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RESEARCH ARTICLE

First Report of Zoonotic *Cryptosporidium parvum* Subtype IIaA15G2R1 in Dogs in Türkiye

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ABSTRACT

Cryptosporidium (*C.*) is an opportunistic protozoan causing gastrointestinal illness in both humans and animals, leading to acute or chronic diarrhea and even death. The study aimed to investigate the prevalence and subtyping of *Cryptosporidium* spp. in shelter dogs in Van province, Türkiye. For microscopic identification of this parasite, a total of 300 fecal samples were collected and stained with Kinyoun's acid-fast method. For molecular analysis, the positive samples were subjected to DNA extraction and SSU rRNA gene of *Cryptosporidium* spp. *was amplified using* nested PCR. The microscopic examination revealed a 4.67% prevalence of *Cryptosporidium* spp. Sequence analysis indicated all samples were positive to *C. parvum*. In addition, GP60 gene was also amplified and *C. parvum* subtypes IIaA15G2R1 was confirmed by analyzing the obtained sequences. All the sequences of SSU rRNA and GP60 were deposited in GenBank. To our knowledge, *Cryptosporidium parvum* subtypes IIaA15G2R1 have been reported first time in dogs in Türkiye. It is recommended to implement control strategies by awareness campaign, preventing stray dogs from freely entering public areas, and proper disposal of dog feces.

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INTRODUCTION

Cryptosporidium (*C.*) is an opportunistic zoonotic parasite that affects the gastrointestinal tract of various animals and humans (Giangaspero *et al.*, 2006; Machado-Alves *et al.*, 2018; Ranjbar *et al.*, 2018; Ramadevi *et al.*, 2021). It has a ubiquitous geographic distribution (Abe *et al.*, 2002; Dixit *et al.*, 2019) and causes acute or chronic diarrhea, and even death in animals and humans (Fayer, 2004; Gharieb *et al.*, 2018; Swain *et al.*, 2019).

Cryptosporidium contains more than 30 species and at least 50 genotypes (Jian *et al.*, 2014; Ranjbar *et al.*, 2018). However, previous studies have repeatedly indicated that most canine GIT infections are caused by various *Cryptosporidium* species (Fayer, 2004; Ranjbar *et al.*, 2018). Among them, *C. parvum* is among the most common pathogenic species infecting many mammals (Gharieb *et al.*, 2018; Ranjbar *et al.*, 2018). The contamination occurs by ingestion of infective oocysts via the fecal-oral route, food and water sources (Mirzaei, 2012; Bhagat *et al.*, 2018; Abbas *et al.*, 2022). *Cryptosporidium* spp. significantly impacts the gastrointestinal system of its hosts, leading to a range of clinical manifestations such as diarrhea and abdominal

discomfort, as well as subclinical infections where symptoms may be less apparent but still contribute to an overall disease burden (Mirzaei, 2012; Tavalla *et al.*, 2017; Gharieb *et al.*, 2018).

Several epidemiological studies indicate that *Cryptosporidium* is more prevalent in developing countries compared to developed ones. Notably, *C. parvum* accounts for over 90% of cryptosporidiosis cases in humans in these regions (Mirzaei, 2012; Tavalla *et al.*, 2017; Gharieb *et al.*, 2018). While there is existing information on the zoonotic potential of *Cryptosporidium* spp., epidemiological evidence strongly supports that *C. parvum* is a significant zoonotic agent (Xiao *et al.*, 2007; Machado-Alves *et al.*, 2018).

Previously, the prevalence of *Cryptosporidium* spp. in Türkiye has been reported from different animals and humans. But less attention has been given to its occurrence in the dog population (Şimşek *et al.*, 2012). Additionally, these studies only focused on the identification of *Cryptosporidium* spp. Therefore, the current study was designed to estimate the prevalence, molecular characterization and subtyping of *Cryptosporidium* spp., in the dog population in Van, Türkiye.

MATERIALS AND METHODS

Location and Sample collection: The study was conducted in Van province (38°31′52″N 43°24′55″E) located in the Eastern Anatolia Region of Türkiye. A total of 300 fecal samples were collected directly from the rectum of dogs housed in Van Metropolitan Municipality Animal Care and Rehabilitation Center between 2021 to 2022 and stored in labeled containers in a cold chain. After that, the samples were brought to the lab for additional examination.

Microscopic Examination: Fecal samples were colored following Kinyoun's acid-fast technique, and the samples were viewed using a microscope (Leica, Switzerland) at x100 (Çelik *et al.*, 2023).

DNA extraction: After confirmation of positive samples from microscopy, DNA was extraction from positive samples by using a commercially available DNA extraction kit (Gene MATRIX STOOL DNA Purification Kit, EURx, Gdańsk, Poland) by following the manufacturer's instructions. The DNA obtained was stored at -20°C for further analyses.

PCR amplification: SSU rRNA-gene was amplified with previously published primers through Nested PCR using the method described by Xiao *et al.* (2001). Initially, 1325 bp gene region was amplified by following primer pair: forward primer TTCTAGAGCTAATACATGCG and reverse primer CCCATTTCCTTCGAAACAGGA. After that, the second primer set, F (GGAAGGGTTGTATTTATTAGATAAAG) and R (AAGGAGTAAGGAACAACCTCCA) was used to amplify the 826-864 bp gene region. In both reactions, 4 pmol primers (forward and reverse), 4 µL Master Mix (5x FIREPol®, Solis-BioDyne, Estonia), 13.2 µL nuclease-free water in 20 µL of mix were used. After an initial denaturation step at 95°C for 5 minutes, each-cycle comprises denaturation (1 minute at 95°C), annealing (1 minute at 55°C), and elongation (1.30 minute at 72°C) in both reactions. This cycle is then repeated thirty-five times, and is followed by final extension at 72°C for 5 minutes.

Furthermore, 840bp of GP60 gene of *Cryptosporidium parvum* was also amplified to identify the subtype of this parasite using previously reported primer pairs in nested PCR. The primer pair used in the first round PCR was as follows: AL3531 (ATAGTCTCCGCTGTATTC) and AL3535 (GGAAGGAACGATGTATCT), while the primer pair used in the second round was as follows: AL3532 (TCCGCTGTATTCTCTCAGCC) and AL3534 (GCAGAACCAGCATC-3). The amplification protocol for the target GP60 gene was the same as described for the 18S rRNA gene.

Subsequently, the PCR products were separated on a 1.5% agarose gel stained using RedSafe™ Nucleic Acid Staining Solution and images were obtained from Syngene Bioimaging system.

Sequence Analysis and Phylogeny: The PCR-positive samples were forwarded to a commercial laboratory (BM Labosis, Ankara, Türkiye) for bidirectional sequencing. Sequencing of amplicons from the 18S rRNA and gp60 genes was conducted in both directions using the primers employed for Nested PCR, and sequencing was executed utilizing the ABI 3100 Genetic Analyzer.

The DNA sequences were aligned using CodonCode Aligner (CodonCode Corporation), and consensus sequences were derived using ClustalW (Thompson *et al.*, 1997) and BioEdit Sequence Alignment Editor (Hall, 1999). These generated sequences were subsequently compared with homologous sequences archived in GenBank. Multiple sequence alignments were analyzed using BioEdit and MEGA 11.0 software.

The phylogenetic trees for the 18S rRNA gene were constructed using Maximum Likelihood (ML) analysis and the Tamura-Nei (T92) model (Tamura, 1992) in MEGA 11 (Kumar *et al.*, 2016), with Plasmodium vivax utilized as an outgroup. For the GP60 gene, a phylogenetic tree was similarly constructed in MEGA 11 (Kumar *et al.*, 2016) using Maximum Likelihood analysis under the Tamura-Nei (T92) model (Tamura, 1992). Bootstrap analysis comprising 1000 replicates was conducted to evaluate the robustness of the phylogenetic reconstructions.

Statistical Analyses: The data collected in this study were analyzed by using SPSS V16.0 (IBM, Chicago, IL, USA). The relationship between the categorical variables was evaluated using the Chi-square test. Statistical significance was established at a threshold of $P < 0.05$.

RESULTS

The results of the microscopic examination revealed that the prevalence of *Cryptosporidium* spp. was 4.67% (14/300). The prevalence was slightly higher (P>0.05) in female (5.00%) dogs compared to males (4.17%). Moreover, the prevalence of *Cryptosporidium* was higher (P>0.05) in young dogs (less than 1 year; 7.69%)

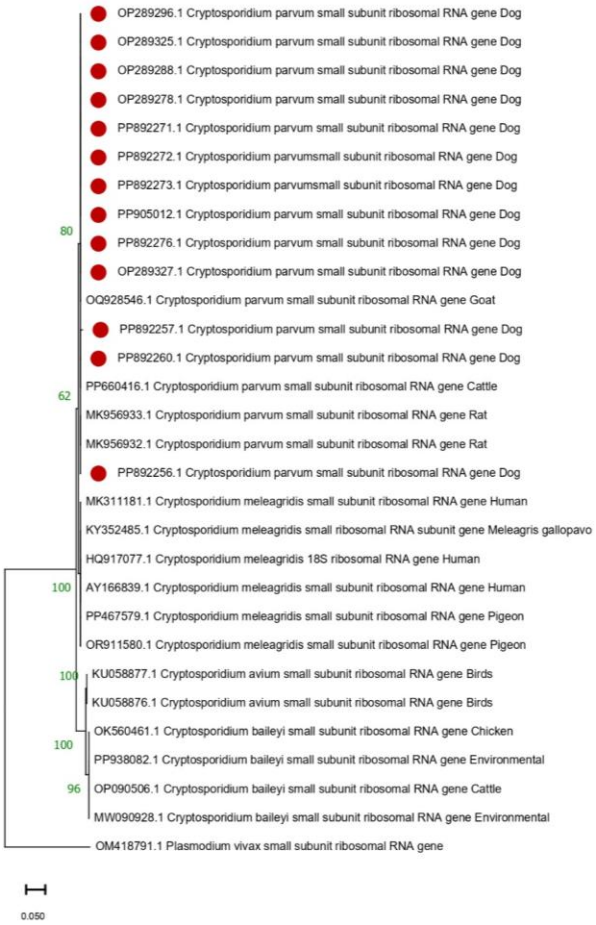

Fig. 1: Phylogenetic analysis of 18S small subunit ribosomal RNA sequence data representing *Cryptosporidium parvum* from dogs. The phylogenetic tree was constructed using Maximum Likelihood analysis with 1000 bootstrap replicates under the T92 model, based on 18S rRNA gene sequences. Evolutionary analyses were performed in MEGA11. The sequence of *Plasmodium vivax* (OM418791.1) was used as an outgroup. The sequences obtained in the present study are indicated by red circle.

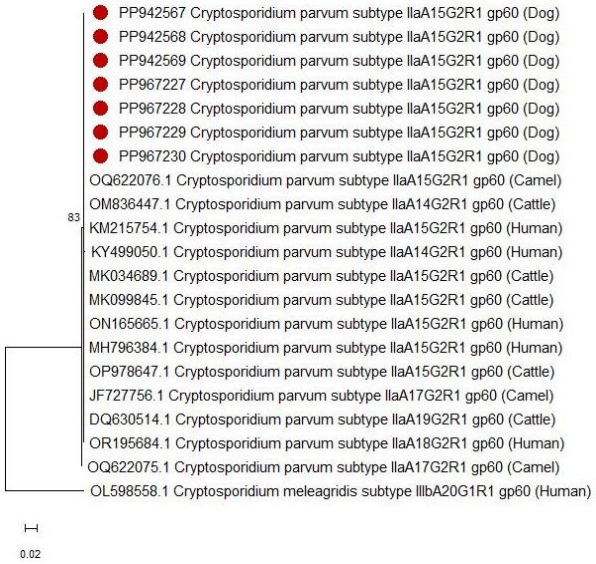

Fig. 2: Phylogenetic tree constructed based on partial sequences of the GP60 gene of *Cryptosporidium parvum* using Maximum Likelihood analysis using the Tamura-Nei model. The sequences of *C parvum* and *C. meleagridis* were subtyped using the reference sequences. The bootstrap value was taken as 1000. The sequences obtained in the present study are indicated by red circles.

compared to adults (above 1 year;3.06%) (Table 1). The positive fecal samples, following microscopic examination, were processed for Nested PCR, which revealed a similar pattern of positivity in all samples.

Sequence analysis of *Cryptosporidium* revealed that all the samples were associated with *C. parvum*. The obtained *C. parvum* 18srRNA sequences were compared with the reference sequences of *C. meleagridis*, *C. baileyi*, and *C. parvum* isolated from different animals and humans. All the sequences *C. parvum* isolates were deposited in GenBank with the following accession numbers: OP289278.1, OP289288.1, OP289296.1, OP289325.1, OP289327.1, PP892256.1, PP892257.1, PP892260.1, PP892271.1, PP892272.1, PP892273.1, PP892276.1, and PP905012.1. (Fig. 1). The comparison of *C. parvum* isolates obtained in this study with studies conducted in other countries is shown in Table 2.

In addition, Of the 13 samples for which sequence analyses were performed, subtype determination could be made for 7 of them. Genotyping at the gp60 locus identified subtype IIaA15G2R1. The isolates have been submitted to GenBank and assigned accession numbers PP942567, PP942568, PP942569, PP967227, PP967228, PP967229, and PP967230 (Fig. 2). The BLAST analysis showed that the *Cryptosporidium parvum* gp60 gene isolates obtained in this study had high similarity to the data sets available in GenBank (Table 3).

Table 1: Prevalence of *Cryptosporidium* spp. among different age and gender groups.

Variable	Examined (n)	Positive (n)	Positive (%)	*P-value*
Gender				
Female	180	9	5.00	0.737
Male	120	5	4.17	
Age (Year)				
0-1	104	8	7.69	0.07
2-3	196	6	3.06	
Total	300	14	4.67	

DISCUSSION

Cryptosporidium spp. are prominent zoonotic protozoan parasites that pose significant health risks across a broad range of vertebrate hosts. these parasites are known to cause symptoms ranging from acute to chronic diarrhea and, in severe cases, can be fetal (Jian *et al.*, 2014). Advances in molecular research have underscored the critical role of zoonotic *Cryptosporidium* spp. and their genotypes in the epidemiology of cryptosporidiosis. and identified them as important factors in human infections (Tavalla *et al.*, 2017). Molecular epidemiological studies consistently identify *C. hominis* and *C. parvum* as the predominant *Cryptosporidium* species affecting humans. Notably, evidence indicates that animals, including dogs, can be significant reservoirs for these parasites, contributing to the spread of human cryptosporidiosis (Ranjbar *et al.*, 2018).

The frequency of *Cryptosporidium* prevalence in dogs worldwide has been reported at different rates; 3.3% in Italy (Giangaspero *et al.*, 2006), 3.8% in China (Jian *et al.*, 2014), 4.6% in Brazil (Machado-Alves *et al.*, 2018), 4.6% in the Czech Republic (Svobodova *et al.*, 1995), 7.4% in Spain (Causape *et al.*, 1996), 9.3% in Japan (Abe *et al.*, 2002), 24% in Egypt (Gharieb *et al.*, 2018), 31.2%

Table 2: The obtained *C. parvum* isolates deposited in GenBank.

Obtained Sequences					Reference sequences from GenBank		
Pathogen	Host	Target Gene	Accession number	Length (bp)	Identity (%)	Accession number	Country
C. parvum	Dog	18S rRNA	OP289278.1	824	100	MK990043.1	Human, China
C. parvum	Dog	18S rRNA	OP289288.1	852	100	MW043436.1	Calf, Bangladesh
C. parvum	Dog	18S rRNA	OP289296.1	826	100	MF671870.1	Cattle, China
C. parvum	Dog	18S rRNA	OP289325.1	839	99.88	AB513881.1	Calf, Egypt
C. parvum	Dog	18S rRNA	OP289327.1	823	100	CP141124.1	Bos taurus, USA
C. parvum	Dog	18S rRNA	PP892256.1	750	100	KT948751.1	Equus caballus, Brazil
C. parvum	Dog	18S rRNA	PP892257.1	723	99.31	PP897362.1	Calf, Slovakia
C. parvum	Dog	18S rRNA	PP892260.1	730	100	PQ047137.1	Human, Romania
C. parvum	Dog	18S rRNA	PP892271.1	752	100	OR229417.1	Goat, China
C. parvum	Dog	18S rRNA	PP892272.1	757	100	OQ928543.1	Goat, South Korea
C. parvum	Dog	18S rRNA	PP892273.1	756	100	CP139736.1	Calf, China
C. parvum	Dog	18S rRNA	PP892276.1	742	100	PP124629.1	Mouse, China
C. parvum	Dog	18S rRNA	PP905012.1	756	100	CP141125.1	Bos taurus, USA

Table 3: The obtained *C. parvum* gp60 isolates deposited in GenBank.

Obtained Sequences					Reference sequences from GenBank	
Pathogen	Host	Target Gene	Accession number	Length (bp)	Identity (%)	Accession number
C. parvum subtypes IIaA15G2R1	Dog	gp60	PP942567	776	99.87	CP029785.1
C. parvum subtypes IIaA15G2R1	Dog	gp60	PP942568	826	100	OQ818286.1
C. parvum subtypes IIaA15G2R1	Dog	gp60	PP942569	818	99.88	MK099848.1
C. parvum subtypes IIaA15G2R1	Dog	gp60	PP967227	841	99.41	OQ818280.1
C. parvum subtypes IIaA15G2R1	Dog	gp60	PP967228	841	99.76	KY990912.1
C. parvum subtypes IIaA15G2R1	Dog	gp60	PP967229	810	99.88	OQ818295.1
C. parvum subtypes IIaA15G2R1	Dog	gp60	PP967230	813	99.63	MK034689.1

in Thailand (Tangtrongsup *et al.*, 2017). Iran is also heavily burdened by cryptosporidiosis prevalence (2-12%) which shares the borders with the province of Van (Kakekhani *et al.*, 2011; Mirzaei, 2012; Tavalla *et al.*, 2017; Ranjbar *et al.*, 2018). However, a limited number of studies have been performed in Türkiye that reported the variable prevalence of cryptosporidiosis from 15.5%-64.7% (Denizhan and Karakuş, 2019; Ayan and Orunç Kılınç, 2020; Öner and Ulutaş, 2022). Although different methods can be used to diagnose the disease (Tavalla *et al.*, 2017), molecular diagnostic methods are extensively employed to achieve precise diagnosis of cryptosporidiosis and to identify species, subspecies, or strains (Şimşek *et al.*, 2012). In this study, the molecular method supported the classical microscopic method and determined a similar ratio of cryptosporidiosis prevalence (4.67%). The prevalence of *C. parvum* recorded in this study is higher than that reported in earlier studies by Giangaspero *et al.* (2006), Jian *et al.* (2014) and Ranjbar *et al.* (2018), while lower than that reported in other previous studies by Svobodova *et al.* (1995), Gharieb *et al.* (2018) and Abe *et al.* (2002). However, similar results were found by Causape *et al.* (1996), Jian *et al.* (2014) and Tangtrongsup *et al.* (2017). This variation could be attributed to geographic location, animal ownership, demographic characteristics, sample size, age of animals, diarrhea incidence, methodologies employed, and the administration of anthelmintics.

Although *C. canis* predominates in the majority of canine infection studies, additional species such as *C. muris*, *C. parvum*, and *C. meleagridis* have also been identified in dogs (Fayer, 2004; Ranjbar *et al.*, 2018). To our knowledge, no prior studies have investigated genetic diversity of *C. parvum* in dogs in Türkiye. In this study, *C. parvum* subtype IIaA15G2R1 was found. Previously, this subtype has also been reported in humans (Alves *et al.*, 2006; Wielinga *et al.*, 2008; Feng *et al.*, 2018), dogs (Rosanowski *et al.*, 2018; Murnik *et al.*, 2022) and farm animals (Alves *et al.*, 2006; Wielinga *et al.*, 2008; Arslan and Ekinci, 2012; Kaupke and Rzeżutka, 2015; Mammeri *et al.*, 2019; Ribeiro *et al.*, 2022). This indicates a risk of zoonotic spread from dogs to humans.

The *C. parvum* isolates (OP289278.1, OP289288.1, OP289296.1, OP289325.1, OP289327.1, PP892256.1, PP892257.1, PP892260.1, PP892271.1, PP892272.1, PP892273.1, PP892276.1 and PP905012.1) obtained in this study were coincide with the results of the study conducted in China (Human, 100%), in Bangladesh (Calf, 100%), in China (Cattle, 100%), in Egypt (Calf, 99.88%), in USA (*Bos taurus*,100%), in Brazil (*Equus caballus*, 100%), in Slovakia (Calf, 99.31%), in Romania (Human, 100%), in China (Goat, 100%), in South Korea (Goat, 100%), in China (Calf, 100%), in China (Mouse, 100%), and USA (*Bos taurus*, 100%).

In addition, the *C. parvum* gp60 isolates (PP942567, PP942568, PP942569, PP967227, PP967228, PP967229, and PP967230) obtained in this study were similar to the results of the study conducted in Germany (Calf, 99.87%), in Portugal (Calf, 100%), in USA (Calf, 99. 88%), in Portugal (Calf, 99.41%), in Mexico (Child, 99.76%), in Portugal (Calf, 99.88%) and Germany (Calf, 99.63%).

Comparing the age factor, Tavalla *et al.* (2017) reported a higher prevalence in adult dogs, while other researchers (Ramirez *et al.*, 2004; Mirzaei, 2012; Gil *et al.*, 2017) have previously reported a higher prevalence among young dogs. While in the present study, a higher prevalence was detected in young animals (7.69%) compared to adults (3.06%). The difference between the groups was not found to be statistically significant. These results are consistent with various published studies (Ramirez *et al.*, 2004; Mirzaei, 2012; Gil *et al.*, 2017).

Upon comparative investigation of *Cryptosporidium* prevalence among gender, Gharekhani (2014) reported a higher prevalence in males, while according to other researchers (Bahrami *et al.*, 2011; Mirzaei, 2012; Olabanji *et al.*, 2016; Tavalla *et al.*, 2017), the prevalence of this parasite was higher in females. However, in this study, a higher prevalence of *Cryptosporidium* was detected in

females (5.00%) compared to males (4.17%), although the difference was not found to be statistically significant. These findings corroborate the previous observation that the females had a higher prevalence of cryptosporidiosis (Bahrami *et al.*, 2011; Mirzaei, 2012; Olabanji *et al.*, 2016; Tavalla *et al.*, 2017). The possible reason for this higher parasite prevalence in females may include reduced immunity during certain periods of physiological cycle (Olabanji *et al.*, 2016).

Conclusions: In this study, zoonotic subtype of *Cryptosporidium parvum* was detected in dogs from the Eastern Anatolia Region of Türkiye. This finding underscores the potential for interspecies transmission of this parasite and highlights the need for continued monitoring and preventive measures to mitigate zoonotic risks.

Ethical Statement: Ethical approval for the current-study was received from the Van YYU-Animal Experiments Local Ethics Committee with the number 2020/09-01.

Conflict of Interest: There is no conflict of interest.

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