Prevalence and molecular characterization of *Cryptosporidium* spp. in calves in the Siirt Province, Türkiye

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Abstract

Cryptosporidiosis, one of the main protozoan infections of the last century, is especially dangerous for calves and causes significant economic losses. This research was carried out to determine the prevalence of *Cryptosporidium* spp. by microscopic and molecular methods and to determine subtypes in 100 calves up to 6 months old and with diarrhoea in the Siirt Province, Türkiye. As a result of the microscopic examination (Kinyoun's acid-fast), *Cryptosporidium* spp. oocysts were found in 8 (8%) of 100 samples. As a result of nested PCR, 826-864 bp specific bands for *Cryptosporidium* spp. were obtained in 13 (13%) of 100 samples. When the DNA sequences of the SSU rRNA gene were compared with the NCBI Basic Local Alignment Search Tool database, it was determined that eight samples sequence analyses showed 100% similarity with the *C. parvum, C. ryanae*, and *C. bovis* samples. The detection of *C. parvum*, which has zoonotic importance in this study, suggests that calves with diarrhoea may be a source of contamination for other animals and humans. Therefore, animal owners and people in close contact with animals should be informed about the public health of cryptosporidiosis.

Cattle, C. parvum, *C. ryanae, C. bovis, nested PCR*

Cryptosporidiosis progresses massively in calves and causes significant economic losses (Sarı et al. 2008). Calf diarrhoea constitutes a significant portion of all calf losses. *Cryptosporidium* factors are in the first place in the aetiology of calf diarrhoea (Sarı and Arslan 2012). *Cryptosporidium* spp., one of the most crucial protozoan infectious agents of the last century, are apicomplexan protozoans that are ubiquitous and cause cryptosporidiosis, capable of infecting various animal species and humans (Lefay et al. 2000; Del Coco et al. 2008; Sarı et al. 2008; Prakash et al. 2009; Zhang et al. 2013; Lombardelli et al. 2019; Santoro et al. 2019).

Cryptosporidiosis is a disease of public health importance that causes gastrointestinal infections in a wide variety of mammals, including humans, cattle, sheep, goats, pigs, and horses worldwide (Santın et al. 2004; Değerli et al. 2005; Nasir et al. 2009;

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Maurya et al. 2013; Regassa et al. 2013). Among susceptible hosts, cattle are considered one of the main reservoir hosts (Zhang et al. 2013) and contribute to zoonotic infection (Lombardelli et al. 2019). More than 16 species and genotypes have been reported in studies performed on cattle (Maurya et al. 2013; Zhang et al. 2013; İpek 2022). Cattle are commonly infected by four *Cryptosporidium* spp., particularly *C. parvum*, *C. bovis*, *C. andersoni,* and *C. ryanae* (Xiao et al. 2004; Liu et al. 2009; Safavi et al. 2011; Venu et al. 2012; Regassa et al. 2013; Lombardelli et al. 2019).

Among these, the zoonotic species *C. parvum* has been reported to cause disease predominantly in pre-weaned calves, *C. bovis* and *C. ryanae* in post-weaned calves and young calves, and *C. andersoni* mostly in young adults (Safavi et al. 2011; Regassa et al. 2013; İpek 2022). *Cryptosporidium* is one of the most common enteropathogens found in calves during the first fortnight (Thompson et al. 2007; Delafosse et al. 2015). The faecal-oral route primarily infects calves, and fewer than 50 oocysts can infect a healthy calf (Fayer et al. 2000). Infections can spread more rapidly when animals are housed in bulk or are overcrowded (Regassa et al. 2013). The infection course may be asymptomatic or display fever, anorexia, diarrhoea, dehydration, lethargy, depression, abdominal pain, and growth retardation.

Many factors affect the severity of the disease, such as the number of oocysts, age, the immune and nutritional status of animals, and the presence of other infections (Xiao et al. 2004; Del Coco et al. 2008; Safavi et al. 2011; Sarı and Arslan 2012; Regassa et al. 2013; Delafosse et al. 2015; Lombardelli et al. 2019; İpek 2022). Infection in cattle is mainly dependent on age. Young calves show the highest prevalence of infection and spread in the organism at the highest intensity (Safavi et al. 2011). It has been reported that pre-weaned calves are predominantly infected with the zoonotic species *C. parvum* (Safavi et al. 2011; Regassa et al. 2013). These calves are an essential source of human cryptosporidial infections (Safavi et al. 2011; Lombardelli et al. 2019; Santoro et al. 2019). In addition, water and foodborne human cryptosporidiosis outbreaks may occur due to manure contamination of infected animals (Del Coco et al. 2008; Sarı et al. 2008; Zhang et al. 2013).

The disease can cause major health problems in children, people with weakened immune systems, people who deal daily with livestock, veterinarians, and those in close contact with infected people (Sevinç 2004; Safavi et al. 2011; İpek 2022).

This study was carried out to determine the prevalence of *Cryptosporidium* spp. in calves in the Siirt Province by microscopic and molecular methods and to determine the subtypes.

Materials and Methods

The study area

This study was conducted between April and May 2021 in the Siirt Province, located in the Southeastern Anatolia region of Türkiye (37°55'N, 41°57'E).

Animal material and sample collection

Large animal breeding in the Siirt Province is carried out using traditional methods, following a family-run business model, and the number of animals in these facilities is quite low. The animal material of the study consisted of 100 calves of different breeds and both sexes, up to 6 months old, with clinical diarrhoea in the Kurtalan district and centre. Samples were taken from the rectum using disposable gloves and placed in individual faeces containers. Afterward, the samples were brought to the laboratory and stored at 4 °C until they were analysed.

Microscopic examination

All samples brought to the laboratory were stained with Kinyoun's acid-fast method and examined under a microscope (Leica, Switzerland) at a × 100 magnification (Plate I, Fig. 1). For Scanning Electron Microscope images, samples were first dropped on glass slides and allowed to dry at room conditions. Then, the dried samples were coated with an Au sputter coater device for 60 s to form a conductive layer on the surface. The coated sample was then placed on the sample holder and taken into the device for images to be taken under a scanning electron microscope (Sigma 300 Zeiss, Germany). The images were detected by the scattered electron detector (Plate II, Fig. 2).

DNA extraction

DNA extraction was performed according to the manufacturer's protocol using a commercial kit (GeneMATRIX Stool DNA Purification Kit, EURx, Gdańsk, Poland) in the samples that were positive in microscopic examination. The obtained DNAs were stored at −20 °C for use in the following steps.

PCR amplification

To amplify the SSU rRNA gene region, nested PCR was performed according to the (Xiao et al. 2001) primers described. Primers 5'-TTCTAGAGCTAATACATGCG-3' and 5'-CCCATTTCCTTCGAAACAGGA-3' were used to amplify the 1325 bp gene region in the PCR step. Primers 5'-GGAAGGGTTGTATTTATTAGATAAAG-3' and 5'-AAGGAGTAAGGAACAACCTCCA-3' were used to amplify the 826-864 bp gene region in the nested PCR step. In both reactions; 5 pmol forward and reverse primer, 200 µM dNTPs, 3 mM $MgCl_2$, 1U Taq polymerase, and 10 × PCR buffer (0.8 M Tris-HCl, pH 8.8, 0.2 M $(NH_4)_2SO_4$ and 0.1% Tween®20) in 25 µl of master mix, nuclease free water and 2 µl of DNA were used. Following a 15-min pre-denaturation at 95 °C in both reactions, each cycle consisted of denaturation (1 min at 95 °C), coupling (1 min at 60 °C), and elongation (1 min at 72 °C): thirty-five cycles and a final elongation of 7 min at 72 °C. Then, 1.5% agarose gel was prepared and stained with RedSafe Nucleic Acid Staining Solution. Next, PCR products were run on agarose gel, and images were obtained on the gel imaging device (Syngene bioimaging system).

Sequence analysis and phylogeny

A commercial firm (BM Labosis, Ankara, Turkey) performed bidirectional sequence analyses of positive PCR samples. The obtained DNA sequences were aligned using a BioEdit program and were ready to be analysed. NCBI Basic Local Alignment Search Tool was used to compare edited DNA sequences and dataset formats to identify assemblages.

Ethical approval

The Local Ethics Committee for Animal Experiments of the Siirt University provided the ethical clearance for the study with the number 2020/04-06.

Results

Cryptosporidium spp. was detected in 8 (8%) of 100 samples with the microscopic examination. Correspondingly, in nested PCR, specific bands of 826-864 bp were detected for *Cryptosporidium* spp. in 13 of the 100 samples (Plate II, Fig. 3). According to the microscopic examination, the distribution for sexes is 9.62% in females and 6.25% in males. As reported by the nested PCR method, the distribution was 15.38% in females and 10.42% in males ($P > 0.05$). According to age groups, the highest prevalence was found in the 8–15 days old group ($P > 0.05$) (Table 1).

Table 1. Distribution of positive samples by sex, age, and analysis methods.

Factor	Examined (n)	Microscopy Positive (n)	Microscopy (%)	*P*	Nested-PCR Positive (n)	Nested-PCR (%)	*P*
Sex							
Male	48	3	6.25	0.535	5	10.42	0.461
Female	52	5	9.62		8	15.38	
Age (day)							
≤ 7	12	1	8.33	0.216	2	16.67	0.128
8–15	36	5	13.89		8	22.22	
16–30	21	2	9.52		2	9.52	
> 30	31	0	0.00		1	3.23	
Total	100	8	8.00		13	13.00	

When the DNA sequences of the SSU rRNA gene obtained in the study were compared with the database in NCBI Basic Local Alignment Search Tool, it was determined that both forward and reverse sequencing products of samples A, B, and D were 100% overlapped with *C. ryanae* and *C. bovis*; both forward and reverse sequencing products of samples C, E, and G were 100% overlapped with *C. parvum* samples. Sample F was analysed only on the forward sequence and overlapped 100% with *C. parvum*; sample H was analysed only on the reverse sequence and overlapped 100% with *C. bovis*, and *C. ryanae* (Table 2).

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

Sample	Access codes of the most similar samples	Species	Similarity (%)
A	MW043439, HQ179573	*C. ryanae*	100
		C. bovis	100
B	MT374189, KU168249	*C. ryanae*	100
		C. bovis	100
C	MT648442, MT649862	*C. parvum*	100
D	MH028032, KU168249	*C. ryanae*	100
		C. bovis	100
E	MT648442, MT043922	*C. parvum*	100
F	MT043922	*C. parvum*	100
G	MT476898, MT043922	*C. parvum*	100
H	MT374188, MT002726	*C. bovis*	100
		C. ryanae	100

Discussion

Different results have been obtained in studies conducted to determine the prevalence of bovine cryptosporidiosis in different countries of the world. It has been reported to be at 25% in Mexico (Maldonado-Camargo et al. 1998), 17.9% in France (Lefay et al. 2000), 47.86% in Spain (Castro-Hermida et al. 2002), USA 35.5% (Santın et al. 2004), 27.3–40.6% in Canada (Trotz-Williams et al. 2005; Coklin et al. 2007), 17.0–25.4% in Argentina (Del Coco et al. 2008; Lombardelli et al. 2019), 5.30–47.68% in China (Liu et al. 2009; Zhang et al. 2013), 12% in Norway (Hamnes et al. 2006), 74.8% in Portugal (Martins et al. 2007), 37% in Belgium (Geurden et al. 2007), 37.4% in Ireland (Thompson et al. 2007), 28% in the UK (Brook et al. 2008), 9.05% in India (Prakash et al. 2009), 27.2% in Pakistan (Nasir et al. 2009), 30.2% in Egypt (Amer et al. 2010), 34.83% in Iraq (Khalil 2011), 36.6% in Iran (Safavi et al. 2011), 27.1% in Malaysia (Muhid et al. 2011), 16.3–39.65% in India (Venu et al. 2012; Maurya et al. 2013), 13.8–15.8% in Ethiopia (Regassa et al. 2013; Ebiyo and Haile 2022), 41.5% in France (Delafosse et al. 2015), 22.63% in Estonia (Santoro et al. 2019), and 4.4% in Korea (Lee et al. 2019).

The first study on *Cryptosporidium* spp. in calves in Turkey was carried out by Burgu (1984).

Studies conducted in Turkey reported the following prevalences: 21.88–65.8% in Ankara (Emre et al. 1998; Sakarya et al. 2010), 5.1–32.9% in Kars (Arslan et al. 2001; Aydin et al. 2001; Çitil et al. 2004; Gündüz and Arslan 2017), 7.0–70.3% in Sivas (Değerli et al. 2005; Özçelik et al. 2012; Kuliğ and Coşkun 2019), 22.14% in Hakkari (Göz et al. 2007), 8.13–13.18% in Van (Çiçek et al. 2008; Gül et al. 2008), 3.9–22.8% in Erzurum (Sarı et al. 2008; Güven et al. 2013), 7.2% in Elazig (Özer et al. 1990), 20.7% in Nevşehir (Şimşek et al. 2012), 40% in Kütahya (Akalın 2018), 34% in Kayseri (Yildirim et al. 2020), 37.2% in Burdur (Yildirim et al. 2020), 27.33–39.4%

in Konya (Sevinç 2004; Ekici et al. 2011; Kabir et al. 2020), and 56.25% in Diyarbakır (İpek 2022).

The above mentioned studies indicate a widespread *Cryptosporidium* spp. infection both in Turkey and worldwide. The zoonotic feature of *Cryptosporidium* spp. increases the importance of this parasite in terms of public health.

There are different methods for the detection of *Cryptosporidium* spp. including microscopic, immunological, and molecular techniques (Regassa et al. 2013). The modified Ziehl-Neelsen or Kinyoun staining technique is the gold standard (Sarı and Arslan 2012). Molecular diagnostic methods, which are more specific and sensitive than classical methods, are widely used today for the specific diagnosis of cryptosporidiosis and the identification of species, subspecies, or strains (Şimşek et al. 2012). It is reported that PCR protocols can detect 1–50 oocysts (Sarı and Arslan 2012; Birdane 2017).

This study determined a prevalence of 8% and 13% using Kinyoun's acid-fast and nested PCR methods, respectively. These results found in our study are higher than the findings of some researchers (Güven et al. 2013; Lee et al. 2019), similar to some researchers (Özer et al. 1990; Hamnes et al. 2006; Çiçek et al. 2008; Gül et al. 2008; Liu et al. 2009; Prakash et al. 2009; Regassa et al. 2013; Gündüz and Arslan 2017; Ebiyo and Haile 2022), and lower than findings of some researchers (Arslan et al. 2001; Santın et al. 2004; Değerli et al. 2005; Coklin et al. 2007; Geurden et al. 2007; Martins et al. 2007; Thompson et al. 2007; Del Coco et al. 2008; Şimşek et al. 2012; Lombardelli et al. 2019; İpek 2022). The season, number of samples, number of animals with diarrhoea, age of calves, care and feeding conditions, and methods used can be cited among the potential reasons for the differences between studies.

As a result of the molecular characterization of *Cryptosporidium* spp. in the world, *C. parvum* (Coklin et al. 2007; Geurden et al. 2007; Thompson et al. 2007; Brook et al. 2008; Venu et al. 2012; Zhang et al. 2013; Lombardelli et al. 2019; Santoro et al. 2019), *C. ryanae* (Liu et al. 2009; Muhid et al. 2011; Zhang et al. 2013; Santoro et al. 2019), *C. bovis* (Coklin et al. 2007; Thompson et al. 2007; Brook et al. 2008; Muhid et al. 2011; Venu et al. 2012; Santoro et al. 2019), *C. andersoni* (Santin et al. 2004; Liu et al. 2009; Amer et al. 2010; Muhid et al. 2011), and *C. meleagridis* (Zhang et al. 2013) have been reported. In Turkey, three species have been reported which are *C. parvum* (Şimşek et al. 2012; Güven et al. 2013; Gündüz and Arslan 2017; Akalın 2018; Kabir et al. 2020; Yildirim et al. 2020; İpek 2022), *C. ryanae* (Yildirim et al. 2020; İpek 2022), and *C. bovis* (Kabir et al. 2020; Yildirim et al. 2020).

When the DNA sequences of the SSU rRNA gene obtained in this study were compared with the NCBI Basic Local Alignment Search Tool database, *C. parvum*, *C. ryanae*, and *C. bovis* species were detected, similar to the previous studies carried out in Turkey. In some samples, the sequences showed 100% similarity with both *C. ryanae* and *C. bovis* sequences, which may be due to the recent separation of *C. ryanae* from the deer-like genotype which is considered a subspecies of *B. bovis*.

While some researchers (Bhat et al. 2012; Güven et al. 2013; Maurya et al. 2013) reported a higher prevalence in females in line with the findings of this study, others (Khalil 2011; Şimşek et al. 2012) report a higher prevalence in males. Bejan et al. (2009) revealed no effect of sex in their determination of *Cryptosporidium* oocysts in goats.

According to age groups, the highest prevalence was found in the 8–15 days old group, similar to previous studies (Arslan et al. 2001; Sevinç et al. 2003; Güven et al. 2013)

In conclusion, this study revealed the existence of *Cryptosporidium* spp. in the region and determined its subspecies. The detection of *C. parvum*, which is of zoonotic importance, suggests that calves with diarrhoea may be a source of contamination for other animals and humans. Implementing effective control strategies such as proper farm hygiene is extremely important, as hygienic conditions, feed, and water resources are potential

risk factors for the emergence of the pathogen. For this reason, animal owners and people in close contact with animals should be informed about the public health and economic importance of cryptosporidiosis. In addition, it was concluded that more comprehensive studies are needed that will include water resources in the region as an object of study.

Conflict of interest

The authors state no conflict of interest.

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Fig. 1. *Cryptosporidium* spp. oocysts (black arrow) stained with Kinyoun's acid-fast method (× 100 magnification)

Plate II

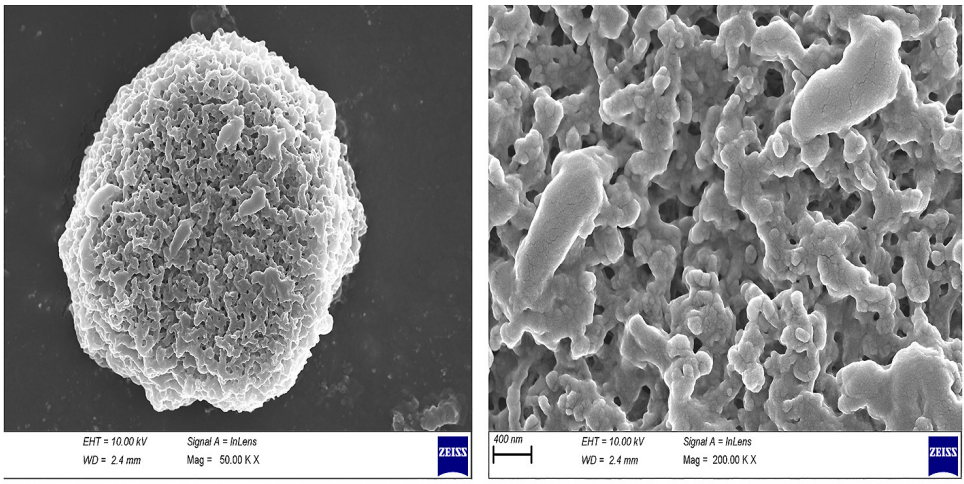

Fig. 2. Scanning electron micrograph of *Cryptosporidium* spp. oocyst identified from faecal samples of *Cryptosporidium* infected calves.

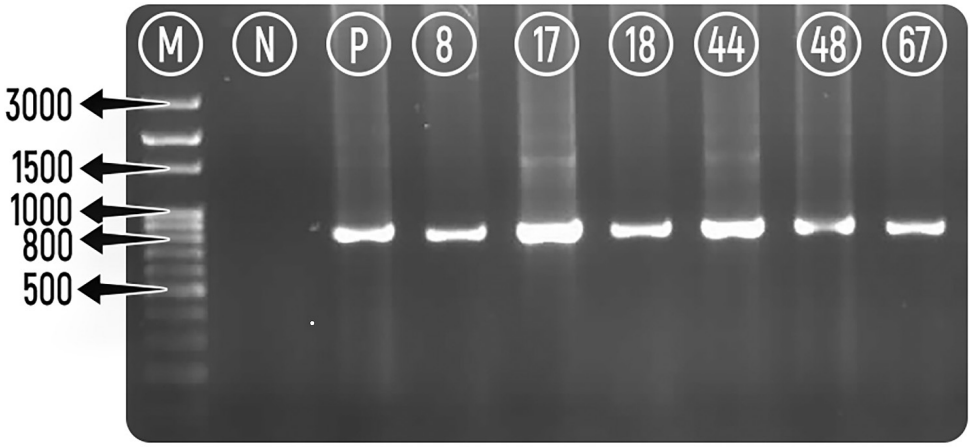

Fig. 3. Nested PCR agarose gel image.
M - marker; N - negative control; P - positive control; 8, 17, 18, 44, 48, 67 positive samples