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Original Article

Molecular Characterization of *Giardia intestinalis* and *Cryptosporidium* spp. Detected in Humans in Ağrı, Türkiye

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Received 11 Aug 2023
Accepted 14 Oct 2023

Keywords:
Cryptosporidium spp.;
Epidemiology;
Genotype;
Giardia intestinalis;
Subtypes;
Turkey

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Abstract

Background: We aimed to determine the prevalence of *Giardia intestinalis* and *Cryptosporidium* spp. in patients who admitted hospital with diarrhea and to gain information about the transmission of these parasites in Ağrı, Türkiye.

Methods: This study included 184 patients who applied to Ağrı-Diyadin State Hospital, Türkiye in 2022. The immunochromatographic card test was used for detection of the *G. intestinalis* and *Cryptosporidium* spp. Nested PCR-RFLP analysis of the COWP gene and sequence analysis of the gp60 gene were used to genotype and subtype *Cryptosporidium* spp., whereas Nested PCR and sequence analyses of β-giardin gene were used genotype *G. intestinalis*.

Results: Of the 184 stool specimens examined, 12 (14.29%) and 7 (3.80%) were positive for *G. intestinalis* and *Cryptosporidium* spp., respectively. The *Cryptosporidium* species were identified as *C. parvum* belonging to the IId subtype family. The *G. intestinalis* were identified assemblages A.

Conclusion: Assemblage A, which is associated with diarrhea, is responsible for giardiasis and *C. parvum* IId subtype, often found in sheep, goats and cattle, is responsible for cryptosporidiosis in Ağrı, Türkiye.

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Introduction

Enteric parasites cause a serious public health problem and significant socioeconomic burden associated with medical and treatment costs in developing countries. Enteric protozoan parasite *Cryptosporidium* spp. and *Giardia intestinalis* are major agents of diarrhea in humans, and *Cryptosporidium* species are the most common protozoan parasite-causing diarrhea worldwide (1). *Cryptosporidium* species and *G. intestinalis* were ranked as the sixth and eleventh most important foodborne parasites worldwide. Both parasites are excreted in the feces of infected hosts and can infect new hosts through feces-contaminated soil, water, feed, and food (2). Between 2011 and 2016, worldwide water-borne protozoan outbreaks were reported to be caused by *Cryptosporidium* species and *G. intestinalis* (3).

Currently, at least 38 *Cryptosporidium* species and more than 40 genotypes of these species have been described. The most common species causing human cryptosporidiosis reported worldwide are *C. parvum* and *C. hominis*. *C. hominis* is predominantly a human pathogen and thus the main cause of anthroponotic transmission. The reservoir of *C. parvum* contains several mammalian species, primarily cattle (4). Some subtype families of *C. parvum* have been noted to be more common in certain host species. Subtype families IIa in cattle, IIc in humans, and IId in sheep and goats are common, but all three-subtype families can infect humans (5).

There are at least eight different genotypes (A–H) of *G. intestinalis* species causing giardiasis. Among these, genotypes A and B have been reported to be associated with diseases in humans and mammals, while genotypes C and D are seen in dogs, E in ruminants, F in cats, G in rodents, and H in marine mammals. Genotypes C–H are often thought to be host-specific (6). Diarrhea cases developing with genotype A are more severe than those with genotype B. The observation of different clinical manifestations in giardiasis developing with different genotypes, as reported in genotypes A and B, suggests that *G. intestinalis* genotype groups may have different pathogenic characteristics, and the differentiation of these may benefit the clinic in terms of treatment (6). In recent years, there have been researchers who have advocated that *G. intestinalis* genotypes are different species (7).

We aimed to determine the prevalence of *G. intestinalis* and *Cryptosporidium* spp. in patients who admitted hospital with diarrhea for the first time. Besides, we wanted to gain information about the transmission of these parasites in Ağrı, Türkiye.

Methods

Study Area

This study was carried out in Ağrı Province in eastern Türkiye in 2022. Rich herbaceous plant species dominate the vegetation in Ağrı. Winters are cold and harsh, but summers are dry and hot. Ağrı is one of the least developed provinces of Türkiye. Small ruminants farming is common in Ağrı. Small ruminants farming is carried out under extensive conditions, in small family farms and according to a traditional structure.

Ethical clearance

The ethical approval for this study was obtained from the Non-Invasive Ethics Committee of the local university (19/11/2021-2021/12-02).

Specimen collection

This study included a total of 184 patients, 75 (40.76%) male and 109 (59.24%) female patients, who applied to Ağrı-Diyadin State Hospital (Fig. 1) with the complaint of diarrhea between October and December 2022. Stool samples were transported to the laboratory in coolers. In the laboratory, *G. intestinalis* and *Cryptosporidium* spp. positive samples were

determined by the cassette test method. Positive samples were kept at 4 °C until DNA isolation by adding 2 ml of distilled water. Age, gender, and diarrhea information of the patients were obtained from the hospital automation system.

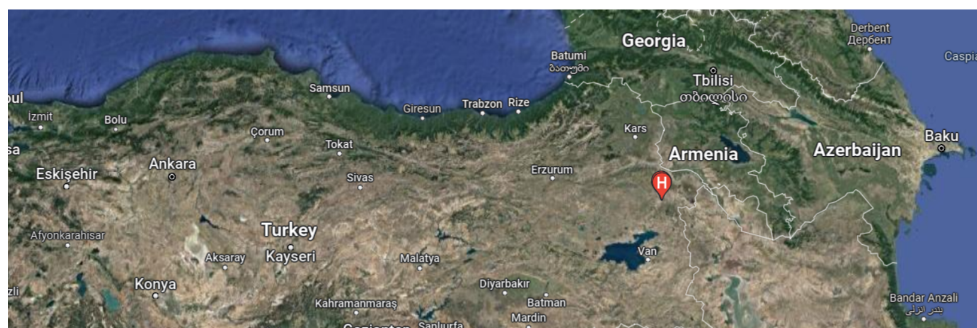

Fig. 1: Location of the hospital (H) where the samples were collected

Identification of G. intestinalis and Cryptosporidium spp.

The immunochromatographic card test (Certest Biotec, Spain) was used for detection of the *G. intestinalis* and *Cryptosporidium* spp. in the stool. This test is a coloured chromatographic immunoassay for the simultaneous qualitative detection of *Cryptosporidium* spp. and *G. intestinalis* antigen in stool samples. The immunochromatographic test used provides results in a short time, does not require experienced personnel and is highly reliable (8). The method was applied as recommended by the manufacturer.

DNA extraction

Positive samples kept at 4 °C by adding distilled water were vortexed. Then, 1 ml of the samples was taken, and 1 ml of phosphate-buffered saline (PBS) was added to it. The mixture was centrifuged at 5000 xg for 5 min, and the supernatant was discarded. DNA isolation was performed from the pellet using a DNA extraction kit (EURx GeneMATRIX Stool DNA Purification Kit, Poland). The method was performed with some modifications to the manufacturer's instructions. After incubation at 70 °C for 5 min according to the manufacturer's instructions, incubation was performed at 95 °C for 45 min in a dry block heater. During the incubation, vortexing was done at 5-min intervals. After incubation, the tubes were placed in a horizontal vortex and vortexed at maximum speed for 30 min. Afterwards, the manufacturer's instructions were applied.

G. intestinalis genotyping

The nested-polymerase chain reaction (PCR) protocol for amplification of the β-giardin gene region in *G. intestinalis* was performed as previously described by Ayan et al. (9) (Table 1). The *G. intestinalis* genotypes were identified by bidirectional DNA sequence analysis of the secondary PCR products.

Cryptosporidium spp. genotyping and subtyping

Cryptosporidium spp.-positive specimens were examined for the identification of species using nested PCR targeting a 553 bp fragment of the *Cryptosporidium* oocyst wall protein (COWP) gene region (10) (Table 1). The nested PCR samples were cut with enzymes and the *Cryptosporidium* species were typed. The Thermo Scientific Fast Digest restriction RsaI enzyme was used for this procedure (8). *C. parvum*-positive specimens were further analyzed using nested PCR targeting a ~850 bp fragment of the 60-kDa glycoprotein (gp60) gene (11) (Table 1). The *C. parvum* subtypes were identified by bidirectional DNA se-

quence analysis of the secondary PCR products of the gp60 gene (Table 1).

DNA sequence analyses

Sequence chromatograms of each strand were examined with SnapGene 4.1 software (GSL Biotech LLC, Chicago, IL, USA) and compared with published sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST; www.ncbi.nlm.n-ih.gov/BLAST).

Table 1: Primers used in this study

Gene region	*PCR*	*Primer*	*Long (bp)*
β-giardin gene	First PCR	G7F (5'- AAGCCCGACGACCTCACCCGCAGTGC-3') G759R (5'-GAGGCCCGCCCTGGATCTTCGAGACGAC-3')	510
	Second PCR	BG1F (5'-GAACGAGATCGAGGTCCG-3') BG2R (5'-CTCGACGAGTTCGTGTT-3')	
COWP gene	First PCR	BCOWPF (5'-ACCGCTTCTCAACAACCATCTTGTCCTC-3') BCOWPR (5'-CGCACCTGTTCCCACTCAATGTAAACCC-3')	553
	Second PCR	Cry-15: (5'-GTAGATAATGGAAGAGATTGTG-3') Cry-9: (5'-GGACTGAAATACAGGCATTATCTTG-3')	
gp60 gene	First PCR	GP60-F1 (5'-ATAGTCTCCGCTGTATTC-3') GP60-R1 (5'-GGAAGGAACGATGTATCT-3')	800-850
	Second PCR	GP60-F2 (5'-TCCGCTGTATTCTCAGCC-3') GP60-R2 (5'-GCAGAGGAACCAGCATC-3')	

Results

Sociodemographic description

Of the 184 study participants, 75 (40.76%) were male, and 109 (59.24%) were female. The mean age of the study participants was 25.86 years. All of the participants reported diarrhea prior to visiting the health center.

Prevalence of G. intestinalis and Cryptosporidium spp. infection

Of the 184 stool specimens, 12 (6.52%) and 7 (3.80%) were positive for *G. intestinalis* and *Cryptosporidium* spp., respectively (Table 2). Concurrence of the *G. intestinalis* and *Cryptosporidium* spp. was detected in one of the specimens.

Table 2: Distribution of the positive samples by gender and age

Variable	*Number of sample (%)*	*Giardia intestinalis*		*Cryptosporidium spp.*	
		Number of Positive (%)	*P*-value	Number of Positive (%)	*P*-value
Gender					
Female	109 (59.24)	6 (5.50)	0.513	6 (5.50)	0.103
Male	75 (40.76)	6 (8.00)		1 (1.33)	
Age(yr)					
≤25	103 (55.98)	8 (7.77)	0.428	4 (3.88)	0.949
>25	81 (44.02)	4 (4.94)		3 (3.70)	
Total	184	12 (6.52)		7 (3.80)	

G. intestinalis genotyping

All 12 of the samples that were *G. intestinal*-positive by immunochromatographic card test were positive via β-giardin PCR (Fig. 2). As a result of the sequence analysis, nine samples were in the assemblage A genotype. The remaining three samples could not be evaluated due to the low sequence quality (Table 3).

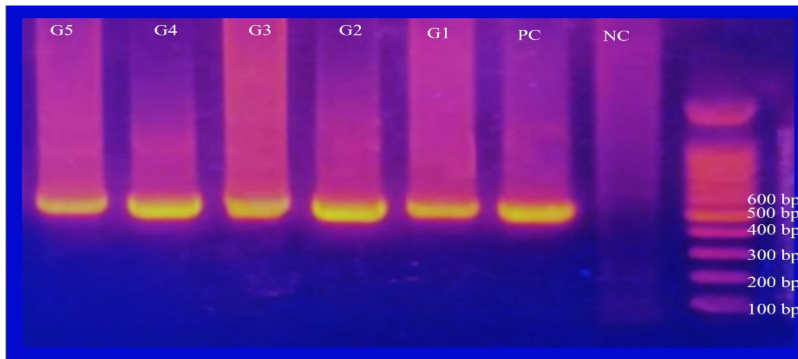

Fig. 2: *G. intestinalis* β-giardin gene region amplification of the 510 bp region using the nested PCR method in agarose gel (marker: 100 bp ladder; NC: negative control, PC: positive control, G1-G5: positive samples)

Cryptosporidium spp. genotyping and subtyping

All of seven of the samples that were *Cryptosporidium* spp.-positive by immunochromatographic card test were positive via COWP PCR (Fig. 3). Because of RFLP analysis of COWP PCR products, all isolates were determined to be *C. parvum* (Fig. 4).

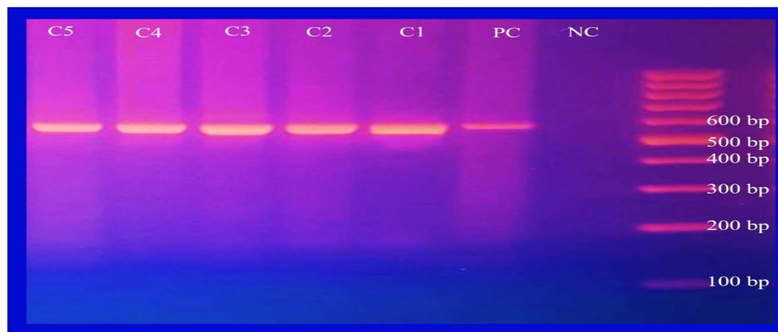

Fig. 3: *Cryptosporidium* spp. COWP gene region amplification of the 553 bp region using the nested PCR method in agarose gel (marker: 100 bp ladder, NC: negative control, PC: positive control, C1-C5: positive samples)

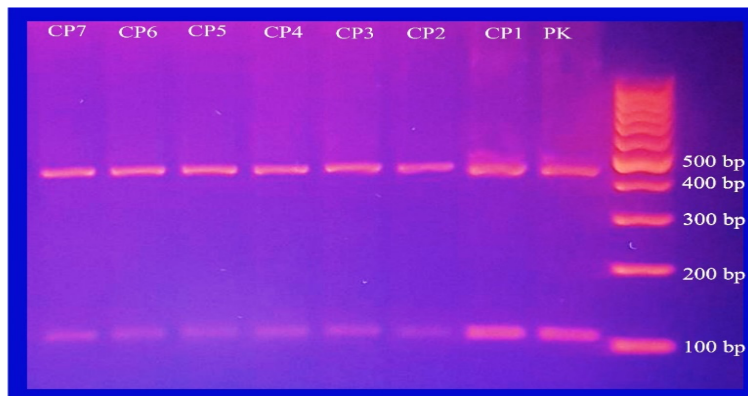

Fig. 4: Bands of *C. parvum* formed as a result of cutting nested PCR amplicons with RsaI enzyme in agarose gel (marker:100 bp ladder, PC: *C. parvum* positive control, CP1-CP7: positive *C. parvum* samples)

Only the IId subtype family was identified within the *C. parvum* by gp60 sequence analysis and subtypes detected included IIdA18G1, IIdA19G1, and IIdA20G1 (Table 3).

Table 3: *G. intestinalis* genotypes and *C. parvum* subtypes detected in positive samples

Sample no	*G. intestinalis genotyping*	*C. parvum subtypes*
5	Assemblage A	IIdA18G1
12	Assemblage A	
17	Assemblage A	
19	Not identified	
37		IIdA19G1
38	Not identified	
53		IIdA18G1
57	Assemblage A	
65	Assemblage A	
93	Not identified	
99		IIdA18G1
108	Assemblage A	
125	Assemblage A	
131		
138	Assemblage A	
153		IIdA20G1
161	Assemblage A	
167		IIdA19G1
177		IIdA18G1

Discussion

In the present study, the rates of *G. intestinalis* and *Cryptosporidium* spp. in Ağrı, Türkiye, were 14.29% and 3.80%, respectively. There is no information in the literature regarding the prevalence of both parasites in Ağrı Province. Few studies have been conducted on the prevalence of *G. intestinalis* and *Cryptosporidium* spp. in neighboring provinces. In Van, *G. intestinalis* and *Cryptosporidium* spp. rates were reported to be 9.3%–12.6% and 0.7%–20%, respectively (8, 12, 13). In a study investigating the prevalence of intestinal parasites in patients with diarrhea in Iğdır, *G. intestinalis* was found at a rate of 2% and *Cryptosporidium* spp. was found at a rate of 3% (14). In Erzurum, *G. intestinalis* was found in 13% of children and *Cryptosporidium* spp. was found in 7.75% (15). *G. intestinalis* was detected in 10.9% of patients with gastrointestinal complaints in Kars, but *Cryptosporidium* spp. was not detected (16). In this study, the incidence of *G. intestinalis*, which was determined for the first time in Ağrı Province, is higher than in neighboring provinces and the rate of *Cryptosporidium* spp. is at a substantial level.

Genotyping of nine samples positive for *G intestinalis* was possible. According to the sequence analysis of the β-giardin gene region, all of the samples belonged to the assemblage A genotype. Assemblages A and B are genotypes with zoonotic characters that can cause infection in humans and some mammals. Worldwide, assembly B is reported to be more common than assembly A. (5, 17). However, the detection rates of human-associated *G. intestinalis* genotypes vary according to the regions where the study was conducted. While assemblage A is more common in the USA,

Mexico, Brazil, Portugal, and Italy, Assemblage B is dominant in England, France, Netherlands, Norway, Nigeria, Argentina, Egypt, and Bangladesh (18, 19). In Türkiye, assemblages A and B were detected in studies conducted in Ankara (20), Sivas (21), Aydın (17), and Manisa (22), and assemblage A was the dominant genotype. These studies showed that assemblage A is the dominant genotype in Türkiye, as in the current study. In a study conducted in Ankara, assemblage A was found in 85% of symptomatic patients, and assemblage B was found in 92% of asymptomatic patients (23). The detection of only assemblage A in this study, in which all of the patients had diarrhea, supports studies (23-25) that found assemblage A to be associated with symptoms such as diarrhea.

All of the isolates obtained from the *Cryptosporidium* spp.-positive samples in this study were *C. parvum*. Worldwide, *C. parvum* and *C. hominis* are the most common species reported in humans (26). In a study conducted in İzmir, 20 isolates were found to be *C. parvum* and one isolate was *C. meleagridis*, and the researchers stated that *C. meleagridis* was detected for the first time in humans in Türkiye (27). In Tekirdağ, four isolates were *C. parvum* and one isolate was *C. hominis*, and the researchers stated that *C. hominis* was detected for the first time in humans in Türkiye (28). In the Van region, 30 isolates were *C. parvum* (8). Studies on the speciation of *Cryptosporidium* spp. isolates obtained from humans in Türkiye have shown that *C. parvum* is the dominant species. Since calves, sheep, and goats play a major role in transmission, *C. parvum* is the dominant species responsible for cryptosporidiosis infections, especially in people in rural areas who are in frequent contact with farm animals (29). In the Ağrı Province, where this study was conducted, people have more contact with animals because livestock is their main source of livelihood. Therefore, it is believed that *C. parvum* infections are transmitted from farm animals. *C. parvum* subtypes detected in the study also supported this opinion.

With gp60 sequence analysis, seven isolates detected in this study belonged to the IId subtype family. The IId subtype family is frequently found in sheep and goats (30). Therefore, the importance of IId in human infections may be related to the importance of small ruminants, which are commonly infected with *C. parvum* IId subtypes (31). In some studies, the IId subtype family was found to be common in cattle (30).

Conclusion

Cryptosporidiosis and giardiasis gain more importance in places with low socioeconomic status, inadequate infrastructure and insufficient attention to personal hygiene, studies on the typing of *Cryptosporidium* species and *G. intestinalis* are limited in Türkiye and studies in this field should be increased. Assemblage A, associated with diarrhea, is responsible for giardiasis infections; *C. parvum* is responsible for cryptosporidiosis infections; and sheep and goats or cattle play a major role in the transmission of the infection in Ağrı.

Acknowledgements

The study has received financial support from Van Yüzüncü Yıl University Scientific Research Projects Presidency (Project No: TSA-2022-9937)

Conflict of Interest

The authors declare that there is no conflict of interest.

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