IIaA13G2R1 is the most common *Cryptosporidium parvum* Subtype among Calves with diarrhea in Türkiye

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ABSTRACT

Cryptosporidium spp., an intracellular extra cytoplasmic localized protozoan, is one of the leading infectious agents in the etiology of neonatal diarrheal syndrome of ruminants. Cryptosporidiosis is a common disease seen all over the world. However molecular epidemiologic studies are limited on distribution of *Cryptosporidium parvum* subtypes in the world. To date, nearly 20 subtypes of *C. parvum* have been described. Gene sequence analysis of the glycoprotein 60 structural protein (GP60) is used to identify subtypes. It is reported that the GP60 gene sequence shows a high degree of genetic polymorphism among the strains. In this study, it was aimed to determine the subtypes of *C. parvum* parasite in cow and calf stool samples by using GP60 gene sequence analysis. A total of 109 stool samples from calves (<30 days) found to be *Cryptosporidium* spp. positive by Kinyoun Acid Fast staining were further studied for subtyping. Samples were obtained from farms in 2 regions of Türkiye (Burdur/Yesilova and Aydin provinces). DNA isolation from feces was done with the QIAamp Stool Mini Kit. *Cryptosporidium* specie identification was done by 18S gene sequences analysis. Nested PCR was performed to amplify GP60 gene and sequences of amplicons were used to identify subtypes. The repeat regions at the serine residue in the GP60 sequences were used to define the subtypes. The results of *C. parvum* subtyping study showed that IIaA13G2R1 subtype was the most common subtype. IIaA13G2R1 was detected in 73% (80/109) of 109 stool samples, followed by IIaA14G1R1 with 15 samples (14%), IIaA2R1 with 12 samples (11%), and IIaA16R1 and IIaA5G1R1 subtypes with one sample each (1%). In subtype studies based on the GP60 sequence, IIaA15G2R1 was found to be the most common in the world, however this subtype was not found among our samples. In our study, two new subtypes were found, IIaA2R1 and IIaA5G1R1 subtypes. These subtypes were defined for the first time. The subtype, IIaA16R1 was determined to be new in Türkiye. Molecular epidemiology studies are important for better understanding the dissemination of subtypes. IIaA13G2R1 is the most common subtype in Türkiye and the IIaA15G2R1 the most common subtype in the world were absent among our samples. The number of studies on *Cryptosporidium parvum* subtyping in Türkiye is limited. Our study, the first subtyping study was carried out among cattle in Aydın province. The IIaA2R1 and IIaA5G1R1 subtypes that we detected in our study were defined for the first time in the world and IIaA16R1 in Turkey.

Keywords: *Cryptosporidium*; Subtyping; GP60; IIaA13G2R1.

INTRODUCTION

Cryptosporidium is an important agent in the etiology of diarrhea in newborn calves and are frequently isolated from stool samples (1,2). Cryptosporidiosis is a disease caused by the parasite *Cryptosporidium*, which is characterized by diarrhea in young animals during their first weeks of life. Diarrhea causes economic losses directly and indirectly, but also affects the welfare of animals (3). Cryptosporidiosis is characterized by diarrhea that causes intense fluid loss and is life-threatening in young animals. Since the parasite also

causes intestinal hyperplasia, it causes serious economic losses even if the infection has been overcome. At the same time, sick animals pose a threat to humans (4). *Cryptosporidium* spp. is also an enteric protozoan seen in humans and many animals (5). Among *Cryptosporidium* species, *C. parvum*, *C. bovis*, *C. ryanae* and *C. andersoni* species are common in cattle (5). While *Cryptosporidium bovis* and *Cryptosporidium ryanae* parasitize only cattle. *C. parvum* species gain zoonotic importance because the environment can be contaminated with this parasite infecting humans (6).

Epidemiological studies using genetic markers are required to better elucidate the zoonotic potential of *Cryptosporidium* (7).

Cryptosporidium spp. are transmitted by fecal-oral ingestion of environmentally resistant oocysts, which are excreted abundantly with feces (8). After fecal-oral transmission, the parasite infects host enterocytes and enters the sexual reproduction phase, which allows for the formation of different genetic families and subtypes. Genotyping studies using molecular methods allow the determination of the subtypes of the parasite. As a result of determining the subtypes, the host specificity of the parasite, its transmission methods, traceability of infection sources and their potential to become zoonotic, have become more explicable. (9).

Restriction fragment length polymorphism (RFLP) analysis performed after nested PCR with small subunit rRNA (SSUrRNA) primers is widely used in *Cryptosporidium* genotyping studies. In addition, proteins in the genome of *Cryptosporidium* such as heat shock proteins, thrombospondin-associated adhesive proteins, *Cryptosporidium* oocyst wall protein and actin genes are also used in genotyping studies (9).

The genomic sequence of GP60 (Cpgp40/15) exhibits a highly variable polymorphic structure even in the same *Cryptosporidium* species (10, 11). Therefore, sequence analysis of this gene is a frequently applied as the subtyping method (12). In *C. parvum*, of which nearly 20 subtype families have been described, the most common subtype families are IIa, IId and IIc. The IIa subtype family is most commonly found in neonatal calves and is implicated in most zoonotic infections caused by *Cryptosporidium*. IId is very common in sheep and goats and has a zoonotic character. The IIc subtype family is generally found in humans and has an anthroponotic character (13, 14). There is also a parallelism with the level of development of a country regarding the prevalence of subtype families. While the IIa subtype family is found in industrialized, IIc underdeveloped and developing countries, the IId subtype family is seen in some European and Middle Eastern countries (14).

In our study, stool samples taken from calves (n=109) with diagnosis of *Cryptosporidium* infection and from calves of mothers (n=16) which died due to infection in Burdur/Yeşilova and Aydın provinces in Türkiye were used. This study was intended to explore the responsible *Cryptosporidium* species using SSU rRNA sequence analyses. The subtypes of *C. parvum* isolates were also revealed based on the sequence analysis of the gp60 gene.

MATERIAL AND METHOD

Faecal sample collection

In our study, 109 samples evaluated as *Cryptosporidium spp*. positive by Kinyoun Acid Fast staining taken from calves (n=109 less than 30 days) in Burdur/Yeşilova and Aydın provinces) were used. The clinical picture in the animals from which the samples were taken varied. A total of 109 samples were taken from the calves, 94 of which had severe diarrhea and 15 had diarrhea, while 16 calves with severe diarrhea died due to disease-related causes.

DNA Isolation

DNA isolation from feces was performed according to the manufacturer's instructions using the QIAamp Stool Mini Kit (Qiagen GmbH, Hilden, Germany).

SSU 18s rRNA Amplification

For primary PCR, the 1325 bp gene region was amplified using primers SSU-F2: (5'TTCTAGAGCTAATACATGCG3') and SSU-R2: (5'CCCATTTCCTTCGAAACAGGA3') (15, 16). For this purpose, 50µl PCR mix was prepared as 1X Taq enzyme buffer solution, 0.2mM of deoxynucleotide triphosphatase (dNTP), 0.4pmol of primer (for each), 1.5 Unit of Taq DNA polymerase. Then, using the primary PCR products as template DNA, the 830 bp gene region was amplified using primers SSU-F3: (5'GGAAGGGTTGTATTATTATTAGATAAAG3') and SSU-R4: (5'CTCATAAGGTGCTGAAGGAGTA3') (17). Thermocycling parameters were 4 minutes at 94°C hot start (initial heat activation step), followed by 35 cycles of 45 seconds at 94°C, 90 seconds at 58°C and 1 minute at

72˚C, with a final extension of 7 minutes at 72˚C (15). PCR products were visualized on a 1% agarose gel.

Amplification of the GP60 Gene

Primers GP60-F1 (5'-ATAGTCTCCGCTGTATTC-3') and GP60-R1 (5'-GGAAGGAACGATGTATCT-3') in the first PCR to identify subtypes based on amplification of the GP60 gene; GP60-F2 (5'-TCCGCTGTATTCTCAGCC-3') and GP60-R2 (5'-GCAGAGGAACCAGCATC-3') primers were used in secondary PCR (18, 19) Thermocycling parameters were 10 minutes at 94˚C hot start (initial heat activation step), followed by 35 cycles of 45 seconds at 94˚C, 45 seconds at 50˚C and 1 minute at 72˚C, with a final extension of 7 minutes at 72˚C. Obtained amplicons were visualized on 1.5% agarose gel.

Identification of subtypes based on GP60 sequence analysis.

The terminology developed by Sulaiman *et al*. was used to identify GP60 subtype families and subtypes (9). Roman numeral symbols I, II, III, IV, V, VI, VII, VIII, IX, X and XI are respectively *C. hominis*, *C. parvum*, *C. meleagridis*, *C. fayeri*, rabbit genotype, horse genotype, *C. wrairi* was used to denote subtype families of ferret genotype, mouse genotype I, mink genotype, and opossum genotype I. The name of the GP60 subtypes begins with the subtype family designation (IIa, IIb, IIc, IId, IIe, IIf, IIg,IIh,IIi,IIk and IIl for C. parvum). After subtype families were identified, the TCA repeat at the serine residue was represented by the letter A, the TCG repeat by the letter G, and the ACATCA repeat by the letter T. The repeats in the residue are expressed with the number following the letters A, G or R. DNA sequences from different isolates determined to be the same gp60 subtype were compared with each other. BLAST analysis was performed using the NCBI BLAST N program (20). Representative sequences of every detected C. parvum gp60 subtype were deposited in GenBank, and accession numbers were provided.

Sequencing and construction of the phylogenetic tree

Amplification products of both gene regions were sent to a private company for sequence analysis. The company performed the sequence analysis following purification. Sequences sent to our laboratory in Fasta format were compared with gene bank databases using the BLAST program (20). In order to draw the phylogenetic tree based on the GP60 sequence analysis, the sequences sent to us in fasta format were aligned using the Clustal W program and edited using the Bioedit 7.2.5 program (21, 22). A phylogenetic tree was created using the MEGA 7.0 program (23).

RESULTS

All 109 samples examined were identified as *Cryptosporidium parvum* based on SSU 18s rRNA results. (Table 1)

Identification of subtypes and phylogenetic tree based on GP60 sequence analysis.

All studied samples belonged to family IIa according to DNA sequencing in the GP60 gene region. The most common subtype detected in the studied samples was IIaA13G2R1. The sequences of the subtypes in the studied samples were entered in the Genbank. Sequences from the isolates belonging to each Cryptosporidium species obtained in the study were deposited in the NCBI GenBank database under accession numbers; OR077152, OR077153, OR077154, OR077155 and OR077156. An evolutionary tree showing the kinship relationships of 109 studied samples is shown in Figure 1.

Distribution of subtypes in farms based on GP60 gene sequence analysis

All of the samples (n=29) from Burdur/Yeşilova region were taken from 11 different farms and all were found to be subtype IIaA13G2R1. The diversity of subtypes detected in Aydın region was high, and the most common subtype was IIaA13G2R1 (N=51). IIaA13G2R1 subtype was detected in 10 farms in 5 different locations, IIaA14G1R1 was detected in 2 different locations in 3 farms, IIaA2R1 in 2 different locations in 4 farms and IIaA5G1R1 and IIaA16R1 subtypes were detected in 12 farms. (Table 1-Figure 2) IIaA2R1 and IIaA5G1R1 subtypes were detected for the first time in the world in our study. Subtypes IIaA2R1 has 2 copies of TCA (A), trinucleotide repeat encoding serine, respectively, and one copy of the sequence ACATCA (R1) following the trinucleotide repeat. In subtypes IIaA5G1R1, 5 copies of TCA (A) and 1 copy TCG(G) trinucleotide repeat encoding serine, respectively, and one copy of the sequence ACATCA (R1) following the trinucleotide repeat.

DISCUSSION

Cryptosporidium parvum infections occur in calves no later than 30 days of age (24). *Cryptosporidium* parasite may not cause infection in adults and animals with robust immune systems. These animals act as carriers in the environment and may affect human health. There are molecular methods targeting various genes of the parasite for the determination of *Cryptosporidium* species and genotypes in humans and animals. While 18S small subunit (SSU) rRNA is involved in studies to identify species, a method based on amplification of the GP60 surface protein is often used to determine genotypes because of its high polymorphism (15, 19, 25).

While it has been reported that nine species and two genotypes are responsible for Cryptosporidium infections in cattle (*Bos taurus*) in worldwide studies, calves are generally infected with *C. parvum*, a zoonotic species (6). The rate of *C. parvum* infections in cattle varies in various studies conducted in Europe. In Belgium, England, France, Germany,

Table 1. Age group, species and subtype situations in farms

Farm No	Number of Positive Samples	Age Group	Species	Subtypes
1	10	5-30 days calf	*C. parvum*	IIaA13G2R1
2	2	5-30 days calf	*C. parvum*	IIaA13G2R1
3	3	5-30 days calf	*C. parvum*	IIaA13G2R1
4	3	5-30 days calf	*C. parvum*	IIaA13G2R1
5	1	5-30 days calf	*C. parvum*	IIaA13G2R1
6	2	5-30 days calf	*C. parvum*	IIaA13G2R1
7	1	5-30 days calf	*C. parvum*	IIaA13G2R1
8	2	5-30 days calf	*C. parvum*	IIaA13G2R1
9	1	5-30 days calf	*C. parvum*	IIaA13G2R1
10	2	5-30 days calf	*C. parvum*	IIaA13G2R1
11	2	5-30 days calf	*C. parvum*	IIaA13G2R1
	14	5-30 days calf	*C. parvum*	IIaA13G2R1
	10	5-30 days calf	*C. parvum*	IIaA14G1R1
12	5	5-30 days calf	*C. parvum*	IIaA2R1
	1	5-30 days calf	*C. parvum*	IIaA5G1R1
	1	5-30 days calf	*C. parvum*	IIaA16R1
13	4	5-30 days calf	*C. parvum*	IIaA13G2R1
14	8	5-30 days calf	*C. parvum*	IIaA13G2R1
15	2	5-30 days calf	*C. parvum*	IIaA13G2R1
16	6	5-30 days calf	*C. parvum*	IIaA13G2R1
17	3	5-30 days calf	*C. parvum*	IIaA13G2R1
18	8	5-30 days calf	*C. parvum*	IIaA13G2R1
19	2	5-30 days calf	*C. parvum*	IIaA2R1
	3	5-30 days calf	*C. parvum*	IIaA14G1R1
20	3	5-30 days calf	*C. parvum*	IIaA13G2R1
21	2	5-30 days calf	*C. parvum*	IIaA13G2R1
	2	5-30 days calf	*C. parvum*	IIaA14G1R1
22	1	5-30 days calf	*C. parvum*	IIaA13G2R1
23	2	5-30 days calf	*C. parvum*	IIaA2R1
24	2	5-30 days calf	*C. parvum*	IIaA2R1

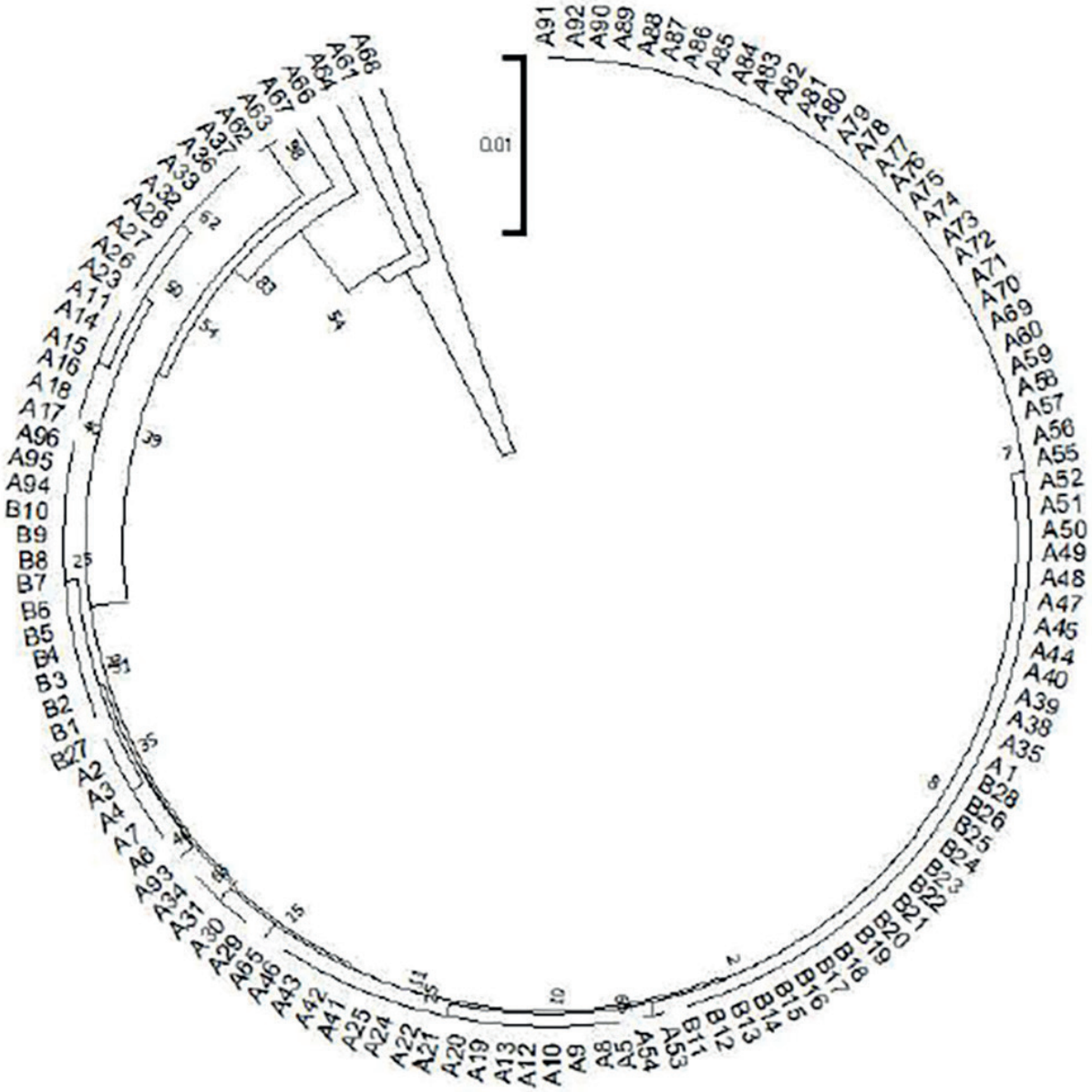

Figure 1. Phylogenetic tree based on the GP60 gene sequence. Each subtype is named along with the Genbank Accession number. The numbers represent the bootstrap values of the bootstrap replicates (parameter Tamura-3, 1000 replicates). Branch lengths show percentage difference in different subtypes

Italy, Netherlands, Poland, Portugal, Romania, Serbia and Montenegro, Slovenia and Spain, the species responsible for the infection in cattle was determined to be predominantly *C. parvum*, while the only species responsible reported for bovine cryptosporodiosis in Italy was *C. parvum* (26). In Türkiye, it has been reported that the species responsible for bovine cryptosporodiosis is most commonly *C. parvum* (24, 27). In our study, all samples for which we performed 18s SSU-rRNA analysis were identified as *C. parvum*.

In subtyping studies based on GP60 sequence analysis, the subtype family belonging to the species, is defined first. In the subtyping studies of *Cryptosporidium parvum*, the most common subfamily with the highest zoonotic potential was determined as IIa. Those with the highest potential to be zoonotic within the IIa subtype family of *C. parvum* are IIaA15G2R1, IIaA18G3R1, IIaA13G2R1, IIaA16G1R1, IIaA20G1R1, IIaA16G3R1, IIaA17G2R1, IIaA17G2R1, IIaA17G2R1, IIaA17G2R1 (28). While the most common

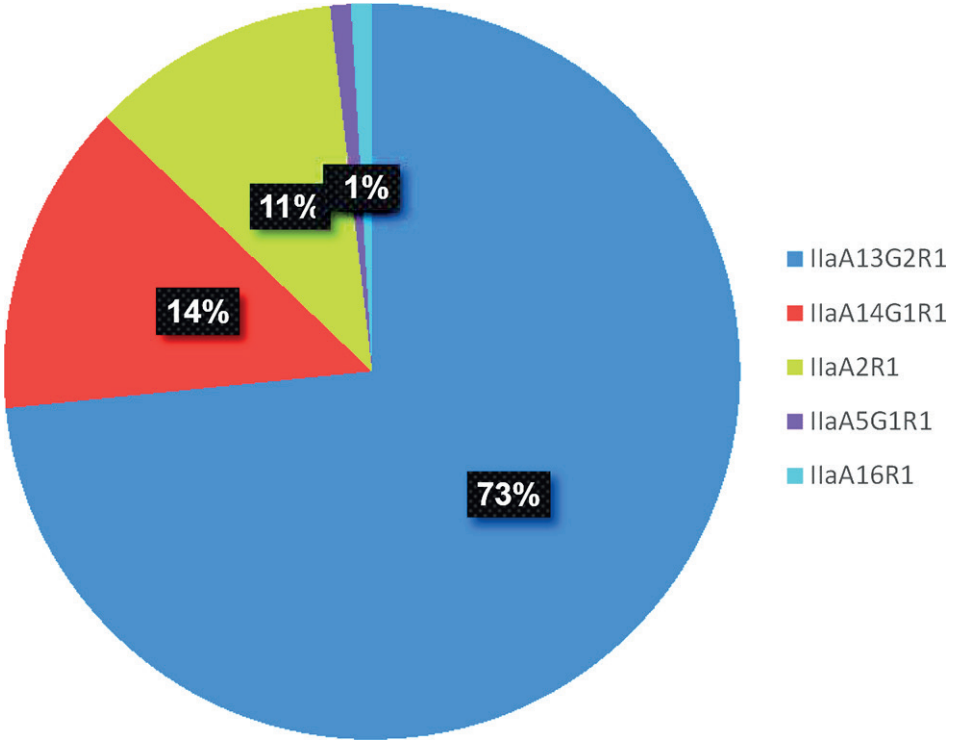

Figure 2. Subtype distribution in our study

subtype family was IIa in subtyping studies conducted in cattle in Türkiye, the most common subtype we found in our study (96/125 (77%)) to be IIaA13G2R1, with a very high zoonotic potential. In the compilation and meta-analysis study conducted by Chen *et al.* on the determination of subtypes in dairy cattle around the world, IIaA13G2R1 subtype with zoonotic character was detected in 187 (8.2%) of 2293 samples (28).

The most common subtype detected in our study (73%) was IIaA13G2R1 with a zoonotic character. Previous studies from Türkiye also showed high rates of this subtype in five provinces in Türkiye (Ankara, Kırıkkale, Çorum, Kırşehir and Balıkesir), with the rates of 87% in calves and among goat kids, where the rate of IIaA13G2R1 was 37.5% (27). In another two province of Türkiye, Burdur and Kayseri, IIaA13G2R1 subtype was also most common with the rate of 88% in calves and heifers (24). In Konya province of Türkiye, IIaA13G2R1 was detected in 100% of the studied calves (29). Another study from Konya province with higher numbers of samples indicated that IIaA13G2R1 subtype was 55.5% in calves and 23% in lambs (30). In our study, the rates of IIaA13G2R1 subtype were 100% in Burdur province, and 63.8% in Aydın. However, in other regions of the world *C. parvum* subtype IIaA13G2R1 was not found to be so common. In a multicenter study covering Belgium, France and the Netherlands, it was found to be 20.5% in Belgium, 6.4% in the Netherlands and 0% in France (31). In Germany, IIaA13G2R1 subtype was reported to be only 0.4% (32).

In Aydın we found IIaA14G1R1 as the second most common subtype (14%). Other studies done in Türkiye also reported high rates of IIaA14G1R1 subtype with 22.3% in Diyarbakır province (33), and with 12% in Burdur and Kayseri provinces (24). European countries subtype IIaA14G1R1 rates were also low, 1.64% in Estonia, 2.9% in Austria, 2% in Poland, 4,2% in Netherlands, except in Cyprus which was 54% (27)/50)) (31, 34, 35, 36). The IIaA14G1R1 subtype is important because it is one of the most frequently detected subtypes in humans and is considered as zoonotic (37).

Two subtypes detected in present study, IIaA2R1 and IIaA5G1R1 subtypes, were reported for the first time in the world. More studies need be done to evaluate the dissemination of these two subtypes.

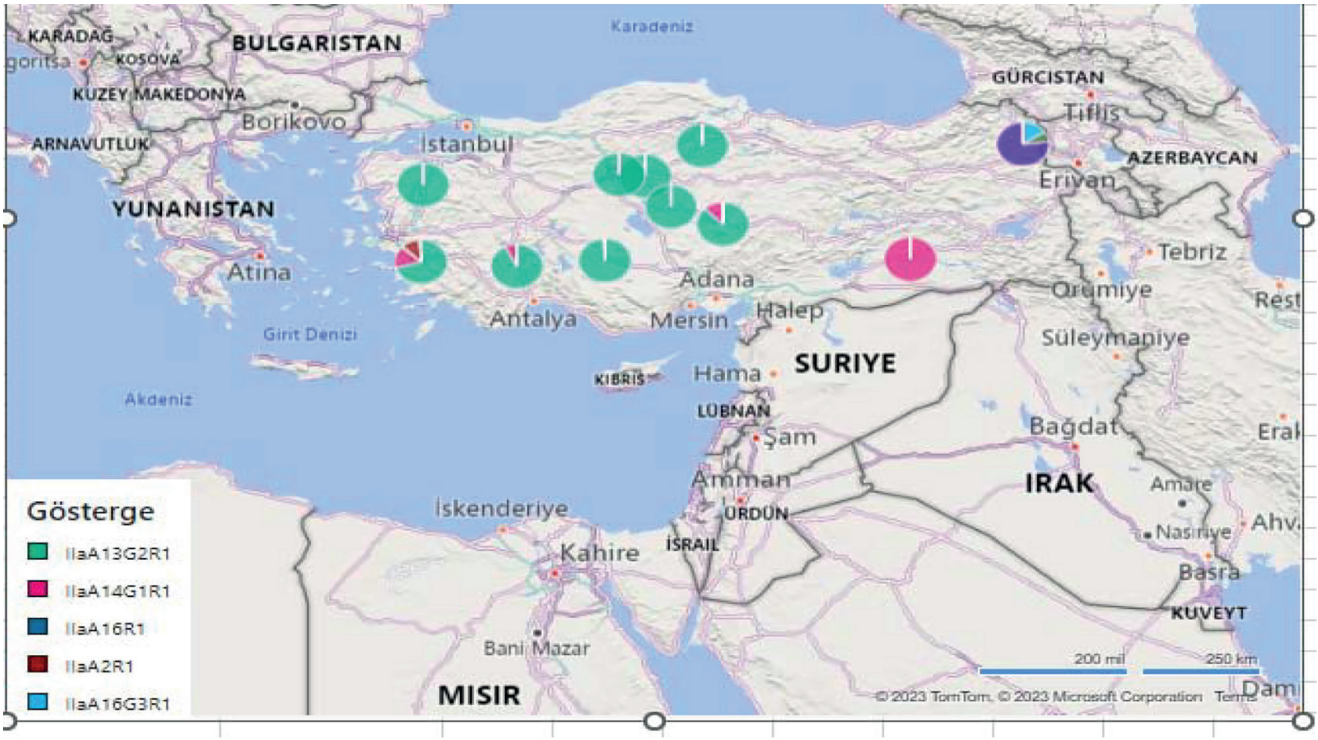

Figure 3. *Cryptosporidium parvum* subtyping in ruminants in Türkiye (24,27,29,30,33,43)

The most common subtype in the world is IIaA15G2R1. Subtype IIaA15G2R1 is the most common subtype, with 87.5% in the United States (38), 87.5% in France (39), 93% in Korea (40), 80% in Austria (35), 53.6% in Uruguay (41) and 50% in Israel (42). In a multicenter study covering Belgium, France and the Netherlands, it was found to be 67.8% in Belgium. 89.4% in the Netherlands and 65.2% in France (31). However in our study none of the samples presented with this subtypes.

CONCLUSION

The number of studies on *Cryptosporidium parvum* subtyping in Türkiye is limited (Figure 3). With our study, the first subtyping study was carried out among cattle in Aydın province. The IIaA2R1 and IIaA5G1R1 subtypes that we detected in our study were defined for the first time in the world and IIaA16R1 in Turkey.

Data availability

All data generated or analyzed during this study are included in this article. Sequences are deposited in GenBank under accession numbers OR077152-OR077156.

Funding

The authors declare that no funds, grants, or other support were received during the preparation of this manuscript.

Author contributions

All authors certified that they have participated to take public responsibility for the content including the manuscript's concept, analysis, writing, and revision.

Conceptualization, methodology and data analysis, writing original draft, review, and editing, resources, Z.E.A. Authors has read and accepted the published version of the article.

Declarations

Competing interests

The authors declare no competing interests.

Conflict of interest

The authors declare no competing interests.

Ethics approval and consent to participate

For this study, necessary documents were obtained from the Animal Experiments Local Ethics Committee of Aydın Adnan Menderes University (ADÜHADYEK 64583101/2017/082).

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