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Original article

Cryptosporidiosis outbreak on a dairy farm: Detection of *Cryptosporidium parvum* as a causative agent in the water source

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Abstract

Diarrhea caused by parasitic agents is common in neonatal calves and leads to significant economic losses in cattle farms worldwide. *Cryptosporidium* spp. is one of the most frequently detected parasitic agents causing diarrhea in neonatal calves. The aim of this study was to investigate the presence of *Cryptosporidium* spp. on a dairy farm which a has major diarrhea problem. Samples were collected from calves, cows, drinking bowls, and two different artesian water sources, as well as from the environment. All fecal samples were investigated using Kinyoun acid-fast stained slides and real-time PCR targeting the *Cryptosporidium* spp. *COWP* gene. In addition, species identification was performed by nested PCR targeting the *Cryptosporidium* spp. *COWP* gene and sequencing. *Cryptosporidium* spp. was detected in 11 calves (30.55%; 11/36) by real-time PCR and the cows were negative. Among real-time PCR positive samples, only five were also found positive by microscopy. Moreover, *Cryptosporidium* spp. was found in one of the two artesian water sources and five environmental samples by real-time PCR. Among these positive samples, eight were sequenced. According to the RFLP pattern, BLAST and, phylogenetic analyses, all sequenced samples were *Cryptosporidium parvum*. These findings show the importance of *C. parvum* as a cause of calf diarrhea on dairy farms.

Key words: neonatal calves, Cryptosporidium parvum, diarrhea, Real-time PCR, calf lose

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Introduction

Cryptosporidium species cause significant intestinal disease in livestock worldwide. Also, it can cause urinary tract infection in calves (Thompson et al. 2007, Fayer et al. 2010). One of the most frequent causes of death in calves is a diarrheal disease (Thompson et al. 2007). In calves younger than 30 days old, *Cryptosporidium* spp., rotavirus, coronavirus, *Escherichia coli*, and *Salmonella* spp. cause severe intestinal damage. Malabsorption, growth retardation, and loss in yield can occur in healing calves due to this intestinal damage (Naciri et al. 1999, Thompson et al. 2007).

Cryptosporidium species are responsible for 30% of diarrhea cases in calves (Şahal et al. 2018). There are 20 *Cryptosporidium* species and more than 60 genotypes. In cattle, it has been reported that ten different *Cryptosporidium* species and genotypes can cause infection (Xiao and Feng 2008). Cattle are most commonly infected with *C. parvum* (previously known as the *C. parvum* genotype II)*, C. andersoni, C. bovis,* and *C. ryanae*. Among these, the most pathogenic species that causes fatal watery diarrhea in calves younger than two months is *C. parvum.* Other species rarely give rise to clinical symptoms (Santín et al. 2004). Moreover, *Cryptosporidium* oocysts have also been detected in the respiratory system, bursa fabricius, and conjunctiva of poultry and in the urinary system of calves (Thompson 2004, Fayer et al. 2010).

In the epidemiology of *Cryptosporidium* infection, oocysts resistant to environmental conditions and standard disinfectants play an important role. Oocysts are small enough to pass through filtration (Silverlås 2010).

Many factors such as immunity, nutrition, environmental factors, and farm management play a major role in the development of *Cryptosporidium* infection in calves. Also, drinking water and food quality, as well as biosecurity, are important factors for the transmission of *Cryptosporidium* oocysts (O'Handley 2007).

In the diagnosis of *Cryptosporidium* spp. infection, microscopy, immunological methods and molecular techniques such as nested PCR and real-time PCR have been used. During molecular diagnosis, *18S rRNA gene, gp60, COWP, β-tubulin gene* and *hsp70* are generally targeted. For example, real-time PCR targeting the *COWP* gene can detect the *C. parvum*, *C. hominis*, *C. baleyi*, *C. felis*, *C. meleagridis*, *C. wrairi*, *C. serpentis,* and *C. muris* and its sensitivity is 4 copies/reaction (Guy et al. 2003). In addition, the COWP gene has also been used in genotyping of *Cryptosporidium* spp. isolates using the RFLP method with the *RsaI* restriction enzyme (Spano et al. 1997, Pedraza-Díaz et al. 2001, Muthusamy et al. 2006, Said et al. 2020).

The present study aimed to investigate the presence of Cryptosporidium in a dairy farm with a diarrhea outbreak using microscopic and real-time PCR methods. In addition, it also aimed to identify species identification in Cryptosporidium positive samples by sequencing the *COWP* gene.

Materials and Methods

Properties of the farm

The dairy farm is located on the Izmir-Ankara road and 1.5 hours away from Izmir. There are about 600 cows and 150 calves on the farm. As a rule, in the farm, cows in the peri-parturient period are taken to a separate paddock for delivery. After birth, calves are fed colostrum using a bottle and transferred to paddocks where they are fed singly until two-months old. At the end of the two months, the calves are placed in bigger paddocks with a capacity of 10-15 animals.

Drinking water for all animals on the farm is provided from two different artesian wells (Fig. 1). Artesian well number 1 coming to the dairy farm is close to the city center, and there is a wastewater treatment plant nearby. Artesian well number 2 is located on a hill far away from the city center. The artesian water comes to the farm via individual pipelines from both artesian wells and is collected into a central tank. The water is then delivered to the calves and cattle without any filtration or disinfection. Two veterinarians work in the farm on a 24/7 shift schedule.

Samples

During the study, 47 stool samples were collected from calves (n: 36) and cows (n: 11) (These samples were designated S1-S47, Table 1). Among the 26 calves younger than two months 12 had clinical diarrhea and the remaining 14 had had diarrhea previously. Antibiotic treatment was applied empirically to all of these calves. The remaining ten calves without diarrhea were older than two months.

Two samples with a volume of 8 liters were collected from the two different artesian wells (the artesian well, close to the city, was designated AW1, and the other one, AW2, Table 2). Drinking water samples with a volume of 50 ml were collected from each drinking bowl in the paddocks with calves younger than two months and 20 samples were pooled. As a result, six pooled samples consisting of one liter of water were generated (These samples were designated WS1-WS6, Table 2). A water sample of two liters was also collected from 6 different paddocks with calves older than 2 months (WS7, Table 2) and another one liter water sample was taken from the delivery paddocks (WS8,

Fig 1. Location of the dairy farm and water resources

Table 2) (Robertson et al. 2009).

Swab samples were obtained from various surfaces on the farm using sterile cotton swabs and then immersed in 1 ml sterile saline in a 50 ml tube (Table 2).

Microscopy

Stool samples were stained by Kinyoun acid-fast dye as it is a reliable, low cost, and simple method performed in the practice setting to detect Cryptosporidium (Kehl et al. 1995, Cole 1997). For this purpose, an average of 5-10 g stool samples in 50 ml tubes were mixed with 10 ml sucrose solution (53 g sucrose, 100 ml water) and centrifuged at 400 × g for 10 minutes (Elmi et al. 2017). Subsequently, the upper liquid collected from the top of each tube was spread on slides and fixed with methanol after drying in the air. The slides were then stained using the Kinyoun acid-fast method according to the manufacturer's protocol (RTA, Turkey). Briefly, the slides were immersed in Kinyoun dye for 2-3 minutes and washed with tap water. The slides were then immersed in acid-alcohol for 5-10 seconds, rinsed with tap water, and immersed in methylene blue for 20-30 seconds. Finally, the slides were washed with tap water, air-dried, and examined under a light microscope using 1000× magnification with immersion oil (Ayinmode and Fagbemi 2010).

DNA extraction

DNA extraction from fecal samples was performed using a stool DNA isolation kit (RTA Labs, Turkey) according to the manufacturer's protocol. During DNA isolation, a 100 µg fecal sample was used and DNA was eluted with 100 µl of elution buffer (Karakavuk et al. 2021). DNA isolation from water samples and environmental samples was performed as described below using a QIAamp Mini kit (Qiagen, USA). Before DNA isolation, the water samples were initially passed through 0.45 µm filters (Corning, USA). The filters were then removed, cut with a sterile scalpel, and put into 50 ml sterile tubes containing 500 µg zirconia beads, 125 µg glass beads, 20 µl proteinase K, 1000 µl saline, and 1000 µl AL buffer (Qiagen, USA). The tubes were then incubated for 4 hours in a shaker incubator at 37°C and 350 rpm. After incubation, the tubes were centrifuged at 3000 rpm for 10 minutes, the supernatants obtained from each tube were collected and 500 µl absolute ethyl alcohol was added to the supernatants. Following this step, the routine DNA isolation method was applied. Swap samples were initially incubated for 1 hour in a shaker incubator at 37°C and 350 rpm before DNA isolation. 20 µl proteinase K and 1000 µl buffer AL were then added and incubated for 10 min at 70°C. After this incubation, the routine DNA isolation method was applied.

Real-Time PCR

During real-time PCR, the primers targeting the 151 bp region of *Cryptosporidium* spp. *COWP* gene were 5'- CAAATTGATACCGTTTGTCCTTCTG -3' (25nt, cowp-P702 forward primer) and 5'- GGCATGTCGAT TCTA ATTCAGCT -3' (23nt, cowp-P702 reverse primer) and the hydrolysis probe was 5'- TGCCATACATT GTTGTCCTGACAAATTGAAT -3'-BHQ (31nt, cowp-P702, labeled at the 3' end with FAM). The PCR reaction with a 20 µl final volume included 5 µl DNA

Table 1. Real-time PCR, Nested PCR and microscopy results in stool samples.

Sample No	Paddock No	Age	Clinic	Crypto Acid Fast	Crypto RT-PCR	Crypto Nested PCR
S1	8	<2 months	Diarrhea	**Positive**	**Positive**	**Positive**
S2	21	<2 months	Diarrhea	**Positive**	**Positive**	**Positive**
S3	17	<2 months	Diarrhea	N	N	N
S4	14	<2 months	Diarrhea	**Positive**	**Positive**	**Positive**
S5	28	<2 months	Diarrhea	N	**Positive**	N
S6	23	<2 months	Diarrhea	N	**Positive**	N
S7	26	<2 months	Diarrhea	N	**Positive**	**Positive**
S8	19	<2 months	Diarrhea	**Positive**	**Positive**	**Positive**
S9	29	<2 months	Diarrhea	N	N	N
S10	30	<2 months	Diarrhea	N	N	N
S11	44	<2 months	Diarrhea	N	**Positive**	N
S12	48	<2 months	Diarrhea	N	**Positive**	N
S13	5	<2 months	P-Diarrhea	N	N	N
S14	123	<2 months	P-Diarrhea	N	N	N
S15	115	<2 months	P-Diarrhea	N	N	N
S16	102	<2 months	P-Diarrhea	N	N	N
S17	116	<2 months	P-Diarrhea	N	N	N
S18	3	<2 months	P-Diarrhea	**Positive**	**Positive**	N
S19	74	<2 months	P-Diarrhea	N	N	N
S20	114	<2 months	P-Diarrhea	N	N	N
S21	107	<2 months	P-Diarrhea	N	**Positive**	**Positive**
S22	2	<2 months	P-Diarrhea	N	N	N
S23	94	<2 months	P-Diarrhea	N	N	N
S24	96	<2 months	P-Diarrhea	N	N	N
S25	99	<2 months	P-Diarrhea	N	N	N
S26	1	<2 months	P-Diarrhea	N	N	N
S27	6-P	>2 months	No Symptom	N	N	N
S28	4-P	>2 months	No Symptom	N	N	N
S29	1-P	>2 months	No Symptom	N	N	N
S30	3-P	>2 months	No Symptom	N	N	N
S31	4-P	>2 months	No Symptom	N	N	N
S32	6-P	>2 months	No Symptom	N	N	N
S33	3-P	>2 months	No Symptom	N	N	N
S34	1-P	>2 months	No Symptom	N	N	N
S35	2-P	>2 months	No Symptom	N	N	N
S36	2-P	>2 months	No Symptom	N	N	N
S37	CC	>2 years	No Symptom	N	N	N
S38	CC	>2 years	No Symptom	N	N	N
S39	CC	>2 years	No Symptom	N	N	N
S40	CC	>2 years	No Symptom	N	N	N
S41	CC	>2 years	No Symptom	N	N	N
S42	CC	>2 years	No Symptom	N	N	N
S43	CC	>2 years	No Symptom	N	N	N
S44	CC	>2 years	No Symptom	N	N	N
S45	CC	>2 years	No Symptom	N	N	N
S46	CC	>2 years	No Symptom	N	N	N
S47	CC	>2 years	No Symptom	N	N	N

N: Negative, P-Diarrhea: Previously Diarrhea, CC: Cow compartment, * Calves older than two months stay in compartments with 10-15 calves

Table 2. Real-time PCR and Nested PCR results in environmental samples.

Sample No	Sample source	Crypto RT-PCR	Crypto Nested PCR
AW1	Artesian well water 1	**Positive**	**Positive**
AW2	Artesian well water 2	N	N
WS1	Paddock Waterer 1-20	N	N
WS2	Paddock Waterer 21-41	N	N
WS3	Paddock Waterer 42-60	N	N
WS4	Paddock Waterer 61-80	N	N
WS5	Paddock Waterer 81-100	N	N
WS6	Paddock Waterer 101-120	N	N
WS7	Paddock Waterer Belong to Older Than 2 Months	N	N
WS8	Delivery Room Water	N	N
S1	Paddock 1-10	**Positive**	**Positive**
S2	Paddock 11-21	N	N
S3	Paddock 22-30	**Positive**	N
S4	Paddock 31-40	**Positive**	N
S5	Paddock 41-50	N	N
S6	Paddock 51-60	N	N
S7	Paddock 61-70	**Positive**	N
S8	Paddock 71-80	N	N
S9	Paddock 81-90	N	N
S10	Paddock 91-100	N	N
S11	Paddock 101- 110	**Positive**	N
S12	Paddock 111-120	N	N
S13	Paddock 1 Belong to Older Than 2 Months	N	N
S14	Paddock 2 Belong to Older Than 2 Months	N	N
S15	Paddock 3 Belong to Older Than 2 Months	N	N
S16	Paddock 4 Belong to Older Than 2 Months	N	N
S17	Paddock 5 Belong to Older Than 2 Months	N	N
S18	Paddock 6 Belong to Older Than 2 Months	N	N
S19	Bottle Inside	N	N
S20	Bottle Outside	N	N
S21	Milk Heating Tank (Insıde)	N	N
S22	Milk Heating Tank (Outside)	N	N
S23	Colostrum Cabinet (Inside)	N	N
S24	Colostrum Cabinet (Outside)	N	N
S25	Towel	N	N
S26	Delivery Room Milk Machine (Inside)	N	N
S27	Delivery Room Milk Machine (Outside)	N	N
S28	Medicine Cabinet (Inside)	N	N
S29	Medicine Cabinet (Outside)	N	N
S30	Medicine Room Door	N	N
S31	Colostrum Bottle	N	N

WS: Water samples, S: Swab samples, N: Negative. AW: Artesian well water

template or controls, 1× LightCycler Taqman Master mix, 0.5 µM from each primer, and 0.1 µM probe. The amplification reaction was performed as follows: 10 min initial denaturation step at 95°C, followed by 45 cycles of 10 seconds at 95°C, 15 seconds at 55°C, and 15 seconds at 72°C (Taniuchi et al. 2011).

Positive control plasmid containing *Cryptosporidium COWP* gene fragment was prepared using a pCR 2.1. TOPO TA kit according to the manufacturer's protocol (Thermoscientific, USA) (Döşkaya et al. 2011, Karakavuk et al. 2018). During the PCR reactions 10-fold dilutions of positive control plasmid ranging from 1×10^6 to 10 copies of COWP/µl were used. *Cryptosporidium parvum* negative and positive fecal samples previously determined by PCR and microscopy were used as external controls. In addition, for the negative control, template DNA was replaced with distilled water and used in each run. PCR reactions were prepared in duplicate to analyze PCR inhibition. One reaction contained only DNA sample and the other contained 10 copies of COWP/µl spiked into the DNA sample. Quantification analysis was performed using LightCycler software, Version 4.0, in a 1.5 LightCycler Real Time instrument (Roche).

Nested PCR

All real-time PCR positive samples were also investigated by nested PCR for species identification of *Cryptosporidium* spp. as described (Spano et al. 1997, Pedraza-Díaz et al. 2001). In the initial reaction, BCOWPF (5'-ACCGCTTCTCAACAACCATCTTGT CCTC-3') and BCOWPR (5'-CGCACCTGTTCCC ACTCAATGTAAACCC-3') primers were used to amplify the 769-bp fragment of the *COWP* gene. In the second reaction, a 553-bp gene fragment was amplified from the initial reaction product by cry-15 (5'-GTAGATAATGGAAGAGATTGTG-3') and cry-9 (5'-GGACTGAAATACAGGCATTATCTTG-3') primers. In the initial reaction of nested PCR, 25 µl amplification reaction included 2.5 µl template DNA, 1 µl primers (0.4 mM each), and 5 µl PCR Master Mix (5×, GeneMark). In the second step of nested PCR, a 2.5 µl PCR product from the first reaction was used as a template. The nested PCR was performed using the following protocol for both steps: 3 min initial denaturation step at 94 °C, followed by 30 cycles of 1 min at 94°C, 1 min at 65°C, and 1 min at 72°C, and a final extension of 10 min at 72°C.

Species identification

Nested PCR products of 553 bp belonging to *Cryptosporidium* spp. positive samples were sequenced by ABI3730XL. Generated sequences were aligned using the MUSCLE algorithm and edited by MEGA7.0 Software to find the *RsaI* digestion regions that are used for the identification of *Cryptosporidium* species (Spano et al. 1997, Pedraza-Díaz et al. 2001). BLAST analysis was also performed to compare with reference *Cryptosporidium* samples in the National Center for Biotechnology Information (NCBI).

Phylogenetic analysis

During the phylogenetic analysis, a model test was performed using MEGA7.0 Software by selecting the "Find Best DNA/Protein Models (ML)" option. The phylogenetic tree based on *COWP* sequences belonging to *Cryptosporidium* spp. positive samples was constructed using MEGA7.0 Software based on the Maximum Likelihood method using the Tamura 3 Gamma distributed with Invariant sites (T3+G+I) model with 1000 Bootstrap replications.

Statistical analysis

The data obtained from the study were processed using the Microsoft Excel 2010 program, and statistical analysis was performed using the Graphpad Prism 3 program (GraphPad, San Diego, CA). Among the calves with diarrhea and previous history of diarrhea, the presence of *Cryptosporidium* spp. was compared with a two-tailed unpaired t-test. $p<0.05$ was considered statistically significant unless otherwise stated.

Results

Microscopy

Cryptosporidium spp. oocysts were observed in 5 (19.23%) calves younger than two months during the microscopic examination. Four of them were detected in calves with diarrhea (33.33%) and the remaining one was detected in a calf with a previous history of diarrhea (7.14%). No *Cryptosporidium* spp. oocysts were observed in calves older than two months or cows. Among all animals examined, *Cryptosporidium* oocysts were detected in 10.63% of them (5/47). (Fig. 2, Table 1).

Real-Time PCR

As a result of the investigation of fecal DNA samples, *Cryptosporidium* spp. *COWP* gene was detected in 11 (n: 26; 42,30%) calves younger than two months using real-time PCR. Among these, the *Cryptosporidium* spp. *COWP* gene was observed in 9 (75%) calves with diarrhea, while *Cryptosporidium* spp. *COWP* gene was detected in 2 (14.28%) calves with a previous history of diarrhea. The difference between calves with

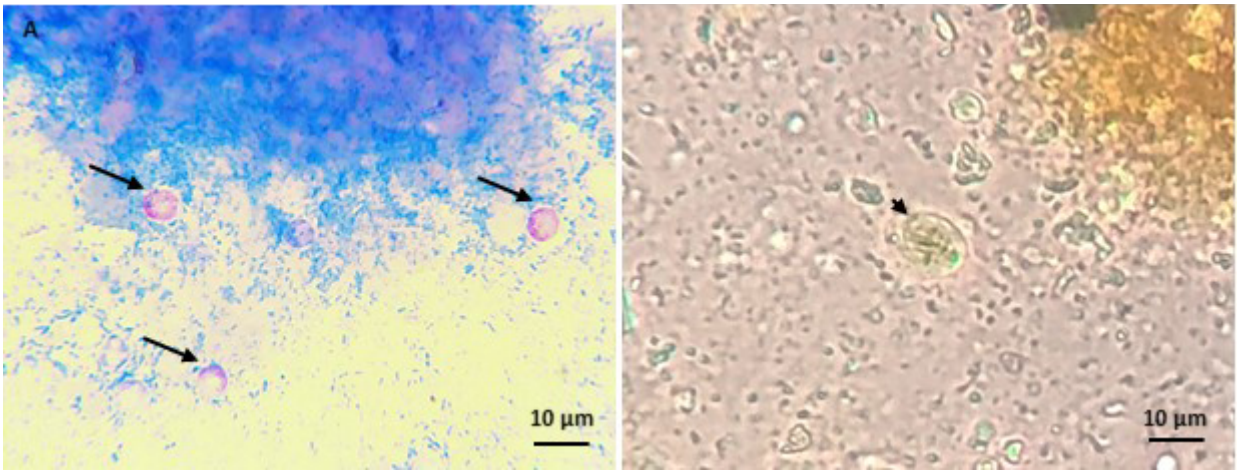

Fig 2. Microscopic examination of feces. **(A)**: *Cryptosporidium* oocysts stained with Kinyoun acid fast (black arrows).

diarrhea and calves with a previous history of diarrhea was statistically significant ($p<0.001$). In terms of the diagnosis of diarrhea caused by *Cryptosporidium* in calves, the real-time PCR method was found to be significantly more sensitive than the Kinyoun acid-fast staining method ($p<0.05$).

The *Cryptosporidium* spp. *COWP* gene was not detected by real-time PCR in calves older than two months and cows. Among all animals examined, the *Cryptosporidium* spp. *COWP* gene was detected in 27.65% (13/47) of animals (Table 1).

In addition, *Cryptosporidium* spp. was not detected in the drinking bowl and artesian well 2 but was detected in artesian well 1. Also, *Cryptosporidium* spp. was detected in paddock (numbered 1-10, 22-30, 31-40, 61-70, 101-110), drinking bowl, and mangers (Table 2).

Nested PCR

Among real-time PCR positive samples, 8 were found positive by nested PCR, of which 6 were detected in fecal samples and the remaining 2 were detected in environmental samples including artesian well 1 and paddock irons (numbered 1-10) (Tables 1 and 2).

Species identification

Sequences of nested PCR positive samples were analyzed, and all isolates were detected to have three *RsaI* digestion regions which resulted in 410, 106 and, 34 bp fragments in size. These fragment sizes were compatible with the expected band pattern for *C. parvum* isolates. Also, BLAST results showed high similarity rates varying from 98.72% to 99.52% among the *Cryptosporidium* spp. isolates detected in this study and *C. parvum* isolates used in phylogenetic analysis the (Table 3). The phylogenetic tree also showed that *Cryptosporidium* spp. positive samples clustered with reference *C. parvum* isolates (Fig 3).

Discussion

In many studies, it has been shown that the prevalence of diarrhea in calves is related to the density of *Cryptosporidium* spp. In addition, it was concluded that the more the farm's hygiene deteriorates, the more *Cryptosporidium* spp.-related diarrhea and calf deaths can increase (Torsein et al. 2011).

The prevalence of *Cryptosporidium* spp. infection in cattle is around 30% worldwide (Hatam-Nahavandi et al. 2019). Parallel to this, the molecular prevalence of *Cryptosporidium* spp. was found as 27.65% in this study. A previous study conducted in Australia reported that the prevalence of *Cryptosporidium* spp. was 22.3% (81/364) in calves (Ng et al. 2011). Another study conducted in Nigeria reported that the prevalence of *Cryptosporidium* was found to be 23.4% (95/406), with an infection rate of 27.4%, 28.1% and 19.9% in cattle less than 6 months, 7-12 months and over 12 months old respectively, using the Kinyoun-acid fast stain method (Ayinmode and Fagbemi 2010). Also, in Thailand, the prevalence of Cryptosporidium oocysts was determined as 51% (102/200) using the Kinyoun--acid fast stain method (Doungmala et al. 2019). In addition to these studies, in India, the prevalence of *Cryptosporidium* spp. was reported as 32.3% in calves with diarrhea according using PCR (Paul et al. 2008). In a different study conducted in India, prevalence was found as 50% (40/80), using modified Ziehl-Neelsen staining (Singh et al. 2006). In one of two different studies conducted in Poland, the prevalence of *Cryptosporidium* spp. in 75 heifers was determined as 30.7%, using modified Ziehl-Neelsen staining (Pilarczyk et al. 2009) while in the other, *Cryptosporidium* spp. prevalence was found to be 17% (119/700), using PCR (Rzezutka and Kaupke 2013). Also, in Canada, the causative agent of diarrhea in 40.6% (203/500) of calves was detected as *Cryptospori-*

Table 3. BLAST results among *Cryptosporidium sp.* isolates and *C. parvum* isolates used in phylogenetic analysis.

Isolates	*C. parvum* COWP (AB514061)	*C. parvum* COWP (KC885900)
Cryptosporidium isolate 1	99.57%	99.14%
Cryptosporidium isolate 2	99.36%	98.93%
Cryptosporidium isolate 4	99.36%	98.93%
Cryptosporidium isolate 7	99.14%	98.72%
Cryptosporidium isolate 8	99.57%	99.14%
Cryptosporidium isolate 22	99.57%	99.14%
Cryptosporidium isolate 23	99.57%	99.14%
Cryptosporidium isolate 24	99.57%	99.14%

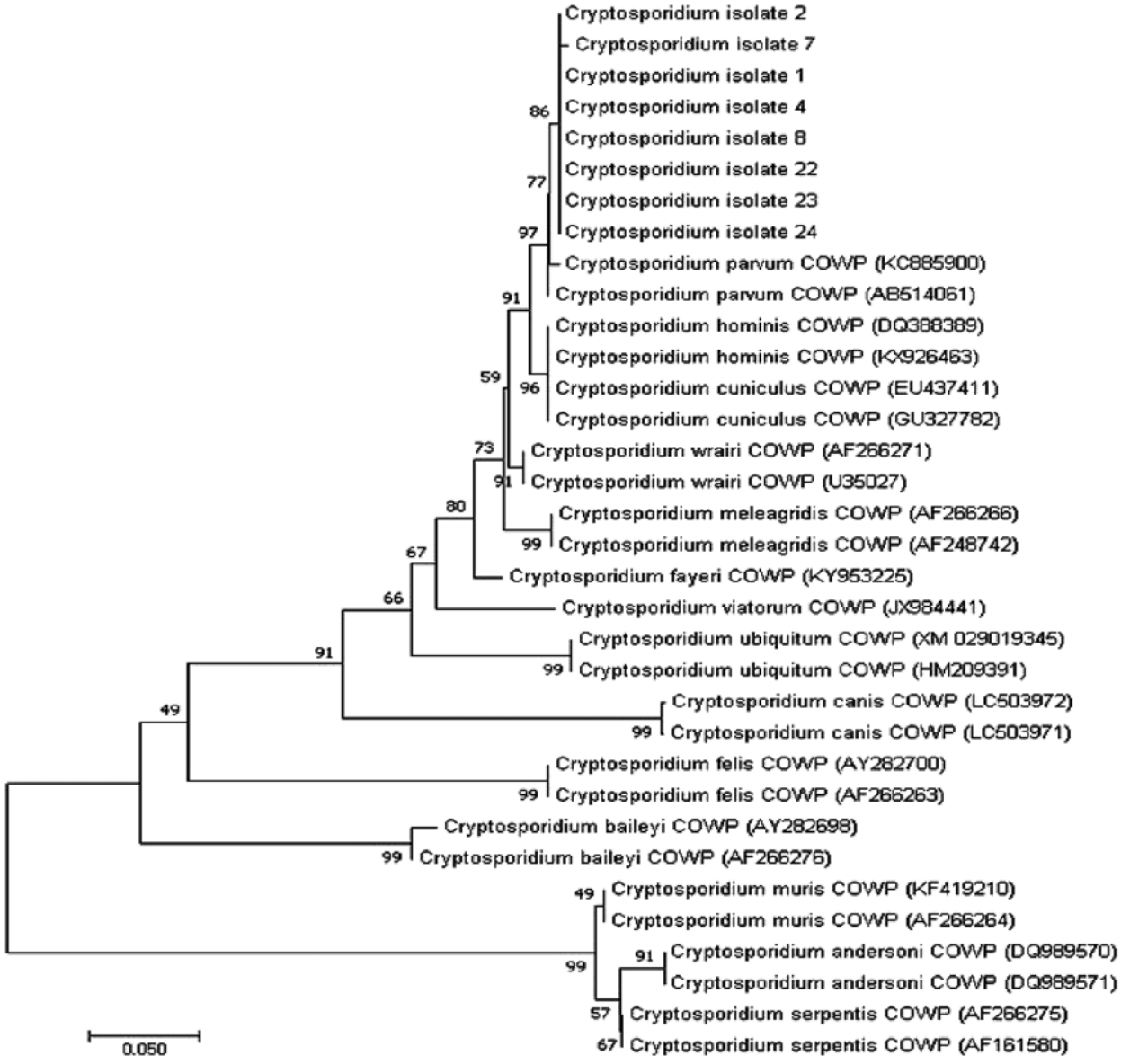

Fig 3. Phylogenetic tree showing the association of *Cryptosporidium* isolates detected in this study with *C. parvum* isolates.

dium spp. by microscopy (Trotz-Williams et al. 2005). A higher prevalence value was reported in a study conducted in Norway, the prevalence of *Cryptosporidium* spp. was determined as 12% (167/1386) in calves and 53% (72/136) in farmers using immunofluorescent staining (Hamnes et al. 2006). These findings from our results and previous results obtained from different countries/regions demonstrate the seriousness of *Cryptosporidium* spp. infection in cattle and indicate that *Cryptosporidium* spp. prevalence is high worldwide.

A high prevalence of *Cryptosporidium* spp. has also been shown in studies conducted in Turkey. *Cryptosporidium* oocysts were first detected in a cattle in 1984 (Burgu 1984). Later, the prevalence of *Cryptosporidium* oocyst in calf stool samples was found to be 32.9% in Kars, 27.33% in Konya, and 35.8% in Ankara by microscopy (Sevinc et al. 2003, Arslan et al. 2012, Şahal et al. 2018). In another study conducted in Nevşehir, *Cryptosporidium* spp. DNA was found in 20.7% of the stool samples of 150 calves with diarrhea using real-time PCR (Şimsek et al. 2012). In this study, *Cryptosporidium* spp. DNA was found in 75% of calves with diarrhea. This high prevalence rate indicates an outbreak of *Cryptosporidium* spp. in calves in the examined dairy farm. In addition, the detection of *Cryptosporidium* spp. DNA in one of the artesian wells indicates that this water is a potential infection source for the animals.

PCR and microscopy are the most commonly used methods in the diagnosis of *Cryptosporidium* spp. infection in calves, and studies have shown that PCR is more sensitive and specific than microscopy (Morgan et al. 1998; Taniuchi et al. 2011). Similarly, to these studies, the real-time PCR method was found to be significantly more sensitive ($p<0.05$) than microscopy in our research. Also, PCR methods are advantageous because they do not require experienced personnel, many samples are run at the same time, and they do not require the concentration methods used in microscopy (Morgan et al. 1998, Taniuchi et al. 2011).

Cryptosporidium spp. is a zoonotic infection that can be passed from calves/cattle to humans and vice versa. *Cryptosporidium* spp. oocysts were found in 33.47% of human cases with diarrhea using Kinyoun acid-fast staining in İzmir which is the neighboring province of the dairy farm examined in this study (Turgay et al. 2012). Accordingly, it is crucial to check the stool samples of the personnel working on such farms to prevent the spread of the disease to humans as well as other animals.

The high incidence of *Cryptosporidium* infections in newborn calves results from inadequate hygiene practices. Transmission in farms can occur through direct contact with infected animals and poor conditions in terms of medical care. Moreover, water supplies contaminated with *Cryptosporidium* spp. oocytes increase the prevalence of cryptosporidiosis (Ralston et al. 2003).

In this study, *Cryptosporidium* spp. was detected in one of the artesian wells. Drinking water used for calves is brought from this polluted artesian water source through an individual water pipeline. It is supplied to calves and dairy cows without any filtration or disinfection. Due to the lack of sanitation, not only *Cryptosporidium* spp. infection but also other agents that lead to diarrhea can infect calves and cows. Adjacent paddocks facilitate the passage of feces with diarrhea that are infected with *Cryptosporidium* spp. oocysts. Also, relevant environmental samples that are positive for *Cryptosporidium* spp. are another factor that eases the transmission of *Cryptosporidium* spp. oocytes among animals.

Following on our study results and recommendations to the dairy farm manager, water intake from the infected artesian well source was immediately stopped, and a new artesian source was opened within the boundaries of the dairy farm. Meanwhile, the calves were treated with anti-parasitic drugs for *Cryptosporidium*. Later, *Cryptosporidium* spp. was investigated in water samples collected from the new artesian source at 3-month intervals, and *Cryptosporidium* spp. DNA was not detected. As a result of these precautions, calf diarrhea cases have decreased on this farm.

Controlling calf deaths is crucial for a sustainable farm economy. Therefore, it is necessary to take measures against diarrhea, which is the most serious cause of calf deaths. Also, even if death does not occur, intestinal damage resulting from the pathogens that cause diarrhea is a significant cause of economic loss. Diarrhea caused by *Cryptosporidium* spp. is quite common in neonatal calves. Filtration or disinfection applied to drinking water used for calves are very important measures to protect them against *Cryptosporidium* and other waterborne pathogens. In addition, routine parasitological examination and pathogen-specific treatment will play an essential role in controlling the disease and reducing drug costs.

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