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# Cryptosporidiosis in Cattle and Buffaloes in Turkey - Molecular Analysis and Public Health Significance

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## ABSTRACT

***Background*:** Calf diarrhea caused by infectious agents is one of the most significant and frequently encountered health problems in cattle breeding worldwide. Among these infectious agents, *Cryptosporidium* stands out as an important protozoan parasite, particularly affecting neonatal calves and young animals with immature or weakened immune systems. These early-life infections can lead to severe dehydration, weight loss, and even death if not properly managed. *Cryptosporidium* spp. is a zoonotic coccidian parasite known to play a substantial role in both human and animal health, especially in regions where hygiene and sanitation practices are inadequate. Its oocysts are highly resistant to environmental factors and common disinfectants, making control and prevention difficult. Importantly, due to its zoonotic nature, it poses a considerable threat to public health. This study was conducted to investigate the prevalence of cryptosporidiosis in buffaloes and cattle raised in the Van region using various diagnostic methods, and to evaluate its potential public health significance.
***Materials, Methods & Results*:** The fecal materials of the study were collected from 100 buffaloes and 200 cattle from different farms in Van province. The samples brought to the laboratory were stained with the Kinyoun Acid Fast method and DNA extraction was performed from each sample. Smear preparations were prepared from fresh fecal samples. The smear preparations were fixed in pure methanol for 1 min and allowed to dry, then stained in Kinyoun Carbol-Fuxin for 5 min, and then the slides were immersed in 50% ethyl alcohol, shaken, and immediately washed in tap water. The slides were placed in a decolorizing solution containing 1% sulfuric acid for 2 min and washed in tap water, then kept in a flask containing methylene blue for 1 min and washed again in tap water and left to dry. After drying, immersion oil was dripped and examined under a microscope at a magnification of 100x. DNA extraction was performed in all samples using a GeneMATRIX Stool DNA Purification Kit. Nested PCR analysis; Primers were used to amplify the SSU rRNA gene region of the obtained DNAs. In the PCR stage, 5'-TTCTAGAGCTAATA CATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3' primers were used to amplify the 1325 bp gene region. In the nested PCR stage, primers 5'- GGAAGGGTTG TATTTATT-TATTAGATAAAG-3' and 5'-AAGGAGTA AGGAACAACCTCCA-3' were used to amplify the 826-864 bp gene region DNA extraction. The PCR products obtained were stained with RedSafe™ Nucleic Acid Staining Solution and images were obtained on 1.5% agarose gel. Positive PCR products were subjected to bidirectional sequencing at a commercial company (BM Labosis, Ankara, Turkey). As a result of microscopic and PCR analyses, 5.50% and 9.50% positivity were detected in cattle, 3% and 6% positivity in buffaloes, respectively. The infection prevalence was highest in cattle and buffaloes in the 0-6 month age group, 17.31% and 15.38%, respectively. In addition, zoonotic *C. parvum* was isolated from cattle calves.
***Discussion*:** In conclusion buffaloes and cattle, with their large fecal volumes, can easily contaminate the environment and serve as significant reservoirs for this pathogen, which is transmitted through water and food. Therefore, it is crucial to identify the *Cryptosporidium* species present in these animals. For this, epidemiological studies should be conducted and expanded with molecular methods.

**Keywords:** calf, *Cryptosporidium* spp., protozoan, sequence analysis, zoonotic coccidian, neonatal enteritis, Van region.

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## INTRODUCTION

*Cryptosporidium* spp. is a zoonotic coccidian parasite that plays a significant role in human and animal health management, particularly in regions with poor hygiene conditions [7,9]. *Cryptosporidium* spp. causes cryptosporidiosis, mainly in newborns and immunocompromised people, spreading via infected feces or contaminated water and food [6,39].

Cryptosporidiosis considered endemic in cattle worldwide and globally recognized as one of the most important causes of neonatal enteritis in calves [10]. Four *Cryptosporidium* species - *C. parvum, C. bovis*, *C. ryanae*, and *C. Andersoni* - commonly infect cattle. Among them, only *C. parvum* causes disease in newborn calves, while in older animals (> 6 weeks), it leads to asymptomatic oocyst shedding [9,10]. *C. parvum's* resistant oocysts and zoonotic nature make it a major concern. Many human outbreaks are linked to calves, showing they are key reservoirs [10,14,17].

Buffaloes (*Bubalus bubalis*) have long been used for meat, milk, and labor. In Turkey, Anatolian buffaloes originate from the Mediterranean subgroup of river buffalo [4,28,44]. Few studies exist on *Cryptosporidium* in buffaloes. Species reported include *C. parvum*, *C. ryanae*, *C. bovis, C. suis*, *C. scrofarum*, and *C. ubiquitum*. *Cryptosporidium parvum* dominates in young buffaloes, as in calves [5,8]

Stool exams are cheap but less sensitive for *Cryptosporidium* diagnosis; PCR and sequencing provide more accurate epidemiology and genotyping [39,40,42].

This study aimed to determine the prevalence of cryptosporidiosis in buffalo and cattle in Van using various methods and to assess its public health significance by identifying species through sequence analysis.

## MATERIALS AND METHODS

### *Study area and sample collection*

In this study, fecal samples were collected from 200 cattle and 100 buffaloes from different farms in Van province. The collected samples were placed in individual sample containers, and age and species information were recorded. The samples brought to the laboratory were stained with the Kinyoun Acid Fast method and DNA extraction was performed from each sample. The extracted DNA samples were then stored at -20°C.

### *Microscopic examination*

Smear preparations were prepared from fresh fecal samples. The smear preparations were fixed in pure methanol for 1 min and allowed to dry, then stained in Kinyoun Carbol-Fuxin for 5 min, and then the slides were immersed in 50% ethyl alcohol, shaken, and immediately washed in tap water. The slides were placed in a decolorizing solution containing 1% sulfuric acid for 2 min and washed in tap water, then kept in a flask containing methylene blue for 1 min and washed again in tap water and left to dry. After drying, immersion oil was dripped and examined under a microscope at a magnification of 100x.

### *DNA extraction*

DNA extraction was performed in all samples using a GeneMATRIX Stool DNA Purification Kit[1] according to the manufacturer's protocol. The DNAs obtained were stored at -20°C until the next steps. Molecular analyses were performed at Van Yüzüncü Yıl University, Faculty of Veterinary Medicine, Genetic Laboratory.

### *Nested PCR analysis*

Primers described by Xiao *et al*. [49] were used to amplify the SSU rRNA gene region of the obtained DNAs. In the PCR stage, 5'-TTCTAGAGCTAATA CATGCG-3' and 5'-CCCATTTCCTTCGAAA-CAGGA-3' primers were used to amplify the 1325 bp gene region. In the nested PCR stage, primers 5'-GGAAGGGTTG TATTTATTTATTAGATAAAG-3' and 5'-AAGGAGTA AGGAACAACCTCCA-3' were used to amplify the 826-864 bp gene region DNA extraction. The PCR products obtained were stained with RedSafe™ Nucleic Acid Staining Solution[2] and images were obtained on 1.5% agarose gel. Positive PCR products were subjected to bidirectional sequencing.

### *Sequence analysis and phylogeny*

A commercial firm[3] performed bidirectional sequence analyses of positive PCR samples. The duplex DNA sequences of *Cryptosporidium* spp. specific PCR products performed with DNA samples isolated from bovine feces and buffalo feces were aligned by the Bioedit program and the ends were trimmed [22]. Each aligned sequence was analyzed with NCBI BLAST to determine how closely it was related to which Cryptosporidium species [2]. In addition, all sequences obtained and selected sequences from the NCBI genbank database including AB089285, AF093492, AF093493, AF093498, AF093502, AF112574, AF112576, AF115378, AF159113, AF513227, AH006572, EU162751, EU410344, EU553556, GQ337962, HM116388, HM243548, JF710244,

JF710261, JN846705, KM199749, KP730318, Phylogenetic analysis was performed with the dataset created using the accession coded 18s rRNA gene sequences of KU679364, KU892566, KX345063, AF093495, EU553550, MH028031, MK982466, MT835148, ON986852, KF854255 (Outgroup, *Sarcocystis neurona*) and KT184354 (Outgroup, *Eimeria tenella*). For this purpose, the dataset was aligned in the Bioedit program and fit tests were performed on 286 different variation models using the Maximum likelihood statistical method with the ModelFinder program integrated into the IQTREE web server [27,46]. To reveal the evolutionary history, the best-fitting model according to the Bayesian Information Criterion (BIC) was automatically selected by the IQTREE web server and the phylogenetic tree was constructed according to this model. The FreeRate model was used to model evolutionary rate differences in regions of variation [50]. The phylogenetic tree was validated using the Hasegawa approximate likelihood ratio test (SH-aLRT) with 1000 replications and Ultrafast bootstrap (UFBoot) [20,35]. Branch lengths were generated based on the number of changes in each region

*Statistical analysis*

The data obtained in the study were analyzed using the SPSS V16.0[4] program. The relationship between grouped variables was calculated using chi-square test. The difference was considered statistically significant when $P < 0.05$.

## RESULTS

As a result of microscopic and PCR analyses, 5.50% and 9.50% positivity were detected in cattle, 3% and 6% positivity in buffaloes, respectively. When the age groups were analyzed, a statistically significant difference was detected between the 0-6 months age group and the age group older than 18 months according to PCR results in buffaloes and both native and PCR results in cattle ($P < 0.05$) [Table 1]. According to both microscopic and PCR results in both cattle and buffaloes, the highest positivity was detected in the 0-6 months age group, while the lowest positivity was detected in the group older than 18 months (Table 1). It was determined that the prevalence in aged 0-6 months was 17.31% although *C. parvum* was dominant in this age group (Tables 1 & 2). 10% and 8% positivity were found in 7-12 and 13-18 months of age, respectively. The prevalence in >18 cattle was found to be quite low (2.08%) [Table 1]. It was determined that the prevalence in aged 0-6 months was 15.38% and also 3.85% and 4.17% positivity was found in 7-12 and 13-18 months of age, respectively in buffalo. No positivity was detected in buffaloes older than 18 months (Table 1).

DNA samples obtained from buffalo feces were sent for sequencing and only 1 sample could be sequenced. When the DNA sequences of the SSU rRNA gene obtained in the study were compared with the database in the NCBI Basic Local Alignment Search Tool, it was observed that sample 1 overlapped (99.87%) with *C. ryanae* samples (Table 2). As with the results obtained from the NCBI Genbank database, the sample was found to be related to *C. ryanae*, as can be seen in the phylogenetic tree constructed using the appropriate model (Figure 1).

After sequence analysis of PCR-positive cattle feces, only 8 samples could be read correctly. When the DNA sequences of the SSU rRNA gene obtained in the study as a result of sequence analysis were compared with the database in the NCBI Basic Local Alignment Search Tool, it was determined that samples 4, 5, 8, 9 overlapped 100% with *C. bovis*, sample 2 overlapped 100% with *C. ryanae*, samples 6 and 7 overlapped 100% with *C. parvum* samples. In addition, sample 3 was 99.65% overlapping with *C. parvum* samples (Table 2). According to the Bayesian Information Criterion, the General Time Reversible model (GTR+F+I+G4) was found to be the most appropriate model for the dataset and this model was used to construct the phylogenetic tree. As can be seen in the phylogenetic tree constructed using the appropriate model, as in the results obtained from the NCBI Genbank database, samples 4, 5, 8, and 9 are related to *C. bovis*, sample 2 to *C. ryanae*, samples 3, 6 and 7 to *C. parvum* (Figure 1).

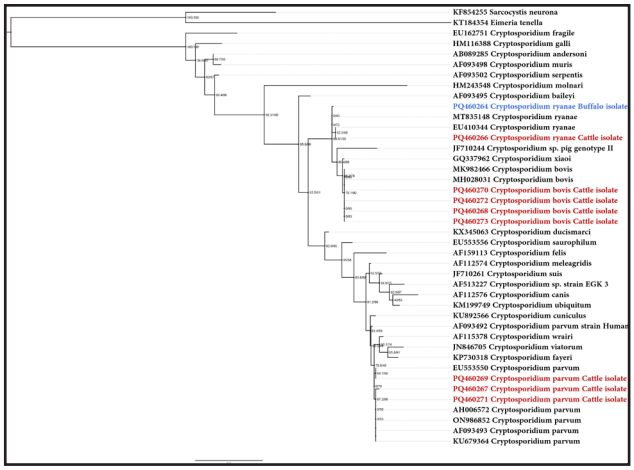


**Figure 1.** Phylogenetic analysis results.

**Table 1.** Microscopic and Nested PCR analysis results in cattle and buffaloes of *Cryptosporidium* spp. and positivity rates according in age groups.

| Parameters | (n) | Native Positive | | *P* | PCR Positive | | *P* |
|---|---|---|---|---|---|---|---|
| | | (n) | (%) | | (n) | (%) | |
| Cattle | 200 | 11 | 5.50 | 0.33 | 19 | 9.50 | 0.30 |
| Buffalo | 100 | 3 | 3.00 | | 6 | 6.00 | |
| Cattle Age (month) | | | | | | | |
| 0-6 | 52 | 7 | 13.46$^{ab}$ | | 9 | 17.31$^{ab}$ | |
| 7-12 | 50 | 3 | 6.00$^{a}$ | | 5 | 10.00$^{a}$ | |
| 13-18 | 50 | 1 | 2.00$^{a}$ | | 4 | 8.00$^{a}$ | |
| >18 | 48 | 0 | 0.00$^{ac}$ | | 1 | 2.08$^{ac}$ | |
| Buffalo Age (month) | | | | | | | |
| 0-6 | 26 | 2 | 7.69$^{a}$ | | 4 | 15.38$^{ab}$ | |
| 7-12 | 26 | 1 | 3.85$^{a}$ | | 1 | 3.85$^{a}$ | |
| 13-18 | 24 | 0 | 0.00$^{a}$ | | 1 | 4.17$^{a}$ | |
| >18 | 24 | 0 | 0.00$^{a}$ | | 0 | 0.00$^{ac}$ | |
| Overall | 300 | 14 | 4.67 | | 25 | 8.33 | |

Different letters (a,b,c) indicate significant differences between groups ($P < 0.05$). a,b: No difference between groups with same letter.

**Table 2.** Comparison results of the study samples generated using the NCBI Basic Local Alignment Search Tool.

| Sample no/Genbank Accession no | Access codes for the most similar example | Species | Similarity ratio % | Age range determined in this study (month) |
|---|---|---|---|---|
| 1/PQ460264 | MT835148 | *C. ryanae* | 99,87 | 7-12 |
| 2/PQ460266 | MT374189 | *C. ryanae* | 100 | 13-18 |
| 3/PQ460267 | ON986852, ON515730 | *C. parvum* | 99,65 | 0-6 |
| 4/PQ460270 | MH028031, MK982466, MK880572 | *C. bovis* | 100 | 0-6 |
| 5/PQ460268 | MH028031, MK982466, MK880572 | *C. bovis* | 100 | 7-12 |
| 6/PQ460269 | OL689400, EU553550 | *C. parvum* | 100 | 0-6 |
| 7/PQ460271 | MT374186, MT043934 | *C. parvum* | 100 | 0-6 |
| 8/PQ460272 | MH028031, MK982466 | *C. bovis* | 100 | >18 |
| 9/PQ460273 | MH028031, MT043859 | *C. bovis* | 100 | 13-18 |

## DISCUSSION

Calf diarrhea caused by infectious agents is one of the biggest problems encountered in cattle breeding. Fifty percent of calf diarrhea occurs in calves up to 1 month of age and has a high mortality rate [29]. *C. parvum* is one of the causative agents especially affecting newborn calves and calves with low immunity. It is also important because it is zoonotic and threatens public health [24,39]. So far, *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* have been reported in cattle [15,30]. Similarly, *C. parvum*, *C. bovis*, and *C. ryanae* were detected in cattle in this study. There are previous studies investigating *Cryptosporidium* agents in cattle in Turkey.

Yildirim *et al*. [51]. examined 550 cattle feces collected from Central Anatolia and the Mediterranean region for *Cryptosporidium* by microscopic and PCR methods and found 134 (24.4%) positivity by microscopic method and 195 (35.5%) positivity using PCR. In a study conducted in the Erzurum region,

*Cryptosporidium* spp. was detected using the PCR method in 3.9% of 307 calf samples [21]. Similarly, in another research Ertaş and Ayan [13], in their study on a total of 100 calf fecal samples, reported detecting *Cryptosporidium* spp. in 34 samples (34%) using the Kinyoun acid-fast staining method in microscopic examination, while they detected *Cryptosporidium* spp. in 38 samples (38%) using the Nested PCR method.

There are also important studies on this subject from around the world. Nasir *et al*. [37]. found PCR positivity in 25.6% of 500 stool samples, and Santin et al. [41]. in 19.2% of 190 samples. In our study, 12 (5.5%) by microscopic and 19 (9.50%) by PCR method were positive and the PCR method was found to be more sensitive in the diagnosis of cryptosporidiosis as stated in previous studies.

There are many studies investigating the age distribution of *Cryptosporidium* prevalence in cattle. In some of these studies [14,31,52], it was determined that the rate was higher in weaned and adult cattle, while in some studies [18,32,48], it was determined that the prevalence was higher in calves in the unweaned period. In addition, many studies have reported that *C. parvum* is the predominant *Cryptosporidium* species in dairy calves. Mammeri *et al*. [34] detected the presence of zoonotic *C. parvum* subtype families (IIa, IId) in fecal samples from 35 naturally infected calves with diarrhea (≤45 days old) in France and stated that calves are likely to be an important reservoir for *C. parvum*. In Scotland that *C. parvum* was the predominant *Cryptosporidium* species in calves aged 6 weeks to 6 months, but *C. bovis* and *C. ryane* species were also detected in calves aged 4 weeks to 6 months. These researchers also found that the prevalence of *Cryptosporidium* was higher in calves than in adults. Doungmala *et al*. [11] collected a total of 200 fecal samples from newborn dairy calves between 1 and 28 days of age in Muang, Khon Kaen region of Thailand and microscopically detected Cryptosporidium oocysts in 102 (51%) of 200 samples using modified Kinyoun's acid-fast staining technique, and also identified *C. bovis* (n = 11) and *C. ryanae* (n = 11) and unidentified strains by PCR and sequence analysis of positive samples. These researchers reported that the incidence of *Cryptosporidium* was higher in calves aged 4 weeks. In our study, it was determined that the prevalence was higher in cattle calves aged 0-6 months (17.31%) and although *C. parvum* was dominant in this age group, *C. bovis* species were also seen. In addition, 10% and 8% positivity were found in 7-12 and 13-18 months of age, respectively. Again, in this study, the prevalence in >18 cattle was found to be quite low (2.08%). Similar to our results, Amer *et al*. [3] did not detect *Cryptosporidium* in adults in their study. It has been reported that different results may be due to age distribution, presence of diarrhea, breast milk intake rates, defense mechanisms, feeding patterns, shelter conditions and oocyst release [53]. *Cryptosporidium* is more prevalent in young animals and that the decrease in occurrence with age can be attributed to the development of acquired immunity due to repeated exposure and also to the development of sufficient intestinal flora that provides local immunity [23]. In cattle, *C. ryanei* has been reported to occur mostly in young animals, although it has been detected in calves [16]. Fayer *et al*. [16] experimentally infected calves with *C. bovis* and argued that this species can also be seen in calves, but some studies argue the opposite and report that *C. bovis* is more common in older animals [43]. In our study, *C. ryanei* was detected in 13-18 months old, and *C. bovis* was detected in both 0-6 months old and >18 months old (Table 2).

There are a few studies on *Cryptosporidium* species in buffaloes: 52% of 571 buffalo feces in Egypt, and 5.7% *Cryptosporidium* in India [33,36]. In this study, 3% positivity was determined by microscopic and 6% positivity by Nested PCR method in buffaloes. Studies investigating the distribution of *Cryptosporidium* in buffaloes according to age are quite insufficient and species distributions are not sufficiently known. In the existing studies, there are different results in buffaloes as well as in cattle. The prevalence of *Cryptosporidium* was higher in adults [26,42], while othres authors [38] argued that the prevalence was higher in 0-6 months of age. El-Khodery and Osman [12] collected 458 fecal samples from buffalo calves up to 3 months of age in Egypt and found 14.19% *Cryptosporidium* spp. positivity. These researchers stated that the most risky group in terms of *Cryptosporidium* was 0-15 days old buffalo calf. A high prevalence of 52% was detected in mallards, and the prevalence was very high in buffalo calves younger than 1 month of age [36]. The prevalence of *Cryptosporidium* of 34.48% in cattle and 36.06% in buffaloes was reported in the Mumbai region of India and these researchers explained that the risk was higher in 0-1-month buffalo calves and calves [25].

In our study, *C. ryanae* was detected in buffaloes and also the prevalence of 15.38% was determined in 0-6 months buffalo calves, while no positivity was detected in those >18 months. Although cattle infected with *C. ryanae* have been detected by molecular methods in many countries [15]. Buffaloes infected with *C. ryanei* were 1st observed in India, [19,47], in Australia [1], in Egypt [3], and in Thailand [26]. This study confirms that *C. ryanei* infects ruminants other than cattle and is likely to infect buffaloes worldwide.

## CONCLUSIONS

*Cryptosporidium* species, which infect humans and many animal species and cause serious economic losses in livestock, were detected in cattle and buffaloes in Van region in Turkey. What makes this agent important is that *C. parvum* species under this genus is zoonotic and causes public health problems. Buffaloes and cattle easily contaminate the environment with their large fecal volumes and are great reservoirs for this agent, which is transmitted through water and food. Therefore, it is important to know the *Cryptosporidium* species in these animals. For this purpose, epidemiologic investigations should be conducted and disseminated by molecular methods. This study has contributed to the literature on buffaloes where there are insufficient studies in terms of *Cryptosporidium* and it has been determined that buffaloes pose a danger to the people of this region as well as cattle in terms of cryptosporidiosis. It is especially important to educate people engaged in animal husbandry in this direction and to make the necessary treatment of carrier animals.

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