some researchers identified *Stx1* in 3.03% and *Stx2* in 9.09% of the 133 *E. coli* isolates they isolated from calves with diarrhea between the ages of 1 day and 3 months, and reported that these genes could be found alone or together with other virulence factors in *E. coli* [12]. In this study, K99 antigen could not be detected by both ELISA and PCR in *E. coli* isolates with the *Stx1* gene. These results showed that other *E. coli* isolates other than *E. coli K99* can also cause diarrhea in calves [12,19]. In this study, similar to the findings of other researchers [1,24,26], the *Stx1* gene was detected in only 2 of the *E. coli* isolates and the *eaeA* gene, while *Stx2* gene could not be detected in any *E. coli* isolates.

The intimin encoded by the *eaeA* gene provides the relationship between bacteria and intestinal epithelial cells of STEC strains. It has generally been detected at low rates in studies, and its pathogenesis is not fully known [24]. In this study, the *eaeA* gene was detected in only 4 (5%) of 79 *E. coli* isolates. The researchers reported the *eaeA* gene in 15.15% of the *E. coli* isolates they isolated from calves with diarrhea [12]. In Turkey, it was announced 8 *eaeA* (57.1%) genes in 14 *E. coli* O157:H7 isolated from 457 feces collected from cattle and calves [19]. The result of this study was found to be quite high compared to our findings. This may be due to the high number of isolates in our study. Additionally, the *eaeA* gene is the most important virulence factor in O157:H7 strains [19]. Researchers reported that this gene was detected at a higher rate in *E. coli* strains isolated from calves with diarrhea than in calves without diarrhea.

CONCLUSIONS

As a result, in this study, it was determined that neonatal calf diarrhea in Burdur province was mostly caused by rotavirus, and among bacterial factors, *E. coli* was the most isolated, and being responsible for diarrhea in the 1st 5 days. It is important to identify the cause of diarrhoea in newborn calves as soon as possible. Therefore, the factors need to be promptly and precisely recognized in order to control the instances of diarrhea in newborn calves in order to minimize economic losses. On farms where calves with diarrhoea were found, it was reported that cattle were not vaccinated in late pregnancy. Vaccinating pregnant cows against BRV, BCoV and *E. coli* in the last 3 months of their pregnancy will greatly reduce calf losses. For this purpose, it has come to the conclusion that training should be given to animal owners and veterinarians about how important vaccination is in preventing calf losses.

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***Declaration of interest*.** All authors have no conflicts of interest. The authors alone are responsible for the content and writing of the paper.

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