Investigation of a Cryptosporidiosis Outbreak Caused by *Cryptosporidium parvum* in a Dairy Farm

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Abstract

Background

Diarrhea caused by parasitic agents is common in neonatal calves and causes significant economic losses in cattle farms worldwide. *Cryptosporidium* spp. is one of the most frequently detected parasitic agents causing diarrhea in neonatal calves. Also, *Giardia intestinalis* is shown to cause diarrhea in calves. This study aimed to investigate the presence of *Cryptosporidium* spp. in calves (n:36), cows (n: 11), drinking water and two different artesian water supplies as well as in environmental swap samples (n:32) obtained from the manger, silage, bottle, and doorknob in a dairy farm which has big diarrhea problems. For this purpose, all fecal samples investigated with using direct microscopy for routine parasitological screening. Then, the presence of *Cryptosporidium* spp. was examined in feces samples and other environmental samples using Kinyoun acid-fast stained slides and Real-Time PCR targeting *Cryptosporidium* spp.. In addition, Real-Time PCR positive samples were investigated by nested PCR and subsequently by sequencing, BLAST, and phylogenetic analysis for species identification by MEGA7.

Results

Giardia intestinalis was detected in 10 feces samples (21,27%; 10/47) during routine microscopic examination. Among them, 9 belonged to calves older than two months without diarrhea, and one belonged to an adult cow. *Cryptosporidium* spp. was found in 11 calves (30.55%; 11/36) by Real-Time PCR, whereas no cows were found positive. Among the PCR positive samples, only five of them were detected as positive by microscopy. *Cryptosporidium* spp. positivity value was found higher in younger than two month old calves with diarrhea (9/12; 75%). Also, *Cryptosporidium* spp. was found in one of two water supplies and five of environmental samples by real-time PCR. Of the 18 real time positive samples, 8 were found positive by nested PCR and all positive samples were detected to be *Cryptosporidium parvum* by sequencing, BLAST, and phylogenetic analysis.

Conclusions

Our findings showed the importance of *C. parvum* infection in diarrhea cases occurred in calves. Besides the correct diagnosis and treatment of *Cryptosporidium* spp. contaminated water resources, and hygiene measures are very important for preventing the cryptosporidiosis in dairy farms.

Background

Cattle production is a very important and vital agricultural sector in Turkey. In 2019, the total cattle population in Turkey was reported to be 18 million (1). Approximately 6 million calves born each year in Turkey. Among them 15% die due to for various reasons before they reach the age of one. When the economic value of this loss is examined in Turkey, the average cost of a calf is considered to be

approximately 500 euros; the national financial loss is at least 450 million euros (1). One of the most important causes of death in calves is a diarrheal disease (2). In calves younger than 30 days old, *Cryptosporidium* spp., rotavirus, coronavirus, *Escherichia coli*, and *Salmonella* spp. cause enormous intestinal damage. Due to intestinal damage, malabsorption, growth retardation, loss in yield can be detected in healing calves (2, 3).

Among the pathogens causing diarrhea, *Cryptosporidium* species are responsible for 30% of them in calves (1). 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 (4). Cattles are most commonly infected with *C. parvum*., *C. andersoni*, *C. bovis*, and *C. ryanae*. But, 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 (5). Moreover, *Cryptosporidium* oocysts can also be detected in the respiratory system, bursa fabricius, and conjunctiva of poultry and urinary system of calves (6, 7).

In the epidemiology of *Cryptosporidium* infection, oocysts that are resistant to environmental conditions and standard disinfectants play important role. The oocysts are small enough to pass through filtration and, as low as nine oocysts in humans and 50 oocysts in calves can cause diarrhea (8).

Many factors such as immunity, nutrition, environment statues, and farm management play a role in the development of *Cryptosporidium* infection in calves. Also, drinking water and food quality, as well as biosecurity, are important for the transmission of *Cryptosporidium* oocysts (9).

Giardia intestinalis is another important parasite infecting a wide range of vertebrates, including humans and domestic animals. It has been found in the feces of calves and dairy cattle worldwide (7, 10). Giardiasis can cause a wide variety of clinical findings in cattle. Main clinic findings are severe persistent diarrhea, mucoid and fatty stool, weight loss, and growth retardation (10).

This study was carried out in a dairy farm located in Aegean Region of Turkey housing 150 calves and 600 cows where a diarrhea outbreak occurred and our study group was invited to investigate the farm and animals and to give scientific advice. Upon this request, we were contacted by veterinarians working in this farm and we went to the farm to find the causative agent of diarrhea. For this purpose, stool samples from calves and cows in the farm, samples from drinking water and feed containers, swabs samples from door handles, feeding bottles and breast milk, and water samples from two sources of artesian water were collected. In these samples, the presence of *Cryptosporidium* spp. oocysts and *Giardia intestinalis* cyst or trophozoite was investigated by direct microscopy and Kinyoun acid-fast stained slides. In addition, the presence of *Cryptosporidium* spp. was investigated by Real-Time PCR. This innovative and in-depth outbreak analysis method has been tried for the first time in a dairy farm in our country. If this approach becomes successful, it may be an outbreak analysis model that can be applied to many of our farms with similar infection problems.

Results

Microscopy

Cryptosporidium spp. oocysts were observed in 5 of 26 (19.23%) calves younger than two months during the microscopic examination of Kinyoun acid-fast stained preparations. Four of them were detected in calves with diarrhea (n: 12; 33.33%), and the remaining one was detected in a calf with previous history of diarrhea (n: 14; 7.14%). *Cryptosporidium* spp. oocysts were not observed in calves older than two months and cows by microscopy. Among all animals examined, *Cryptosporidium* oocysts were detected in 10.63% (5/47). (Fig. 2A, Table 1).

Table 1
Real-time PCR, Nested PCR and Microscopy Results in Stool Samples

Sample No	Paddock No	Age	Clinic	Native Lugol	Crypto Acid Fast	Crypto RT-PCR	Crypto Nested PCR
D1	8	< 2 months	Diarrhea	N	Positive	Positive	Positive
D2	21	< 2 months	Diarrhea	N	Positive	Positive	Positive
D3	17	< 2 months	Diarrhea	N	N	N	N
D4	14	< 2 months	Diarrhea	N	Positive	Positive	Positive
D5	28	< 2 months	Diarrhea	N	N	Positive	N
D6	23	< 2 months	Diarrhea	N	N	Positive	N
D7	26	< 2 months	Diarrhea	N	N	Positive	Positive
D8	19	< 2 months	Diarrhea	N	Positive	Positive	Positive
D9	29	< 2 months	Diarrhea	N	N	N	N
D10	30	< 2 months	Diarrhea	N	N	N	N
D11	44	< 2 months	Diarrhea	N	N	Positive	N
D12	48	< 2 months	Diarrhea	N	N	Positive	N
D13	5	< 2 months	P-Diarrhea	N	N	N	N
D14	123	< 2 months	P-Diarrhea	N	N	N	N
D15	115	< 2 months	P-Diarrhea	N	N	N	N
D16	102	< 2 months	P-Diarrhea	Giardia	N	N	N
D17	116	< 2 months	P-Diarrhea	N	N	N	N

Sample No	Paddock No	Age	Clinic	Native Lugol	Crypto Acid Fast	Crypto RT-PCR	Crypto Nested PCR
D18	3	< 2 months	P-Diarrhea	N	Positive	Positive	N
D19	74	< 2 months	P-Diarrhea	N	N	N	N
D20	114	< 2 months	P-Diarrhea	Giardia	N	N	N
D21	107	< 2 months	P-Diarrhea	Giardia	N	Positive	Positive
D22	2	< 2 months	P-Diarrhea	Giardia	N	N	N
D23	94	< 2 months	P-Diarrhea	N	N	N	N
D24	96	< 2 months	P-Diarrhea	Giardia	N	N	N
D25	99	< 2 months	P-Diarrhea	Giardia	N	N	N
D26	1	< 2 months	P-Diarrhea	N	N	N	N
D27	6-P	> 2 months	No Symptom	N	N	N	N
D28	4-P	> 2 months	No Symptom	Giardia	N	N	N
D29	1-P	> 2 months	No Symptom	N	N	N	N
D30	3-P	> 2 months	No Symptom	Giardia	N	N	N
D31	4-P	> 2 months	No Symptom	Giardia	N	N	N
D32	6-P	> 2 months	No Symptom	N	N	N	N
D33	3-P	> 2 months	No Symptom	N	N	N	N
D34	1-P	> 2 months	No Symptom	N	N	N	N
D35	2-P	> 2 months	No Symptom	N	N	N	N

Sample No	Paddock No	Age	Clinic	Native Lugol	Crypto Acid Fast	Crypto RT-PCR	Crypto Nested PCR
D36	2-P	> 2 months	No Symptom	N	N	N	N
D37	CC	> 2 years	No Symptom	N	N	N	N
D38	CC	> 2 years	No Symptom	N	N	N	N
D39	CC	> 2 years	No Symptom	N	N	N	N
D40	CC	> 2 years	No Symptom	N	N	N	N
D41	CC	> 2 years	No Symptom	N	N	N	N
D42	CC	> 2 years	No Symptom	Giardia	N	N	N
D43	CC	> 2 years	No Symptom	N	N	N	N
D44	CC	> 2 years	No Symptom	N	N	N	N
D45	CC	> 2 years	No Symptom	N	N	N	N
D46	CC	> 2 years	No Symptom	N	N	N	N
D47	CC	> 2 years	No Symptom	N	N	N	N
N: Negative, P-Diarrhea: Previously Diarrhea, CC: Cow compartment, * Calves older than two months stay in compartments with 10–15 calves.							

During the direct microscopic examination of fecal samples, *G. intestinalis* cysts were observed in 6 of the 26 (23.07%) calves younger than two months. In this group, *G. intestinalis* positive samples were detected in 6 calves that had diarrhea previously (n: 14; 42.85%). *G. intestinalis* cysts were observed in 3 calves (n: 10, 30%) older than two months without diarrhea. In addition, *G. intestinalis* cysts were detected in one of the 11 cows (9.09%). Among all animals examined, *G. intestinalis* cysts were found in 21.27% (10/47) of animals (Fig. 2B, Table 1).

Real-Time PCR

As a result of the investigation of fecal DNA samples, *Cryptosporidium* spp. DNA was detected in 11 of 26 (42,30%) calves younger than two months using Real-Time PCR. Among these, *Cryptosporidium* spp. DNA was observed in 9 of 12 (75%) calves with diarrhea, while *Cryptosporidium* DNA was detected in 2 of 14 (14,28%) with previous history of diarrhea. The difference between calves with diarrhea and calves with previous history of diarrhea was statistically significant ($P < 0,001$). In terms of the diagnosis of *Cryptosporidium* in diarrhea calves, Real-Time PCR method was found to be significantly sensitive than the Kinyoun acid-fast staining method ($P < 0,05$).

Cryptosporidium spp. DNA was not recognized by Real-Time PCR in calves older than two months and cows. Among all animal examined, *Cryptosporidium* spp. DNA was detected in 27.65% (13/47) of animals (Table 1).

In addition, while positivity was not detected in paddock drinkers and artesian water 2, interestingly high positivity was found in artesian water 1 (Crossing point: 33.92). This water source was located near the city center and next to the wastewater treatment plant (Fig. 1). Also, *Cryptosporidium* spp. DNA was found on paddock irons (numbered 1–10, 22–30, 31–40, 61–70, 101–110), waterer, and feeders at the edges (Table 2).

Table 2
Real-time PCR and Nested PCR Results in Environmental Samples

Sample No	Artesian well water No/Paddock No	Crypto RT-PCR	Crypto Nested PCR
SK1	Artesian well water 1	Positive	Positive
SK2	Artesian well water 2	N	N
SK3	Paddock Waterer 1–20	N	N
SK4	Paddock Waterer 21–41	N	N
SK5	Paddock Waterer 42–60	N	N
SK6	Paddock Waterer 61–80	N	N
SK7	Paddock Waterer 81–100	N	N
SK8	Paddock Waterer 101–120	N	N
SK9	Paddock Waterer Belong to Older Than 2 Months	N	N
SK10	Delivery Room Water	N	N
S1	Paddock 1–10	Positive	N
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

Sample No	Artesian well water No/Paddock No	Crypto RT-PCR	Crypto Nested PCR
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 (Inside)	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
SK: Water samples, S: Swap samples, N: Negative			

Nested PCR

Among 18 *Cryptosporidium* spp. positive samples detected by real time PCR, 8 were also found positive by nested PCR. Of the 18 *Cryptosporidium* spp. positive samples, 11 were detected in fecal samples and among these positive samples, 6 were also found positive by nested PCR. Among the remaining 7 real time positive samples detected in environmental samples, artesian water 1 and paddock irons (numbered 1–10) were also found positive by nested PCR (Tables 1 and 2).

Species identification

As sequences belonging to *Cryptosporidium* spp. isolates were analyzed, all isolates three *RsaI* digestion regions. After *RsaI* digestion, obtained fragment lengths were 410, 106 and 34 bp. These fragment lengths were compatible with expected band patten for *C. parvum* isolates. Also, BLAST results showed high similarity rate varying 98.72–99.52% among *Cyptosporidium* spp. isolates and *C. parvum* isolates used in phylogenetic analysis (Table 3).

Table 3

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

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

Phylogenetic analysis

Phylogenetic analysis of the COWP sequences belonging to *Cryptosporidium* isolates showed that all isolates were clustered in the same branch with reference samples of *C. parvum* (Fig. 3). No unexpected or mixed branches containing different *Cryptosporidium* species were found. Nearly all branches of the tree showed high bootstrap values.

Discussion

Calf health is vital for the economical production of cattle breeding, which is widespread all over the world. In order to run a cost-effective operation, it is aimed for each cow to give birth to a calf once a year (19). There are 1.5 billion cattle populations in the world, and the densest countries are Brazil, India, the USA, and China (FAO 2018).

Diarrhea in newborn calves causes great economic losses worldwide. Diarrhea can be triggered by both infectious and non-infectious agents. *Cryptosporidium* spp., Rotavirus, Coronavirus, and *E. coli* are the most common neonatal diarrhea agents in calves (Lorenz et al., 2011; Meganck et al., 2015; Ok et al., 2009).

In a lot of studies, it has been stated that the prevalence of diarrhea in calves is related to *Cryptosporidium* spp. density. In addition, the more general hygiene of the farm is terrible, the more *Cryptosporidium* spp. related diarrhea and calf deaths can be seen (23).

Every year 9 million calves are born in the USA. More than 500,000 (6%) of calves born die before weaning. 56.4% of deaths have been determined as digestive system diseases (USDA 2018). This rate is 6.4% in Canada (24). In a study conducted in Brazil, it was determined that 8.5% of 1451 calves died

before weaning and 44% of deaths were caused by diarrhea (25). Acceptable mortality rate before calving was determined as 5% in calves (DCHA Gold Standards, 2013).

The prevalence of *Cryptosporidium* in cattle in the world is around 30% (26). The prevalence of *Cryptosporidium* spp. was found to be 22.3% (81/364) in Australia in calves (27). In India, the prevalence of *Cryptosporidium* by PCR was 32.3% in diarrhea calves (28). In another study conducted in India, *Cryptosporidium* positivity was 50% (40/80) in calves with diarrhea by modified Ziehl-Neelsen staining (29). In another study conducted in 75 heifers in Poland, the prevalence of *Cryptosporidium* was determined as 30.7% by modified Ziehl-Nielsen staining (30). 2009). In Canada, 40.6% (203/500) of calves with diarrhea were *Cryptosporidium* positive by microscopy (31). In a study conducted with immunofluorescent staining in Norway, the prevalence of *Cryptosporidium* was determined as 12% (167/1386) in calves and 53% (72/136) in farmers (32).

The high prevalence of *Cryptosporidium* spp. have been shown in studies conducted in Turkey (33). *Cryptosporidium* oocysts were first detected in cattle in a study conducted in 1984 in Turkey (34). Later, *Cryptosporidium* oocyst prevalence in calf stool samples was found to be 32.9% in Kars, 27.33% in Konya, and 35.8% in Ankara by microscopy (1, 35, 36).

In another study conducted in Nevşehir, *Cryptosporidium* DNA was found in 20.7% of stool samples of 150 calves with diarrhea using Real-Time PCR (33). In this study, *Cryptosporidium* spp. DNA was found in 75% of calves with diarrhea. This high prevalence value indicates that there is 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 water source has been linked to the constant infection of the daily water supplied to the calves with the parasite.

PCR and microscopy are the most commonly used diagnostic methods in the diagnosis of *Cryptosporidium* in calves, and studies have shown that PCR is more sensitive and specific than microscopy (16, 37). Similar to these studies, 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 concentration methods used in microscopy (16, 37).

Cryptosporidium spp. is a zoonotic infection. It can be passed from calves and cattle to man, and vice versa. *Cryptosporidium* spp. oocysts were found in 33.47% of human cases with diarrhea by Kinyoun acid-fast staining in İzmir. İzmir is the neighboring province of the dairy farm examined in this study (38). For these reasons, it is crucial to follow the stool samples of the personnel working in such farms to prevent the spread of the disease.

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 and water supplies contaminated with *Cryptosporidium* oocytes increase the prevalence of *Cryptosporidium* (39).

Diarrhea related to *Giardia intestinalis* in calves has been reported in many studies (32, 40). In our study, *G. intestinalis* cysts were observed in 21.27% (10/47) all animals examined. It was thought that the high prevalence could be due to the *Cryptosporidium* spp. detected-water source.

In this study, *Cryptosporidium* spp. was detected in one of the artesian water sources. This drinking water used for calves is brought from this polluted 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* but also other agents that cause diarrhea can infect calves and cows. In this study, the microscopic detection of *G. intestinalis* cysts supports insufficient water sanitation. Adjacent paddocks facilitate the passage of diarrhea feces that are infected with *Cryptosporidium* oocysts. Also, relevant environmental samples that are positive for *Cryptosporidium* are another factor that eases the communication of *Cryptosporidium* oocytes among animals.

After the detection of *Cryptosporidium* spp. and *G. intestinalis* in calves, water intake from infected artesian water source was stopped and a new artesian source was opened within the boundaries of the dairy farm. Meanwhile, calves were treated with anti-parasitic drugs for *Cryptosporidium* and *G. intestinalis*. Later, when *Cryptosporidium* spp. was checked in water samples collected from the newly opened artesian source with 3 months intervals, DNA was not detected. As a result of these precautions, it has been reported that diarrhea cases have decreased in calves on this farm.

Conclusion

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

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 in the farm. In this farm, cows which are in peri-parturient period, are taken to a separate paddock for delivery. After birth, the colostrum is given to calves by a bottle, and the calves are transferred to single paddocks in which calves are fed until two months old. At the end of the two months, calves are placed in paddocks with a capacity of 10–15 animals.

Drinking water for all animals in the farm is provided from two different artesian water sources (Fig. 1). The artesian water source 1 coming to the dairy farm is close to the city center, and there is a wastewater treatment plant nearby. The artesian water source 2 is located on a hill away from the city center. The artesian water is coming to the farm with an individual pipeline from both sources and collected in a central tank and delivered to the calves and cattle without any filtration or disinfection. Two veterinarians work in the farm 24 hours a day, seven days a week. Veterinarians helped the project staff to collect feces from animals with diarrhea and from animal with previous history of diarrhea.

Samples

During the study, 47 stool samples were collected from calves (n: 36) and cows (n: 11). Of the 26 calves younger than two months, 12 had clinical diarrhea, and the remaining 14 had diarrhea previously. Antibiotic treatment was applied empirically to all of these calves. The remaining ten calves without diarrhea were older than two months (These samples were called D1-D47, Table 1).

Two samples with a volume of 8 liters were collected from two different artesian water sources (one of these examples is close to the city called SK1, and the other is called SK2, Table 2). Water sample with a volume of 50 ml was collected from each paddock with calves younger than two months, and 20 samples were pooled, and as a results six pool samples consisting of one-liter water were generated (These samples were called SK3-SK8, Table 2). Also, two-liter water sample pool was created from 6 different paddocks with calves older than 2 months (SK9, Table 2). In addition, one-liter of water sample was taken from the delivery paddocks (SK10, Table 2) (11).

The swab samples were obtained from various surfaces in the farm. Swabs were taken into 1 ml saline in 50 ml sterile tube using sterile cotton swabs. Swaps were collected from calf paddocks, waterer and mangers by making pools (each pool covers ten paddocks, these pooled samples were called S1-S18, Table 2). In addition, the swap samples from the inside (S19, Table 2) and outside (S20, Table 2) part of the calf bottle, inside (S21, Table 2) and outside (S22, Table 2) of milk heating tank, the inside (S23, Table 2) and outside (S24, Table 2) of cabinet containing colostrum, towels (S25, Table 2), the inside (S26, Table 2) and outside (S27, Table 2) of milk warmer in the deliver paddock, the inside (S28, Table 2) and outside (S29, Table 2) of medicine cabinet, the medicine cabinet door (S30, Table 2) and colostrum bottle (S31, Table 2) were collected to investigate *Cryptosporidium* spp. by PCR .

Microscopy

For microscopic examination, stool samples were diluted with saline and examined under direct light microscope using 40 × magnification (12). Then, smears of stool samples were stained by Kinyoun acid-fast dye. For this, firstly, tap water was added to the average of 5–10 g stool samples in 50 ml tubes until the upper level of the stool and incubated for 2 hours at room temperature. Then tap water was drained, and the stool was emulsified with a spatula by adding 10 ml sucrose solution (53 g sucrose, 100 ml water). The resulting mixture was filtered through two layers of gauze and centrifuged at 400 × g for 10 minutes (13). 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 with the Kinyoun acid-fast dye

method according to the manufacturer's protocol (RTA, Turkey). Briefly, slides were kept in the Kinyoun dye for 2–3 minutes and washed with tap water. Then, slides were kept in acid-alcohol for 5–10 seconds, rinsed in tap water, and kept in methylene blue for 20–30 seconds. Finally, the slides were washed with tap water air dried and examined under light microscope using 100 × magnification with immersion oil (14).

DNA extraction

DNA extraction from fecal samples was performed by stool DNA isolation kit (RTA Labs, Turkey) according to the manufacturer's protocol. During DNA isolation, 100 µg fecal sample was used, and DNA was eluted with 100 µl elution buffer. DNA isolation from water samples and environmental samples were performed as described with the QIAamp Mini kit (Qiagen, USA). Before DNA isolation, water samples were initially passed through 0.45 µm filters (Corning, USA). Later, the filters were removed, cut with a sterile scalpel and put into 50 ml sterile tube containing 500 µg zirconia beads, 125 µg glass beads, 20 µl proteinase K, 1000 µl saline and 1000 µl buffer AL (Qiagen, USA). Thereafter, the tubes were incubated for 4 hours in a shaker at 37 °C and 350 rpm. After incubation, the tubes were centrifuged at 3000 rpm for 10 minutes, and 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 at 37 °C and 350 rpm before DNA isolation. Later, 20 µl proteinase K and 1000 µl buffer AL were added and incubated for 10 min at 70 °C. After this incubation, routine DNA isolation method was applied (15).

For each sample, 2.5 µl of internal control provided by the manufacturer was added to lysis solution of the Isolation Kits.

Real-Time PCR

Cryptosporidium spp. oocyst wall protein (COWP) gene (GenBank accession no: AF248743.1) was investigated by Real-Time PCR using hydrolysis probes (16). During the Real-Time PCR, the primers targeting the 151 bp region of *Cryptosporidium* spp. COWP gene were 5'-CAAATTGATACCGTTTGTCTTCTG – 3' (25nt, COWP-P702 forward primer) and 5'-GGCATGTCGATTCTAATTCAGCT – 3' (23nt, COWP-P702 reverse primer) and the hydrolysis probe was 5'-TGCCATACATTGTTGTCCTGACAAATTGAAT – 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 template, 4 µl LightCycler Taqman 10XMaster mix with 5 mM MgCl₂, 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 and 10 seconds at 40 °C for cooling (16).

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'-ACCGCTTCTCAACAACCATCTTGTCCTC-3') and BCOWPR (5'-

CGCACCTGTTCCCACTCAATGTAAACCC-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 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, 2.5 µl template from the first reaction product was used like the first reaction. 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

PCR products of 553 bp fragments belonging to *Cryptosporidium* spp. positive samples were sequenced by ABI3730XL. Generated sequences were edited and aligned by MEGA 7.0 software to find the *RsaI* digestion regions that are used for identification of *Cryptosporidium* spp. (17, 18). Also, a BLAST analysis was performed to compare with reference *Cryptosporidium* samples in National Center for Biotechnology Information (NCBI).

Phylogenetic analysis

The phylogenetic analysis was performed by MEGA 7.0 software. The phylogenetic tree based on COWP sequences belonging to *Cryptosporidium* isolates was constructed by MEGA7.0 software according to the Neighbour Joining/Maximum Likelihood method using Kimura 2 Gamma distribution (K2 + G) model with 500 Bootstrap replications.

Statistical analysis

The data obtained during the study were processed with Microsoft Excel 2010 program, and statistical analysis was performed with 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.

Declarations

Ethics approval and consent to participate

All experiments were performed under the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms (Permit number: 2019-047). During the study, permission was obtained from the farm administrative manager for collecting stool samples from calves and cows.

Consent for publication

Not applicable.

Availability of data and materials

The datasets used and/or analysed during the current study are available from the corresponding author upon reasonable request.

Competing interests

The authors declare that they have no competing interests.

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Authors' contributions

MK, ADD drafted the manuscript and assisted with the conception and design of the study, data collection, and data analysis. MK, HC, MD, TK, SEA, AG assisted with study design and protocol development. MK, ADD, MD, YG, CÜ, HC assisted with data analysis. HC, MD, CÜ, YG, ADD assisted with interpretation of the study and assisted in writing and editing the manuscript. MK, MD, HC, ADD delineated the hypothesis, helped conceive and design the study, performed and oversaw the data analyses, and assisted in the writing of the manuscript. All authors read and approved the final manuscript for submission and publication.

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Figures

Figure 1

Phylogenetic analysis of the COWP sequences belonging

Figure 2

Microscopic examination of feces. (A): *Cryptosporidium* oocysts using Kinyoun acid fast staining (Black arrows). (B): *Giardia intestinalis* cysts by native-lugol method (Black arrow head)

Figure 3

Phylogenetic tree showing the association of *Cryptosporidium* isolates with *C. parvum* isolates.