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Original article

Occurence and genotype distribution of *Cryptosporidium* spp., and *Giardia duodenalis* in sheep in Siirt, Turkey

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Abstract

Cryptosporidium spp., and *Giardia duodenalis* are intestinal protozoan parasites known to infect humans and various animals and cause diarrhea. This study aimed at determining the prevalence and genotype of *Cryptosporidium* spp. and *Giardia duodenalis* in sheep in different locations of Siirt province. The fecal material for this study was collected from 500 sheep in different locations of Siirt province, Turkey. Fecal samples obtained from sheep were examined for *Cryptosporidium* spp. by Kinyoun Acid Fast staining and the Nested PCR method. Microscopic and Nested PCR methods revealed a prevalence of 2.4% (12/500) and 3.6% (18/500), respectively. Sequence analysis revealed the presence of *C. ryanae, C. andersoni,* and *zoonotic C. parvum*. In terms of *Giardia duodenalis*, 8.4% (42/500) and 10.2% (51/500) prevalence was determined using Nativ-Lugol and Nested PCR methods, respectively. Using sequence analysis, zoonotic assemblages A and B as well as assemblages E and D were detected. As a result of this study, both the prevalence of *Cryptosporidium* spp. and *Giardia duodenalis* and the presence of species that appear to be host-specific, as well as those known to be zoonotic, were revealed. A large-scale study is needed to understand the impact of these agents on sheep farming and their consequences on human health.

Keywords: *Cryptosporidium* spp., *Giardia duodenalis*, molecular analysis, phylogeny, sheep, Turkey

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Introduction

Cryptosporidium spp., is an important zoonotic parasite that occurs in many geographical regions of the world, usually during the warm and rainy seasons (Soltane et al. 2007, Xiao 2010, Gharekhani et al. 2014, Yang, R. et al. 2014, Dessì et al. 2020). This parasite was first described in sheep in Australia in 1974 in lambs with diarrhea (Barker and Carbonell 1974). Sheep can potentially increase the public health risk of *Cryptosporidium* infections as they can cause contamination of water sources (Yang, R. et al. 2014). Zoonotic transmission can occur by direct contact or a fecal-oral route by contamination of drinking water (Goma et al. 2007, Romero-Salas et al. 2016). Cryptosporidial infections can cause significant economic losses due to high morbidity and sometimes high mortality among livestock (Majewska et al. 2000, Soltane et al. 2007). In addition to diarrhea in humans, it causes chronic and fatal diseases in immunocompromised individuals (Romero-Salas et al. 2016). Among farm animals, ruminants are considered to be important reservoirs of both host-specific and zoonotic *Cryptosporidium* species as they shed a large number of oocysts that cause environmental contamination (Xiao 2010, Dessì et al. 2020).

Giardia duodenalis (syn: *G. intestinalis* and *G. lamblia*) is a gastrointestinal protozoan parasite found worldwide, infecting humans and a wide range of animals (Giangaspero et al. 2005, Santín et al. 2007, Wilson and Hankenson 2010, Jafari et al. 2014, Wang et al. 2016, Jian et al. 2018). Giardia has two morphological forms, trophozoite and cyst. The cyst form is resistant to environmental conditions and is responsible for transmission (Wilson and Hankenson 2010, Jafari et al. 2014). Infected individuals shed up to 10 million cysts per gram of feces and infection occurs after ingestion of up to 10 cysts (Wilson and Hankenson 2010). *Giardia duodenalis* genetically has 8 different (A-H) assemblages. Assemblages A and B are seen in humans and other mammals, while the other assemblages (C-H) are host-specific (Santín et al. 2007, Wilson and Hankenson 2010, Wang et al. 2016, Kiani-Salmi et al. 2019). There is increasing evidence that Giardia is zoonotic and can be transmitted from animals (Wilson and Hankenson 2010).

In this study, the aim was to determine the prevalence and genotypes of *Cryptosporidium* spp. and *Giardia duodenalis* in sheep in different locations of Siirt province.

Materials and Methods

Study Area and Sample Collection

The fecal material for this study was collected from 500 sheep in different locations of Siirt province located in the Southeastern Anatolia Region of Turkey. Fecal samples were collected from the rectum of the sheep with disposable latex gloves and placed in individual sample containers. The samples were then brought to the laboratory for analysis.

Microscopic Examination

All samples were examined for *Cryptosporidium* spp. using the Kinyoun Acid Fast staining method (Rekha et al. 2016), and *Giardia duodenalis* using the Nativ-Lugol method (Aslan Celik et al. 2023). Microscopic analyses were performed at Siirt University, Faculty of Veterinary Medicine, Parasitology laboratory.

DNA Extraction

DNA extraction was performed in all samples using a GeneMATRIX Stool DNA Purification Kit 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

For *Cryptosporidium* spp. analyses, primers described by Xiao et al. (2001) were used in the nested PCR analysis. 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 TATTTATTTATTAGATAAAG-3' and 5'-AAGGAGTA AGGAACAACCTCCA-3' were used to amplify the 826-864 bp gene region.

In the Nested PCR analysis for *Giardia duodenalis* analyses, the 753 bp β-giardin gene region was amplified using the primers described by Caccio et al. (2002) (G7 F5'-AAGCCCGACGACGACCTCACCCGCAGT GC-3' forward and G759R 5'- GAGGCCGCCGCCCT GGATCTTCGAGACGAC-3' reverse). Nested PCR was then performed using the primers described by Lalle et al. (2005) (BG1F 5'- GAACGAGATC GAGGTCCG-3' forward and BG2R 5'-CTCGAC GAGTTCGTGTGTT-3' reverse).

The PCR products obtained were stained with Red-Safe™ Nucleic Acid Staining Solution and images were obtained on 1.5% agarose gel. Positive PCR products were subjected to bidirectional sequencing.

Table 1. Occurence of diseases in sheep according to microscopic and Nested PCR analysis results.

Agent	(n)	Microscopic examination		Nested PCR analysis	
		(n)	%	(n)	%
Cryptosporidium spp.	500	12	2.4%	18	3.6%
Giardia duodenalis		42	8.4%	51	10.2%

Sequence Analysis and Phylogeny

Sequence analyses were performed at a private company (BM Labosis, Ankara, Turkey). The 18 PCR samples positive for *Cryptosporidium* spp. were sequenced forward and reverse. The DNA sequences obtained were analyzed in BioEdit software (Hall 1999). The edited formats of the DNA sequences were compared with the databases using the NCBI Basic Local Alignment Search Tool to determine the assemblages (Altschul et al. 1990). In addition, a phylogenetic tree was constructed with the data set created using the sequences of the 18s rRNA gene obtained from the NCBI GenBank database and the DNA sequences obtained as a result of the study. Data sets were aligned in the BioEdit program and the model test was performed using the Maximum Likelihood statistical method in the IQTREE program and the phylogenetic tree was constructed with 1000 bootstraps according to the BIC optimal model (Minh et al. 2013, Trifinopoulos et al. 2016). The PCR products of nine samples were not in quantities that could be sequenced according to agarose gel electrophoresis results.

The 51 PCR samples positive for *Giardia duodenalis* were sequenced forward and reverse (samples 15 and 16 were only sequenced forward). The DNA sequences obtained were analyzed in BioEdit software (Hall 1999). The edited formats of the DNA sequences were compared with the databases using the NCBI Basic Local Alignment Search Tool to determine assemblages (Altschul et al. 1990). In addition, a phylogenetic tree was constructed with the data set created using the sequences of the B-giardin gene from the NCBI GenBank database and the DNA sequences obtained as a result of the study. The data sets were aligned in the BioEdit program and the model test was performed using the Maximum Likelihood statistical method in the IQTREE program and a phylogenetic tree was created with 1000 bootstraps according to the BIC optimal model (Minh et al. 2013, Trifinopoulos et al. 2016). The PCR products of twenty samples were not in quantities that could be sequenced according to agarose gel electrophoresis results.

Ethical Approval

All applicable international, national, and/or institutional guidelines for animal testing, animal care, and use of animals were followed by the authors.

Results

The prevalence rate of *Cryptosporidium* spp. was 2.4% (12/500) and 3.6% (18/500) as a result of microscopic and Nested PCR analyses, respectively (Table 1). According to the Kappa compatibility test, a significant level of agreement (K: 0.794, 98.8%) was found between the two methods. Comparison of the DNA sequences of the SSU rRNA gene obtained in the study with the NCBI Basic Local Alignment Search Tool showed that one sample overlapped with *Cryptosporidium ryanae*, one with *Cryptosporidium andersoni* and seven with *Cryptosporidium parvum*. The placement of the samples in the phylogenetic tree is shown in Fig. 1.

Microscopic examination and nested PCR analysis of all samples for *Giardia duodenalis* showed a prevalence of 8.4% (42/500) and 10.2% (51/500), respectively (Table 1). According to the Kappa compatibility test, a significant level of agreement (K: 0.893, 98.2%) was found between the two methods. When the DNA sequences of the B-giardin gene obtained as a result of the study were compared with the NCBI Basic Local Alignment Search Tool database, it was observed that five samples overlapped with assemblage A, six with assemblage B, one with assemblage D and 19 with assemblage E. The placement of the samples in the phylogenetic tree is shown in Fig. 2.

Discussion

Cryptosporidium spp., and *Giardia duodenalis* have been identified as important enteropathogens in various animal species and humans. These agents have also been recognized as the most common causes of waterborne gastroenteritis (Castro-Hermida et al. 2007). Studies have been carried out in many regions of the world to investigate the prevalence of these diseases.

The prevalence of *Cryptosporidium* spp. in sheep was reported to be 23% in Canada (Olson et al. 1997), 10.1% in Poland (Majewska et al. 2000), 11.2% in Tunisia (Soltane et al. 2007), 25% in the USA (Santín et al. 2007), 31% in Spain (Castro-Hermida et al. 2007), 15% in Norway (Robertson et al. 2010), 2.2% in Papua New Guinea (Koinari et al. 2014), 16.9% in Australia (Yang, R. et al. 2014), 11.3% in Iran (Gharekhani et al. 2014), 25% in Brazil (Paz e Silva et al. 2014), 10.1%

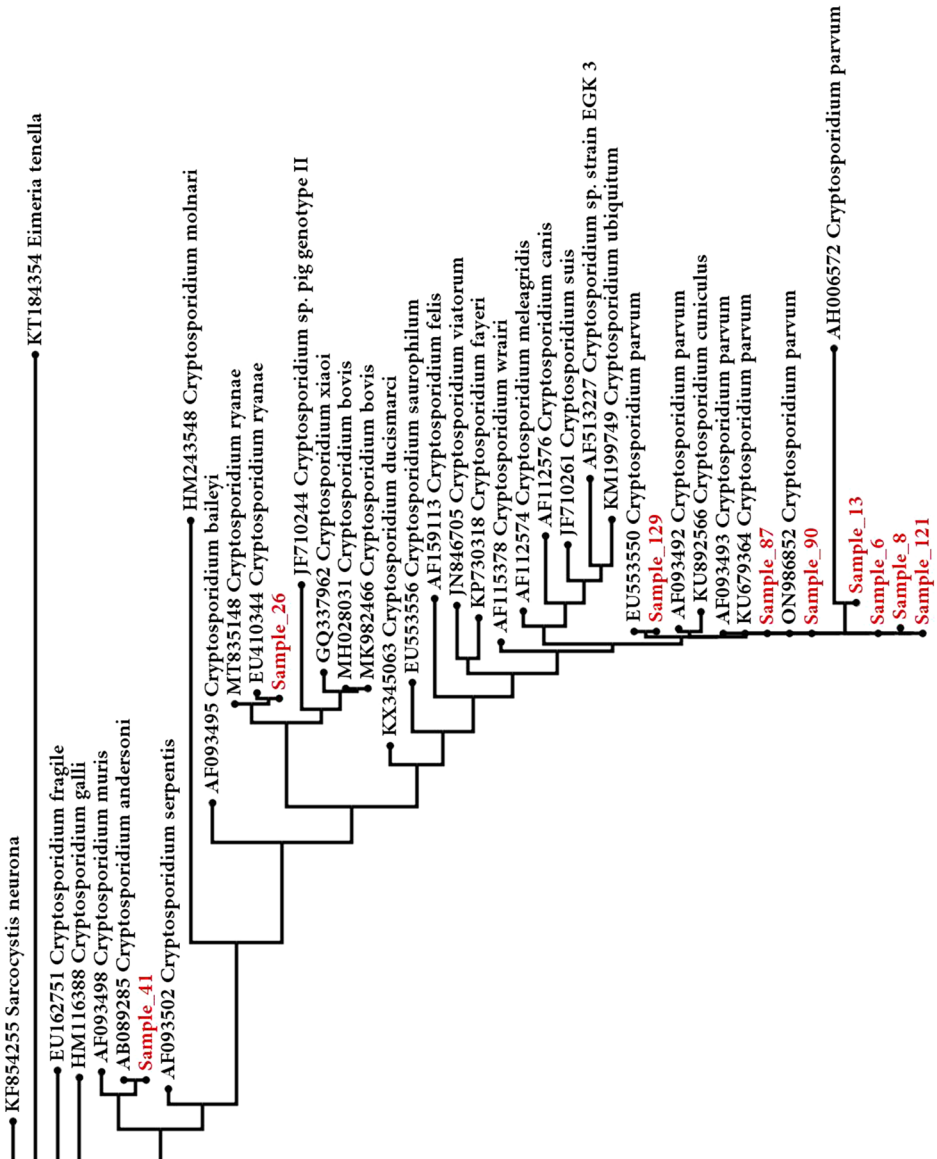

Fig. 1. Phylogenetic relationships of *Cryptosporidium* spp. Isolates from sheep using Maximum Likelihood Method analysis based on SSU rRNA gene region. Numbers in nodes represent Bootstrap values (1000 replicates). *Eimeria tenella* and *Sarcocystis neuroma* were used as outgroups.

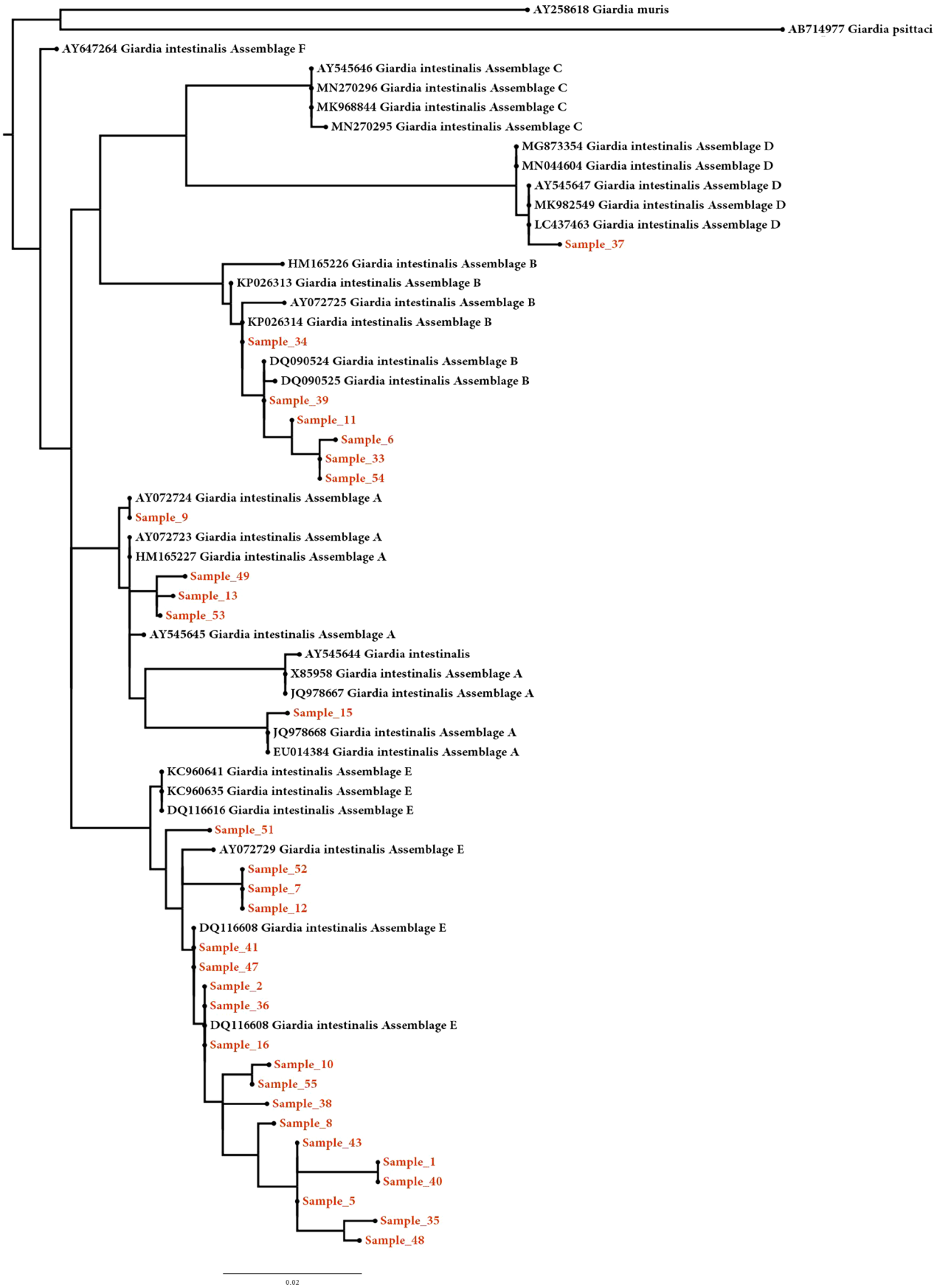

Fig. 2. Phylogenetic relationships of *Giardia duodenalis* isolates from sheep using Maximum Likelihood method analysis based on β-giardin gene region. Numbers in the nodes represent Bootstrap values (1000 bootstrap). *Giardia psitacci* and *Giardia muris* were used as outgroups.

in Italy (Dessì et al. 2020), and 9.6-46.5% in Turkey (Ozmen et al. 2006, Ulutas and Voyvoda 2004). Different methods such as Ziehl-Neelsen staining (Majewska et al. 2000, Soltane et al. 2007, Gharekhani et al. 2014, Majeed et al. 2018, Dessì et al. 2020), Kinyoun Acid Fast staining (Janoff and Reller 1987, Mondebo et al. 2022), ELISA (Goma et al. 2007), and PCR (Majewska et al. 2000, Goma et al. 2007, Koinari et al. 2014) are used for the diagnosis of *Cryptosporidium*. In this study, a prevalence of 2.4% was determined by microscopic analysis using the Kinyoun Acid Fast staining method and 3.6% by the Nested PCR method. These results are similar to the findings of the study conducted by Koinari et al. (2014). The reasons for the differences between the studies can be listed as different geographical conditions, sampling time, sample size, gender, animal species, breeding method, hygiene conditions, nutritional stress, and methods used.

Currently, more than 10 *Cryptosporidium* species (*C. xiaoi, C. ubiquitum, C. parvum, C. andersoni, C. fayeri, C. ryanae, C. scrofarum, C. hominis, C. suis,* and *C. bovis*) have been identified that can infect sheep (Fayer and Santín 2009, Koinari et al. 2014, Yang, F. et al. 2022). Of these, *C. parvum, C. xiaoi* and *C. ubiquitum* are the most common species and *C. parvum* are zoonotic (Castro-Hermida et al. 2007, Santín et al. 2007, Fayer and Santín 2009, Koinari et al. 2014, Dessì et al. 2020, Yang, F. et al. 2022). As a result of the sequence analyses performed in this study, seven samples were identified as zoonotic *C.parvum*, one sample as *C.ryanae*, and one sample as *C.andersoni*. These results are similar to the findings of other studies (Castro-Hermida et al. 2007, Fayer and Santín 2009, Dessì et al. 2020, Yang, F. et al. 2022).

The prevalence of *Giardia duodenalis* in sheep was reported to be 38% in Canada (Olson et al. 1997), 1.5% in Italy (Giangaspero et al. 2005), 33% in Spain (Castro-Hermida et al. 2007), 12% in USA (Santín et al. 2007), 23% in Norway (Robertson et al. 2010), 34% in Brazil (Paz e Silva et al. 2014), 6.2%-19.8% in Iran (Jafari et al. 2014, Kiani-Salmi et al. 2019), 6.56% in China (Wang et al. 2016), and 8.3%-36.6% in Turkey (Ozmen et al. 2006, Kızıltepe and Ayvazoğlu 2022). Different methods such as Native-lugol (Kılınç et al. 2023), ELISA (Wilson and Hankenson 2010), IFAT (Castro-Hermida et al. 2007), and PCR (Giangaspero et al. 2005, Yang et al. 2014, Wang et al. 2016, Jian et al. 2018) are used in the diagnosis of giardiosis. In this study, 8.4% prevalence was determined as a result of microscopic analysis by the Nativ Lugol method and 10.2% by the Nested PCR method. The results of this study are similar to the findings of other studies (Santín et al. 2007, Wilson and Hankenson 2010, Wang et al. 2016, Kızıltepe and Ayvazoğlu 2022).

To date, three assemblages (A, B, E) have been isolated in sheep. Among these, the predominant assemblage is E, which is reported to have a significantly higher prevalence than assemblages A and B (Giangaspero et al. 2005, Santín et al. 2007, Wang et al. 2016, Yang, F. et al. 2022). As a result of the sequence analyses performed in this study, it was determined that 19 samples were Assemblage E, six samples were zoonotic Asemblage B and five samples were zoonotic Asemblage A. These results support the findings of other studies (Giangaspero et al. 2005, Santín et al. 2007, Wang et al. 2016, Yang, F. et al. 2022).

Although assemblage D is reported to be seen especially in canids (Ballweber et al. 2010), there are also studies reporting that it is seen in other animals (Sahraoui et al. 2019, Koçhan et al. 2020). In this study, one sample was found to be assemblage D. This result supports the studies conducted by Sahraoui et al. (2019) and Koçhan et al. (2020). The reason for the detection of assemblage D may be due to the fact that the area where this sample was taken was contaminated by a large number of infected canids. It has also been reported that intensive contact between dogs can lead to assemblage D transmission (Ryan and Cacciò 2013, Sahraoui et al. 2019).

Conclusion

In conclusion, the prevalence of *Cryptosporidium* spp. and *Giardia duodenalis*, which are enteric pathogens, was determined in this study. The presence of zoonotic and host-specific species was also revealed. This supports the idea that sheep may be a reservoir for human infection. Although the prevalence was found to be low, these animals can cause contamination of the environment by spreading cysts/oocysts. Animal owners and people who have intensive contact with animals should be made aware of these diseases. A large-scale epidemiological study is needed to understand the impact of these agents on sheep breeding and the real consequences for human health.

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