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RESEARCH ARTICLE

# Etiological and Predisposing Factors in Calves with Neonatal Diarrhea: A Clinical Study in 270 Case Series [1]

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**Abstract:** This study was carried out to find out the etiology and predisposing factors of calves having diarrhea from Kayseri province and its neighboring cities between January 2016 and September 2019. A total of 270 neonatal diarrheic calves were included to this study. Comprehensive information was obtained by face-to-face interviews with the animal owners about administrative practices such as the vaccination status of the dams, farm type, colostrum intake status. The etiological agents were determined using the lateral flow immunochromatographic test kits. As a result of this investigation, out of 270 diarrheic cases; 21.9% (59) *Cryptosporidium* spp., 15.6% (42) *E. coli* K99+, 14.1% (38) bovine coronavirus (BCoV), 10.4% (28) bovine rotavirus (BRV), 9.3% (25) *Cryptosporidium* spp.+BRV, 8.5% (23) BRV+BCoV were found. Interms of shelter type; 85.2% (230) were traditional and 14.8% (40) were modern type. Regarding the colostrum intake situation; 7.4% (20) received no colostrum, 11.1% (30) received insufficient colostrum and 81.5% (220) received colostrum adequately and on time. Additionally, 36.7% (99) calf mothers were vaccinated and 63.3% (171) were unvaccinated. Compared to those born in autumn, calves born in winter; 6.5-fold, in the spring season; 3.6-fold and in summer; 5.2-fold more likely to develop diarrhea caused by *E. coli* K99+. These findings may generate valuable information not only for the clinicians and researchers but also animal health experts, policy makers, farmer etc.

**Keywords:** *Calf, Diarrhea, Etiology, Predisposing factor*

# Neonatal İshalli Buzağılarda Etiyolojik ve Predispoze Faktörler: 270 Olgu Serisinde Klinik Bir Çalışma

**Öz:** Bu çalışma, Ocak 2016-Eylül 2019 tarihleri arasında Kayseri ili ve çevre illerinden ishal olan buzağıların etiyolojisi ve predispozan faktörlerinin belirlenmesi amacıyla yapılmıştır. Bu çalışmaya toplam 270 yenidoğan ishalli buzağı dâhil edildi. Hayvan sahipleri ile yüz yüze görüşme yapılarak anaların aşılanma durumu, çiftlik tipi, kolostrum alma durumu ve şekli gibi yönetimsel uygulamalar hakkında kapsamlı bilgi alındı. Etiyolojik ajanlar, lateral flow immünokromatografik test kitleri kullanılarak belirlendi. Bu inceleme sonucunda 270 ishal olgusundan; %21.9 (59) *Cryptosporidium* spp., %15.6 (42) *E. coli* K99+, %14.1 (38) bovine koronavirus (BCoV), %10.4 (28) bovine rotavirus (BRV), %9.3 (25) *Cryptosporidium* spp.+BRV, %8.5 (23) BRV+BCoV bulundu. Barınak türü açısından; %85,2'si (230) geleneksel, %14.8'i (40) modern tipti. Kolostrum alma durumu ile ilgili olarak; %7.4'ü (20) hiç kolostrum almamış, %11.1'i (30) yetersiz kolostrum ve %81.5'i (220) kolostrumu yeterli ve zamanında almıştır. Kolostrum alma durumu açısından; sahiplerinin verdiği bilgilere göre %7.4'ü (20) hiç kolostrum almamış, %11.1'i (30) yetersiz kolostrum, %81.5'i (220) ise kolostrumu yeterli ve zamanında aldığı kaydedildi. Ayrıca annelerin %36.7'si (99) aşılı, %63.3'ü (171) aşısızdı. Sonbahar mevsiminde doğanlara kıyasla, kış mevsiminde doğan buzağıların; 6.5 kat, bahar mevsiminde; 3.6 kat ve yaz mevsiminde; 5.2 kat daha fazla *E. coli* K99+'un neden olduğu ishal geliştirme olasılığı olduğu görüldü. Bu bulgular sadece klinisyenler ve araştırmacılar için değil, aynı zamanda hayvan sağlığı uzmanları, politikacılar, çiftçiler vb. için de değerli bilgiler üretebilir.

**Anahtar sözcükler:** *Buzağı, İshal, Etiyoloji, Predispoze faktör*

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# Introduction

Neonatal calf diarrhea (NCD) is one of the most important problems in calf rearing [1]. In the etiology of NCD; *Cryptosporidium* spp., bovine rotavirus (BRV), bovine coronavirus (BCoV), enterotoxigenic *Eschericia coli* (ETEC) K99+ and *Giardia* spp. are commonly reported endemic microorganisms [2,3]. These infectious agents cause diarrhea in calves alone or as mixed infections [2]. Two of these, *Cryptosporidium* spp. and BRV are the most abundant enteropathogens in the feces of calves with diarrhea [4].

Many factors are known to contribute to calf diarrhea. In most cases, there is interaction between environmental conditions, management practices and microorganisms [5]. It is stated that many factors such as the number of animals in the farm, colostrum intake problems such as not giving colostrum in time, adequately or not at all, vaccination problems such as not vaccinating pregnant mothers against infectious diarrhea agents (BRV, BCoV and ETEC K99+), umblical cord problems such as not performing umbilical cord disinfection are all effective in the formation of diarrhea in newborn calves [6,7]. In addition, farm type, shelter structure, season and age are the predisposing factors affecting the emergence and severity of the disease [8]. Furthermore, it is stated that many administrative factors such as unsuitable shelter conditions (crowding, ventilation, lighting, temperature and relative humidity) and poor cleaning and disinfection of vehicles used in collective breeding shelters are also effective in the formation of diarrhea [5].

Various laboratory methods [virus isolation, bacterial culture, polymerase chain reaction (PCR), enzyme-linked immunosorbent assay (ELISA), direct microscopy of fecal smear (acid-fast stain)] have been used to detect entero-pathogens from stool samples [8,9]. These procedures are reliable; however, they are time consuming, expensive and require specialized knowledge. Lateral flow immuno-chromatographic (LFI) diagnostic kits; It is widely used in clinics, animal hospitals and in the field to detect major enteropathogens from stool samples taken from calves with diarrhea in a fast, easy and inexpensive way [10,11]. Since highly selective antigen-antibody reaction and mono-clonal antibodies as detector antibody used mainly in these tests, their specificity and sensitivity are generally over 98% [12].

The incidence of major enteropathogen that cause neonatal calf diarrhea may vary according to countries, regions, farm types, and sampling locations [3,4,8,11,13]. The incidence and distribution of enteropathogens that cause diarrhea in calves have been extensively studied in stool samples collected from farms or barns and have been reported by many researchers [3,4,12,14]. However, in animal hospitals where sick calves are taken for diagnosis, treatment and improvement of their general condition, there is still a lack of epidemiological data on the etiology and predisposing factors of diarrhea in calves brought especially from traditional and modern farms.

In this study, we conducted a cross-sectional and questionnaire-based study using multivariate analyzes to determine the factors that predispose to diarrhea in calves brought to the animal hospital from different parts of Kayseri and its surrounding provinces. So, it was aimed to investigate the prevalence of major enteropathogens that play role in the etiology of neonatal calf diarrhea. In addition, risk factors that predispose to diarrhea and affects on general condition was another aim of the study. Furthermore, data obtained from traditional and modern farms were also compared.

# Material and Methods

## Ethical Statement

This study was approved by the committee of HADYEK-Local Ethics Committee for Animal Experiments Office of Erciyes University (Approval no: 13/10).

## Study Design

A cross-sectional study was performed to determine major etiological and predisposing factors on neonatal calves having diarrhea brought from Kayseri (n=194) and the neighbouring cities [Sivas (n=14), Nevşehir (n=28), Yozgat (n=15), Niğde (n=7), Kırşehir (n=12)] to the Erciyes University, Faculty of Veterinary Medicine, Animal Hospital between January 2016 and September 2019.

## Target Population and Sampling

The target population was 1-35 days old diarrheic calves brought from traditional and modern farms. Regions of the farms where the calves are brought, accounted for approximately 11.2% of national large animal registries [15]. Each calf in the present study represents a different farm. Calves included in the study; were randomly selected from the calves brought to the animal hospital for the diagnosis. Only clinical signs (abnormally frequent, soft or watery consistency and bad odor) of diarrhea were determined as inclusion criteria. No other inclusion criteria were established. The number of samples used for questionnaire in the study was determined according to Krejcie and Morgon table [16].

## Animals

A total of 270 diarrheic neonatal calves from 270 different farms aged between 1-35 days (116 calves were aged between 1-7 days, 84 calves were aged between 8-14 days, 49 calves were aged between 15-21 days, 5 calves were aged between 22-28 days and 16 calves were aged between

29-35 days), in different breeds (187 Simmental, 53 Holstein, 21 Brown Swiss, 5 Cross-breed, 2 Charolais, 1 Belgian Blue, 1 Limousin), from both sex (153 male, 117 female) were the animal materials of the present study. Only one animal from each farm was included to the study.

## Data Collection

Comprehensive information was obtained by face-to-face interviews with the animal owners about administrative practices such as demographic, managerial and health factors presumed to be associated with diarrhea in calves. Demographic data contained; race, age and sex of the calves. Among the administrative factors; there were questions such as the type of farm (modern or traditional), the status of receiving colostrum, the number of animals in the farm, the way colostrum was given, umbilical cord disinfection applied or not, vaccination status of mothers, starting time of diarrhea. Information was taken from a total of 270 persons (ranchers or business executives, animal owners) whom animals used in this study. Those who did not responded to our notification or gave false information, discarded from this study which are not within 270 animal owners.

The data of the date (month and year) when the calves were obtained retrospectively from the patient registration system of the Erciyes University (Patient Registration System, ERUVetO; V.15042019/2015, Kayseri, Türkiye).

## Clinical Examination

Calves included in the study were subjected to a complete physical examination including rectal temperature (°C), pulsation (bpm), respiratory rate, hydration status, suckling reflex, general condition assessment, stool consistency and color. Hydration status of calves were evaluated according to demeanor, recession of the globe into the orbit and skin tent duration (sec) described by Smith [17].

The general clinical conditions of the calves were categorized according to the non-invasive five-point sequential scale clinical evaluation scoring table developed by Sayers et al.[18]. According to this table, diarrheic calves in the present study were categorized as clinically normal, mild, moderate, severe, and comatose.

In the present study, fresh feces taken from a calf was considered diarrheic if it is abnormally frequent, soft and watery consistency and had bad odor. According to the consistency of the stool and presence of blood in its content; it was also classified as muddy (pasty, faeces spreading across the bottom of the container, but not liquid), loose (but stays on the top of floor), watery (liquid faeces) and hemorrhagic (stools that contain mostly blood and are nearly red in color). Stool colors were also recorded (yellow and its tones, white and its tones, green, brown, red, black and gray).

## Pathogen Detection

Samples were taken from diarrheic calves into sterile stool containers by rectal stimulation. From these stool samples, lateral flow immunochromatographic (LFI) test kits (Anigen Rapid BoviD-5 Ag Test Kit, Bionote, Inc. Korea) were performed to detect antigens against *E. coli* K99$^+$ [Sensitivity (sen); 97.8%, specificity (spe); 99.0%], BRV (sen; 99.0%, spe; 98.0%), BCoV (sen; 98.4%, spe; 98.0%), *Cryptosporidium* spp. (sen; 98.2%, spe; 99.0%) and *Giardia* spp. (sen; 92.1%, spe; 99.1%). During the analysis, the instructions in the user manual of the test kit were followed and the results were evaluated qualitatively (positive or negative). Samples with negative results for the above 5 antigens (*E. coli* K99$^+$, BRV, BCoV, *Cryptosporidium* spp. and *Giardia* spp.) were classified as "undiagnosed".

## Data Management and Analysis

Descriptive statistics and frequency distribution variables were performed using SPSS for Windows Release 25.0 (SPSS Inc, Chicago, IL, USA). In order to determine the distribution of etiological agents by age range, calves were divided into five different age groups as 1-7 days, 8-14 days, 15-21 days, 22-28 days and 29-35 days. Calving seasons were defined as winter (December-February), spring (March-May), summer (June to August), autumn (September-November). Data were coded into variables using uniform definitions. The relationship between categorical variables was evaluated using the Pearson's chi-square ($\chi 2$) test (and Fisher's exact test). For variables with more than two categories, row (r) x column (c) (r>2 or c>2) chi-square test was used. This analysis included preliminary explorations, including pairwise analyses for relationship of binary variables using the chi-square test. This was followed by multivariable modelling using mixed effects logistic regression. Three main research questions were addressed. The first was to determine the predisposing factors associated with neonatal calf diarrhea caused by major enteropathogens (at least one or more; ETEC K99$^+$, BRV, BCoV, *Cryptosporidium* spp., *Giardia* spp.). The association between the predisposing factors and the presence/absence of major pathogen induced diarrhea were also analysed using the logistic regression model. The second was to identify the predisposing factors associated with neonatal calf diarrhea caused by each of the enteropathogens. The association between the predisposing factors and the presence/absence of each pathogen was analyzed using the same method. The third was to determine the predisposing factors that affect the general condition categories (normal, mildly affected, moderately affected, severely affected) of calves with diarrhea. Predisposing factors associated with general condition of diarrheic calves were analyzed using ordinal logistic regression model.

The predisposing factor with logistic regression analysis was achieved using three steps. Initially, the interrelationships of all variables taken individually with the occurrence of diarrhea were tested in a univariate model. Then, any variable with a p value <0.2 was considered eligible for the next step. In the third step, a final multivariate model was fitted with all the variables that had remained significant during the two previous steps. Odds ratios (ORs) with 95% confidence intervals were calculated to assess the likelihood of association. The graph showing the intersections of different etiological agents was created using the online Venn Diagram software (UGent, Genomics, & 927, 2020). For all analyses, a p value of <0.05 was considered to be statistically significant.

# Results

## Animal Population

A total of 4389 calves were registered to the Veterinary Teaching Hospital between at January 2016 and September 2019. The total number of diarrheic neonatal calves were 2545 (58%) (Patient Registration System, ERUVetO; V.15042019/2015, Kayseri, Turkey). The number of diarrheic calves included in this study were 270 (10.6%).

## Descriptive Data Analysis

One or more than one etiological agent was determined positive in 238 out of 270 diarrheic calves used in the present study. A single etiological agent in 63.7% (172/270), two etiological agents in 23.3% (63/270), three etiological agents in 1.1% (3/270) were detected in the diarrheic calves *(Table 1)*. However, in 11.9% (32/270) diarrheic calves, major antigens (BRV, BCoV, *E. coli* K99$^+$, *Cryptosporidium* spp., *Giardia* spp.) could not be detected.

It was seen that 56.7% (153/270) of diarrheic calves in this study were male and 43.3% (117/270) were female. Diarrheic calves investigated in the present study were 66.7% (187/270) Simmental, 19.6% (53/270) Holstein, 7.8% (21/270) Swiss Brown, 1.9% (5/270) cross-breed, 0.74% (2/270) Charolais, 0.4% (1/270) Belgian Blue and 0.4% (1/270) Limousin race. There was no statistically significant relationship between the categories of variables of breed and etiological agent ($\chi 2$= 53.115, P=0.986).

It was noted that 81.5% (220/270) of the calves included in the study received colostrum fully and in time, 11.1% (30/270) received less, and 7.4% (20/270) did not receive it at all. In diarrheic calves, which were stated to have never received colostrum; mostly *E. coli* K99$^+$ (35.0%), in diarrheic calves, which were stated to have received less colostrum; mostly *Cryptosporidium* spp. (20.0%), in diarrheic calves, which were stated to receive colostrum fully and in time, mostly *Cryptosporidium* spp. (18.6%) were diagnosed.

In calves given colostrum with a feeding bottle; at most, *Cryptosporidium* spp. (18.5%), then respectively; *E. coli* K99$^+$ (14.8%), BCoV (13.6%), BRV (13.0%), *Cryptosporidium* spp. +BRV (9.9%), BRV+BCoV (8.0%) were diagnosed. In calves given colostrum by suckling; at most, *Cryptosporidium* spp. (19.0%), then respectively; *E. coli* K99$^+$ (18.0%), BCoV (16.0%), BCoV+BRV (10.0%), *Cryptosporidium* spp. +diarrhea cases due to BRV (9.0%) were observed.

Considering the number of calves brought to the hospital according to the seasons; In the winter season (december to february) 85, spring season (march to may) 133, summer season (june to august) 34, autumn season (September to November) 18 calves with diarrhea were included to the study.

Considering the stool colors of the calves with diarrhea in the present study; 60.4% (163/270) were yellow, 11.9% (32/270) white and its tones, 9.3% (25/270) green, 6.7% (18/270) brown, 4.5% (12/270) red, 3.7% (10/270) black and 3.7% (10/270) were gray. There was no statistically significant correlation between stool color of the calves with diarrhea and categories of etiological diagnosis variables.

The general conditions of the calves with diarrhea included in the study; 6.7% (18/270) were normal, 25.2% (68/270) were mild, 42.2% (114/270) were moderate and 25.9% (70/270) were severe. *Cryptosporidium* spp. were the mostly (44.4%, 8/18) detected patogen in calves having a healthy general condition, coronavirus was the mostly (19.1%, 13/68) detected patogen in the calves having mild general condition, *Cryptosporidium* spp. were the mostly (21.1%, 24/114) detected pathogen in calves having moderate general condition and *E. coli* were the mostly (27.1%, 19/70) detected patogen in the calves having severe general condition. Mean body temperatures, respiratory rate and heart rate of diarrheic calves were 38.3°C (32.8-41.6), 40/min (IQR; 28-48, min-max;10-160) and 107.9±24.9 (IQR; 93.5-120, min-max; 36-180) bpm, respectively.

## Etiologic Agents and Age Ranges

As a single etiological agent from stool samples taken from diarrheic calves; at most, *Cryptosporidium* spp. (21.9%, 59/270), more than one etiological agent; at most, *Cryptosporidium* spp.+rotavirus (9.3%, 25/270) were detected *(Table 1, Fig. 1)*.

When etiological agents are examined according to age range, in 1-7 days-old calves; mostly, *E. coli* K99$^+$ (33.6%, 39/116) cases of diarrhea were observed. In 8-14, 15-21 and 22-28 days-old calves, it was mostly; *Cryptosporidium* spp. [27.4% (23/84), 38.8% (19/49) and 40% (2/5), respectively] cases of diarrhea were determined. In 29-35 days-old calves, at most; cases of diarrhea caused by coronavirus (31.3%, 5/16) were detected *(Table 1, Fig. 2)*. Conversely, *Giardia* spp. were observed only sporadically (1.9%).

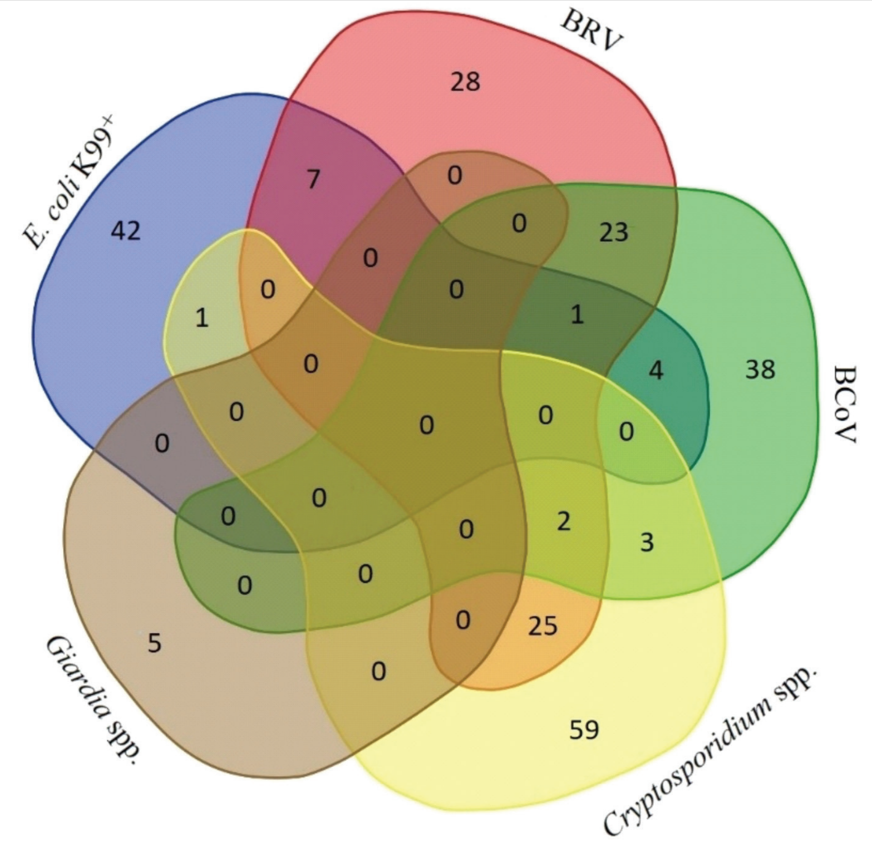


**Fig 1.** The intersections of major etiological agents that cause diarrhea in calves using Venn Diagram. BCoV; bovine coronavirus, BRV; bovine rotavirus

***Table 1.** Distribution of the major etiological agents in diarrheic calves in accordance to age groups and farm types*

| Calf Diarrhea Agent and Co-infections | Total, (n=270) % (n) | Farm Type | Agent Frequency and the Occurrence in Age Groups | | | | |
|---|---|---|---|---|---|---|---|
| | | TF/MF % (n) | 1-7 d (n=116) % (n) | 8-14 d (n=84) % (n) | 15-21 d (n=49) % (n) | 22-28 d (n=5) % (n) | 29-35 d (n=16) % (n) |
| | **100 (270)** | **85.2 (230/270)/ 14.8 (40/270)** | **43.0 (116/270)** | **31.1 (84/270)** | **18.1 (49/270)** | **1.9 (5/270)** | **5.9 (16/270)** |
| Single-infected | | | | | | | |
| *Cryptosporidium* spp. | 21.9 (59) | 22.6 (52) /17.5 (7) | 10.3 (12) | 27.4 (23) | 38.8 (19) | 40 (2) | 18.8 (3) |
| ETEC K99+ | 15.6 (42) | 16.5 (38) /10.0 (4) | 33.6 (39) | 1.2 (1) | 4.1 (2) | 0 (0) | 0 (0) |
| BCoV | 14.1 (38) | 15.7 (36) /5.0 (2) | 13.8 (16) | 14.3 (12) | 10.2 (5) | 20 (1) | 31.3 (5) |
| BRV | 10.4 (28) | 8.7 (20) /20.0 (8) | 10.3 (12) | 11.9 (10) | 10.2 (5) | 0 (0) | 0 (0) |
| *Giardia* spp. | 1.9 (5) | 1.7 (4) /2.5 (1) | 1.7 (2) | 2.4 (2) | 2.0 (1) | 0 (0) | 0 (0) |
| Dual-infected | | | | | | | |
| *Cryptosporidium* spp.+BRV | 9.3 (25) | 8.3 (19)/15.0 (6) | 5.2 (6) | 15.5 (13) | 10.2 (5) | 0 (0) | 6.3 (1) |
| BRV+BCoV | 8.5 (23) | 9.1 (21) /5.0 (2) | 10.3 (12) | 9.5 (8) | 4.1 (2) | 0 (0) | 6.3 (1) |
| ETEC K99+ +BRV | 2.6 (7) | 3.0 (7) /0 (0) | 4.3 (5) | 2.4 (2) | 0 (0) | 0 (0) | 0 (0) |
| ETEC K99+ +BCoV | 1.5 (4) | 1.7 (4) /0 (0) | 2.6 (3) | 1.2 (1) | 0 (0) | 0 (0) | 0 (0) |
| *Cryptosporidium* spp.+BCoV | 1.1 (3) | 0.9 (2) /2.5 (1) | 0 (0) | 1.2 (1) | 2.0 (1) | 0 (0) | 6.3 (1) |
| *Cryptosporidium* spp.+ETEC | 0.4 (1) | 0.4 (1) /0 (0) | 0 (0) | 0 (0) | 2.0 (1) | 0 (0) | 0 (0) |
| Multi-infected | | | | | | | |
| *Cryptosporidium* spp.+BCoV+BRV | 0.7 (2) | 0.9 (2) /0 (0) | 1.7 (2) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| BRV+BCoV+ETEC K99+ | 0.4 (1) | 0 (0) /2.5 (1) | 0.9 (1) | 0 (0) | 0 (0) | 0 (0) | 0 (0) |
| Undiagnosed | 11.9 (32) | 10.4 (24) /20.0 (8) | 5.2 (6) | 13.1 (11) | 16.3 (8) | 40 (2) | 31.3 (5) |

*Data were expressed as % positive (number of positive/number of total cases), undiagnosed, those are not positive for enterotoxigenic E. coli (ETEC) K99+, bovine rotavirus (BRV), bovine coronavirus (BCoV), Cryptosporidium spp., and Giardia spp.; **TF:** traditional farm (n=230); **MF:** modern farm (n=40); d, days*

### Occurrence of Etiological Agents According to Farm Type (Traditional/Modern)

It was determined that 85.2% (230/270) of the diarrheic calves came from traditional farm and 14.8% (40/270) of them came from modern farms. One or more antigens of *Cryptosporidium* spp., BRV, BCoV, *E. coli* K99$^+$ and *Giardia* spp. were determined as positive in 206 out of 230 diarrheic calves brought from traditional farms. In calves with diarrhea brought from traditional farms; at most, *Cryptosporidium* spp. 22.6% (52/230) cases of diarrhea were detected *(Table 1)*. One or more antigens of *Cryptosporidium* spp., BRV, BCoV, *E. coli* K99$^+$ and *Giardia* spp. were determined as positive in 32 out of 40 diarrheic calves brought from modern farms. In calves with diarrhea brought from modern farms; mostly BRV 20.0% (8/40) cases of diarrhea were seen *(Table 1)*. Moreover, to compared to those brought from traditional farms (undiagnosed; 10.4%, 24/270), it was seen to be more diarrhea cases classified as 'undiagnosed' (20%, 8/40) in diarrheic calves brought from modern farms. In the present study, in terms of animal numbers kept in the traditional farms were as follow: 1-10 animal (n=21 farms), 11-25 animal (n=59 farms), 26-50 animal (n=71 farms), 51-100 animal (n=52 farms), 101-250 (n=24 farms), 251-500 (n=3 farms). Furthermore, in the modern farms, number of animals kept in the farms were as 1-50 animal (n=13 farms), 51-100 animal (n=11 farms), 101-250 animal (n=12 farms), 250-1000 animal (n=4 farms).

### Logistic Regression Model Results for Calves with Major Pathogen-Induced Neonatal Diarrhea

With the univariate logistic regression model, 4 predisposing factors (umbilical cord disinfection status, farm type, dam vaccination status, age group) associated with neonatal diarrhea (P<0.2) caused by major enteric pathogens in calves were determined. At the last stage, 2 variables showed a significant relationship with neonatal diarrhea originating from the major enteric pathogen. According to the final model results; compared to calves born from mothers that were vaccinated with *E. coli* K99$^+$, BRV and BCoV antigens in the last period of pregnancy, the probability of developing major pathogen-induced diarrhea in calves born from unvaccinated mothers increased by 3.5-fold. In terms of age groups, it was determined that 15-21, 22-28, 29-35-days-old calves probability of developing major enteric pathogen-induced neonatal diarrhea increased 4.7-fold, 14.4-fold, 8.7-fold respectively compared to the 1-7-days-old age group.

### Predisposing Factors According to the Logistic Regression Model Significantly Associated with Calf Diarrhea Caused by Each Pathogen

With the univariate logistic regression model, the predisposing factors associated with each of the major enteropathogens [*Cryptosporidium* spp., (7 variables), *E. coli* K99$^+$ (7 variables), BRV (4 variables), BCoV (3 variables)] causing neonatal diarrhea (P<0.2) in calves were determined separately. These variables were included in the final multivariate logistic regression model. At the last stage, variables (P<0.05) showing a significant relationship with neonatal diarrhea caused by each of these pathogens are as follows: for *Criptosporidium*; the onset of diarrhea, months of birth, the consistency of feces and dam vaccination status, for *E. coli* K99$^+$ onset of diarrhea, season of birth, and colostrum intake status, for BRV; age groups and the number of animals in the farms, for BCoV; the farm type and age groups were found important.

### Predisposing Factors According to the Logistic Regression Model Significantly Associated with Calf Diarrhea Caused by *Cryptosporidium* spp., *E. coli*, BRV and BCoV

It was observed that the probability of diagnosing

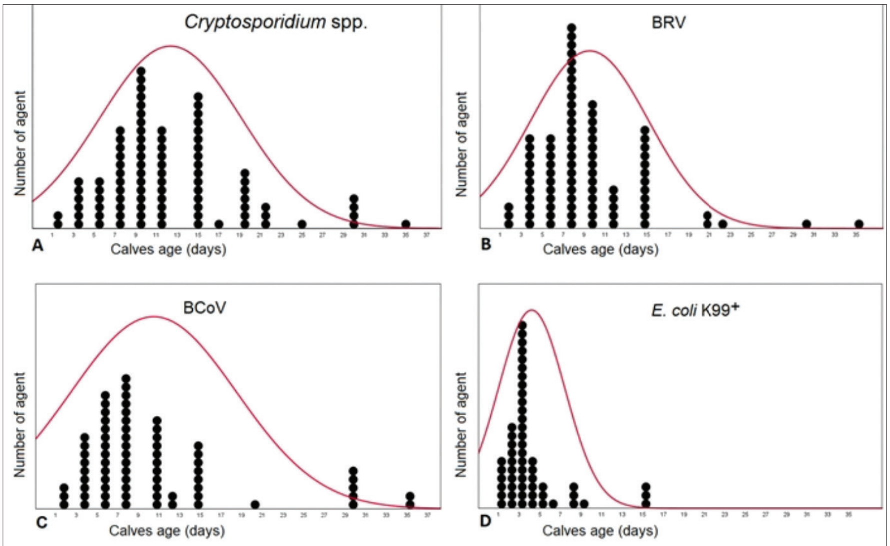


**Fig 2. (A)**; *Cryptosporidium* spp. (n=90), **(B)**; bovine rotavirus (n=86) (BRV), **(C)**; bovine coronavirus (BCoV) (n=71), **(D)**; *E. coli* K99$^+$ (n=55) distribution with concern to age in neonatal calves with diarrhea. Black dots (•) show number of positive cases for each antigen. Red line shows distribution curve

*Cryptosporidium* spp-induced diarrhea increased by 0.4-fold in calves with a diarrhea onset time of "72 h and more" (B:-1.051, OR: 0.350, 95% CI, 0.177 to 0.689, Wald $\chi2$= 9.234, P= 0.002) compared to calves with a diarrhea onset time of "24 h and before". Compared to the calves born from mothers that were vaccinated, the probability of *Cryptosporidium* spp-induced diarrhea increased by 1.9-fold in calves born from unvaccinated mothers. Futhermore, It was determined that the probability of diarrhea caused by *Cryptosporidium* spp. increased 0.3-fold in calves with sludge-like consistency compared to those with watery stools. Additionally, it was also determined that the probability of getting diarrhea caused by *Cryptosporidium* spp. in calves born in the winter season was 14-fold increased in 8-28 days old calves compared to the autumn season.

It was determined that calves did not receive colostrum at all were 0.1-fold more likely to develop *E. coli* K99$^+$-induced diarrhea, compared to those who received full and on-time colostrum. The probability of developing diarrhea caused by *E. coli* K99$^+$ from calves with a diarrhea onset time of "72 h and more" compared to a diarrhea onset time "<24 h and previous", was found to be; 0.1-fold higher. It was determined that calves born in winter season; 6.5-fold, spring season; 3.6-fold and summer season; 5.2-fold more likely to develop *E. coli* K99$^+$-induced diarrhea, compared to those born in the autumn season.

Compared to the 29-35-days-old age group, 7-14-days-old calves were 0.2-fold more likely to develop rotavirus-induced neonatal diarrhea. It was determined that every 1 animal increase in the total number of animals in the farm increased the probability of calves getting rotavirus 1.0-fold.

Calves from traditional farms were 0.3-fold more likely to be diagnosed with coronavirus-induced neonatal diarrhea compared to calves brought from modern farms. Compared to the 29-35-days-old age group, 15-21-days-old calves were 4.0-fold more likely to develop coronavirus-induced neonatal diarrhea.

### Predisposing Factors According to the Ordinal Logistic Regression Model Significantly Associated with General Conditions (Normal, Slightly Affected, Moderately Affected, Severely Affected) of Diarrheic Calves

As a result of this analysis, it was determined that especially absence of suckling reflex, rectal temperature, season of birth, starting time of diarrhea were effective on the general condition of the calf (P<0.05) *(Table 2)*. When compared with the calves having "good suckling reflex", each increase in the number of calves 'without suckling reflex' was found to worsen the general condition of the calves by 6.8-fold (P<0.001) and in calves having 'poor suckling reflex" by 2.7-fold (P<0.001). Compared to autumn, calves born in summer had a 3.0-fold higher risk of being severe in the general condition categories (P<0.05). An increase in rectal temperature (for each °C) was associated with decrease in the odds of general condition, with an odds ratio of 0.765 (P=0.001). An increase in *E. coli* K99$^+$ positive case (expressed in numbers) was associated with an increase in the odds of general condition, with an odds ratio of 1.846 (P=0.069, without statistically significance).

### Chi-square ($\chi2$) Test Results

There was no statistically significant relationship between the dam vaccination status and the type of farms. It was observed that the vaccination rates of pregnant animals

*Table 2. Predisposing factors associated with general conditions of diarrheic calves in ordinal logistic regression model*

| Variable Description | | Estimate | SE | Sig. | OR | 95% CI | |
|---|---|---|---|---|---|---|---|
| Suckling reflex | No reflex | 1.971 | 0.393 | <0.001 | 6.801 | 3.261 | 14.182 |
| | There is but not good | 1.059 | 0.278 | <0.001 | 2.721 | 1.603 | 4.619 |
| | Good (Reference category) | | | | | | |
| Season of birth | Winter | 0.673 | 0.506 | 0.184 | 1.960 | 0.727 | 5.284 |
| | Spring | 0.425 | 0.485 | 0.381 | 1.530 | 0.591 | 3.957 |
| | Summer | 1.113 | 0.568 | 0.050 | 3.043 | 1.000 | 9.265 |
| | Autumn (Reference category) | | | | | | |
| Starting time of diarrhea | <3 h | -1.405 | 0.992 | 0.157 | 0.245 | 0.035 | 1.715 |
| | 12 h | 0.194 | 0.409 | 0.636 | 1.214 | 0.545 | 2.706 |
| | 24 h | 1.209 | 0.411 | 0.003 | 3.350 | 1.497 | 7.497 |
| | 48 h | -0.201 | 0.325 | 0.536 | 0.818 | 0.433 | 1.547 |
| | 72 h and more (Reference category) | | | | | | |
| Rectal temperature (°C) | | -0.268 | 0.083 | 0.001 | 0.765 | 0.655 | 0.893 |
| *E. coli* K99$^+$ Yes/No | | 0.613 | 0.337 | 0.049 | 1.846 | 0.954 | 3.573 |

***SE:** Standart Error, **Sig:** significance, **OR:** odds ratio, 95% **CI:** 95% confidence interval, $R^2$= 0.246 (Cox & Snell), $R^2$= 0.269 (Nagelkerke). Model: $\chi2$ (11)= 76.288* in traditional (36.5%) and modern farms (37.5%) were similar. In calves born from mothers vaccinated (36.7%); at most, *Cryptosporidium* spp. (22%), then respectively; *Cryptosporidium* spp.+rotavirus (14.1%), *E. coli* K99$^+$ (13.1%), coronavirus+rotavirus (11.1%), rotavirus (10.1%), coronavirus (10.1%) diarrhea were determined. In calves born from unvaccinated mothers (63.3%); mostly *E. coli* K99$^+$ (17%), then respectively; Coronavirus (16.4%), *Cryptosporidium* spp. (15.8%), rotavirus (9.9%), rotavirus + coronavirus (7%), *Cryptosporidium* spp. + rotavirus (6.4%) diarrhea were observed.

It was noted that 75.6% of the calves included in the study had umbilical cord disinfection and 24.4% were not. In calves undergoing umbilical cord disinfection; at most, *Cryptosporidium* spp. (16.7%), then respectively; *E. coli* K99$^+$ (14.7%), coronavirus (11.8%), *Cryptosporidium* spp., rotavirus (11.3%) infections were seen. For those who did not have umbilical cord disinfection; at most, *Cryptosporidium* spp. (22.7%), then respectively; coronavirus (21.2%), *E. coli* K99$^+$ (18.2%), rotavirus (10.6%), *E. coli* K99$^+$+rotavirus (6.1%) infections were recorded. A statistically significant relationship was observed between the type of farms and the application of umbilical cord disinfection (P=0.007). The rate of umbilical cord disinfection in modern farms (92.5%) was higher than in traditional farms (72.6%).

When looking at the way of taking colostrum, it was noted that 60% of calves received colostrum by feeding bottle and 40% of them received colostrum by suckling. A statistically significant relationship was observed between colostrum intake (suckling, feeding bottle) and colostrum intake status [(did not receive, received less, received fully and on time) (P=0.021)]. It was stated that 85.8% of the calves given colostrum with a feeding bottle received the colostrum fully and on time. In calves given colostrum by suckling, the rate of taking colostrum fully and on time was 75.0%. The proportion of calves "received less" colostrum with a feeding bottle was 6.8%, while the same ratio of those who received colostrum by suckling was recorded as 17.6%. In calves receiving colostrum with a feeding bottle; at most, *Cryptosporidium* spp. (18.5%), then respectively; *E. coli* K99$^+$ (14.8%), coronavirus (13.6%), rotavirus (13.0%), *Cryptosporidium* spp.+rotavirus (9.9%), rotavirus+coronavirus (8.0%) infections were seen. In calves received colostrum through suckling; at most, *Cryptosporidium* spp. (19.0%), *E. coli* (18.0%), coronavirus (16.0%), coronavirus + rotavirus (10.0%), *Cryptosporidium* spp. + rotavirus (9.0%) infections were seen.

A statistically significant relationship was observed between the suckling reflex and the general condition of the calves (P<0.001). A statistically significant relationship was observed between the general condition of the calves and the type of farm where the calves were brought (P=0.014). It was observed that the proportion of calves with mild general condition was lower in calves from traditional farms (21.7%) than those from modern farms (45.0%). There was no statistically significant relationship between the onset of diarrhea and the type of farm (P=0.079). A statistically significant relationship was observed between the seasons and the type of farm where calves were brought (P=0.05). It was observed that the ratio of calves with diarrhea brought to our clinic in the winter season was higher in modern farms (47%) than in traditional farms (28.7). In the spring season, the ratio of calves with diarrhea brought to our clinic in traditional farms (51.7%) was higher than in modern farms (35.0%).

## Discussion

In this study, the incidence of major enteropathogens and the distribution of these pathogens according to 5 different age groups in neonatal diarrheic calves brought from two different farm types (traditional/modern farm) in Kayseri and its surrounding provinces were determined. Predisposing factors associated with neonatal calf diarrhea caused by each pathogen were recorded. In addition, predisposing factors affecting the general condition of calves with diarrhea were also revealed.

Considering the fact that in the present study, the animal owners applied to the animal hospital in order to determine the etiology, treatment and improvement of the general condition of the diarrheic cases that did not improve and/or had a severe course, the underrepresentation of treatment-responsive or cured diarrheic cases from the main population could be a potential source of error. The same disadvantage is often present in other studies investigating the etiology of neonatal calf diarrhea [3,8]. In addition, 270 stool samples from calves with diarrhea were analyzed with lateral flow immunochromatographic (LFI) test kits in the present study. As a result of these analyses, 5 etiological agents causing diarrhea (BRV, BCoV, *E. coli* K99$^+$, *Cryptosporidium* spp., *Giardia* spp.) could not be determined in 32 calves with diarrhea. Failure to confirm the accuracy of stool samples determined as positive or negative by LFI test kits with more sensitive diagnostic methods such as "virus isolation, conventional and/or real-time PCR, bacterial culture, etc" and toxin isolation [heat] for ETEC K99$^+$-stable enterotoxin-a (STa), heat-labile enterotoxin-IIc (LTIIc)] is another weakness of the present study. Recent studies have focused on the toxins (Sta, LTIIc) secreted from this bacterium which is responsible for the main pathogenic effect, rather than the diagnosis of *E. coli* in the feces of calves with diarrhea [19,20]. In the current study, these sensitive diagnostic methods were not preferred because they were expensive and take a long time between examination and diagnosis of sick calves. Thus, the reason choosing LFI test kits was that they are faster (<5 min), inexpensive and have the advantage of starting early treatment against the agent. Once the enteropathogens are diagnosed in a short time, measures such as segregation of affected animals and disinfection of contaminated clinics, animal hospitals, farms and/or shelters can be implemented quickly to prevent the spread of infection to other animals. In addition, it is a fact that this diagnostic method has high specificity and sensitivity rates due to the use of monoclonal antibodies as detector antibodies in these tests [12]. Another weakness of the present study is that the accuracy of the answers given to the questions that asked face to face could not be confirmed. The farms where the calves were brought from could not be visited. Because of the economic or other concerns of the calf owners, there may be a possibility of giving wrong answers to the questions posed to hide the current situation.

*Cryptosporidium* spp. (21.9%, 59/270) induced diarrhea cases were observed mostly as a single etiological agent in diarrheic calves, in the current study *(Table 1)*. While the results of the present study were similar to the values reported by many researchers [8,21,22], but higher than the values reported by some other researchers [3,10,23]. This situation can be explained by the age of calves, number of samples, difference in farm type, differences in the hygiene and management practices in farms, climate and geografical differences in which the study was conducted.

When more than one etiological agent taken into consideration, at most; *Cryptosporidium* spp. + BRV (9.3%) combination were seen, in the present study *(Table 1)*. In calves less than 30 days old, BRV was the most reported etiologic agent in addition to *Cryptosporidium* [1,4]. When antigen positivity rates examined (including mix infections) in stool samples, the most common antigen was *Cryptosporidium* spp. (33.3%), followed by BRV with 31.9% *(Table 1)* in our study. As a matter of fact, it is stated that there is a positive correlation between *Cryptosporidium* spp. and BRV infections [24]. In addition, it has been recently reported that *Cryptosporidium* spp. is the major causative factor in neonatal calf diarrhea and is a risk factor for the occurrence of BRV [24,25].

In this study, the diagnosed *Giardia* spp. ratio (1.8%) were lower than studies conducted in our country [26] and other countries [27,28]. The prevalence of enteropathogens may vary depending on the countries, regions, climates, farms and the application of management and hygiene measures. For this reason, it may be more beneficial for veterinarians to evaluate enteropathogens such as BRV, BCoV, *E. coli* K99$^+$ and *Cryptosporidium* spp. rather than *Giardia* spp. in the etiology of neonatal diarrhea in the said region.

The incidence of diarrhea caused by *Cryptosporidium* spp. was 1.9-fold higher in calves born from unvaccinated dams compared to calves born from vaccinated dams ($P<0.05$) in the present study. This may be related to the reduction in diarrhea caused by ETEC K99$^+$ and BCoV due to vaccination. Diarrhea cases due to *E. coli* K99$^+$ (13.1%) and BCoV (10.1%) in calves born from vaccinated dams compared to calves born to unvaccinated dams *[E. coli* K99$^+$ (17%) and BCoV (16.4%)] were found to be lower. BRV incidence rates in calves born from vaccinated and unvaccinated dams were very close to each other. Frequent mutation and antigenic variation of rotaviruses due to recombination may also occur. Thus, BRV vaccines may require frequent surveillance and further characterization of circulating rotaviruses in the field [29,30]. Additionally, the high prevalence of BRV-induced diarrhea in vaccinated patients may be associated with the higher incidence of *Cryptosporidium* spp. related diarrhea. Because *Cryptosporidium* spp. and BRV are risk factors for the formation of each other [1,24]. Furthermore, *Cryptosporidium* spp. can increase the proliferation of viral agents, especially rotaviruses, in the digestive tract by causing malabsorptive diarrhea in calves [31].

The probability of catching *Cryptosporidium* spp.-induced diarrhea was higher in calves born in the winter season compared to the autumn season in calves aged 8-28 days in this study. This result is in agreement with the results reported by Hamnes et al.[32]. On the other hand, in the current study, the majority of calves were brought to the hospital in winter (31.5%) and spring (49.2%) seasons. Therefore, the reason for the high incidence of diarrhea caused by *Cryptosporidium* spp. in winter observed in this study, may be due to patient load in winter season [33]. Similarly, Sanford et al.[33] reported high patient load during the winter months. In contrast, some researchers speculated higher in other seasons than winter [34,35]. A possible explanation for our findings is that during the winter months, reduced cleaning routines may result in a heavier pathogen load in the farms [32]. As a matter of fact, crowding, lower temperature and higher humidity in winter months increase the level of infectious agents that elevate the risk of developing diarrhea [32,36,37].

Consistent with the literature in the current study, diarrhea cases due to ETEC K99$^+$ (33.62%) were the most common in calves aged 1-7 days. This result is compatible with the results of other studies conducted in our country (22.58%-32.1%) [11,21] but, higher than the values reported from different parts of the world (1.4-17.4%) [1,38]. The reason for our results can be attributed to the type of farm (mostly traditional farms; 85.2%), inadequate shelter, hygiene and management practices in the farms, as stated by Cho and Yoon [39]. In addition, it was determined that preventive vaccination against enteropathogens *(E. coli*, rotavirus, coronavirus) in these farms was low (36.7%). So, *E. coli* K99$^+$ (17%) related diarrhea were seen at most in calves born from unvaccinated dams which can be atributed to low vaccination rate.

Colostrum management is one of the most important preventive measures in reducing infectious calf diarrhea [40]. In the current study, it was determined that the probability of diarrhea caused by *E. coli* K99$^+$ was higher in calves that colostrum was not given compared to calves given full and timely colostrum. According to the results of this study, *E. coli* K99$^+$ (35.0%) related diarrhea was the most common diarrhea in calves that colostrum was not given. Inadequate quality and quantity of colostrum given in the first colostrum feeding is an important reason for the failure of passive immunity transfer [41].

In the present study, it was determined that calves born in the winter season were 6.5-fold more likely to develop diarrhea caused by *E. coli* K99$^+$ compared to the autumn season. Similar findings were also reported by other researchers [38,42]. During the winter months, more crowded shelters, lower ambient temperature and higher indoor air humidity may increase the level of infectious agents such as *E. coli* [38,43].

In our study, compared to the 29-35-days-old age group, 7-14-days-old calves were 0.2-fold more likely to develop rotavirus-induced neonatal diarrhea. Similar results were also reported by other researchers [1]. It was determined that every 1 animal increase in the total number of animals in the farms increased the probability of calves getting rotavirus 1.0-fold, in the present study. The increase in the number of animals on farms may increase transmission of infectious agents by adult cattle or healthy-looking calves [6,44], and could be the reason for above results. In addition, it is a fact that vaccination rates against BRV were low in the study population. This is due to the prevailing belief that vaccination causes an increase in costs.

In this study, calves from traditional farms were 0.3-fold more likely to be diagnosed with coronavirus-induced neonatal diarrhea compared to calves brought from modern farms. In another study, it was reported that coronaviruses are more common in group housing systems compared to individual housing systems [44]. It has been reported that the spread of coronavirus in adult cattle increases during birth, therefore newborns are susceptible to infections and their mothers plays a major role in the exposure of calves to the agent in the first days of their lives. In addition, this disease is more common in animals raised or housed indoors for a long time, especially in winter months [45]. The reason of getting high rate of coronavirus infection in the present study, could be due to keeping calves at the same environment with adult animals especially in traditional farms.

When compared with the calves having "good suckling reflex", each increase in the number of calves "without suckling reflex" was found to worsen the general condition of the calves by 6.8-fold and in calves having 'poor suckling reflex" by 2.7-fold. It has been reported that the suckling reflex in calves with diarrhea is closely related to base excess (BE) values [31,46]. Furthermore, dehydration, metabolic acidosis and increased serum D-lactate concentration are common findings in calves with diarrhea with or without a reduced suckling reflex [31].

In the present study, an increase in rectal temperature (for each °C) in the diarrheic calves was associated with decrease in the odds of general condition, with an odds ratio of 0.765. Boccardo et al.[47] reported that a 1-unit increase in rectal temperature (°C) in calves with diarrhea increased the calf survival rate 1.2-fold. In calves with diarrhea with poor general condition, the prognosis can still be considered favorable if the body temperature is above 38°C. Lower values indicate a poor prognosis [6].

Calves born in summer had 3.0-fold higher risk of being severe in the general condition categories compared to autumn in this study. Indeed, Windyer et al.[48] reported that calves born in summer are 2.0-fold less likely to respond to treatment for neonatal diarrhea compared to calves born in autumn. Similarly, in another study, bovine viral diarrhea virus related diarrheic calves born in summer show 2.7-fold more severe symptoms than calves born in autumn [49]. It has been speculated that many factors such as heat stress and suitable environmental conditions for bacterial growth in summer may play role [49]. Another reason may be related to the workload of animal owners in the summer season.

It was determined that 60% of the calves received colostrum by feeding bottle and 40% by suckling, in this study. Suckling colostrum is the least preferred approach. Because, this approach in the end belived to cause higher rates of passive transfer failure [2,6]. Moreover, in the present study, *E. coli* related diarrhea was seen at higher rates in calves received colostrum by suckling (18.0%) than those received colostrum by feeding bottle (14.8%). In contrast, Mohammed et al.[50] reported that the prevalence of *E. coli* was higher in calves received colostrum by feeding bottle than those received colostrum by suckling. Unlike the present study, Mohammed et al.[50] were carried out their study entirely on modern farms with concern to careless management systems during bottle feeding.

Contrary to the fact that the rate of calves with diarrhea brought from traditional farms in the winter season is lower than that of modern farms, this result can be attributed to various reasons. One of these can be explained by the inadequacy of traditional business owners in transporting their patients to veterinarians due to transportation, distance and adverse weather conditions in winter. On the other hand, it can be concluded that modern business owners are more sensitive to veterinary consultation. Another reason can be explained by the fact that the total number of calves (40) brought from modern processing is lower than the total number of calves (230) brought from traditional farms.

As a result of above findings, etiological and predisposing factors of calf diarrhea have been put forward. These findings may generate valuable information not only for the clinicians and researchers but also animal health experts, policy makers, farmer etc. Investigating the subtypes of the identified etiological agents in future studies will also contribute to the development of vaccines, especially against different serotypes. Further studies with concern to etiology and predisposing factors at different locations in our country and in the world should be performed in future, because, locations and animal movements may also affect such factors. So, precautions specific to each area can be taken.

## Availability of Data and Materials

The data given in this study may be obtained from the corresponding outher on reasonable request.

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## Conflict of Interest

The authors declare that they have no conflict of interest.

## Ethical Statement

This study was approved by the committee of HADYEK-Local Ethics Committee for Animal Experiments Office of Erciyes University (Approval no: 13/10).

## Authors' Contributions

İK, MÇ, VG, ÖA and ACO supervised the study. GE, ET, İKB, KV and ÖD collected the data. GE made the statistics. The first draft of the manuscript was written by GE and İK and all authors contributed to the critical revision of the manuscript and have read and approved the final version.

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