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Original article

First report of *Blastocystis* subtype ST25 in calves in Turkey

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Abstract

Blastocystis spp. is a parasite that causes intestinal infection in humans and other animals. A few studies have been performed in Turkey on the distribution of *Blastocystis* in cattle. In this study, fecal samples were collected from 100 calves and subjected to analysis based on an SSU rRNA gene fragment. The overall prevalence of the disease was determined as 15% (15/100). This rate was 14.04% for females and 16.28% for males. In addition, three *Blastocystis* subtypes were identified: ST10, ST14, and novel subtypes ST25. To our knowledge, the ST25 subtype was reported with this study for the first time in Turkey. The nucleotide sequences (OM920832-OM920839) obtained in this study were deposited in GenBank. The results obtained will be useful for a better understanding of the epidemiology of *Blastocystis* spp., and its effects on public health.

Keywords: *Blastocystis*, calf, molecular analysis, phylogeny, ST25, Turkey

Introduction

Blastocystis spp., is an enteric anaerobic protozoan parasite found in humans, various animals (such as cattle, pigs, poultry), and the environment (Duda et al. 1998, Popruk et al. 2013, Abuodeh et al. 2019, Nemati et al. 2021). *Blastocystis* spp., which is possibly the most common human intestinal parasite with an estimate of one billion infections worldwide (Maloney et al. 2019), was first identified in 1911 and was named “*Blastocystis enterocola*” (Eroğlu 2015). The name was later updated as *Blastocystis hominis* in 1912 (Popruk et al. 2013, Eroğlu 2015). *Blastocystis* species have been given different names for many years when isolated

from humans and animals, such as *B. hominis* and *B. ratti* (Malatyali and Özçelik 2011, Eroğlu 2015). Later a decision was made to use the naming system “*Blastocystis* sp. subtype nn” for the species isolated from humans and animals since genetic studies have shown that there is only one species of *Blastocystis* spp. that infects humans and animals (Malatyali and Özçelik 2011, Popruk et al. 2013, Eroğlu 2015).

Blastocystis spp. has various morphological forms (vacuolar, granular, amoeboid, cyst, multivacuolar and avacuolar) that can be found in fecal samples (Stenzel and Boreham 1996, Popruk et al. 2013). The fecal cyst is the only environmentally resistant infectious form among all forms (Hemalatha et al. 2014). Although the

life cycle of *Blastocystis* spp. is not fully understood (Tan 2008, Popruk et al. 2013), it is usually transmitted via the fecal-oral route, especially through the consumption of infectious cysts taken with contaminated water or food (Moura et al. 2018, Tavur and Önder 2022).

The pathogenicity of *Blastocystis* spp. is both uncertain and controversial (Li et al. 2018, Abuodeh et al. 2019). Pathogenicity is reported to vary depending on the subtype of the parasite and the host's immune status (Aynur et al. 2019). Only a limited number of studies conducted in different regions of the world report that the disease has zoonotic potential (Tan 2008, Abuodeh et al. 2019). It is suggested that the organism is zoonotic, but there is insufficient evidence to either support or refute this suggestion (Duda et al. 1998). Studies have shown a higher prevalence among animal handlers compared to individuals who are not normally in contact with animals (Tan 2008, Abuodeh et al. 2019). *Blastocystis* spp. is encountered in people of various age groups (Moura et al. 2018). Infected people may remain asymptomatic, or may exhibit gastrointestinal symptoms such as abdominal pain, diarrhea, nausea, vomiting, bloating, and anorexia (Dagci et al. 2014, Kamaruddin et al. 2020).

Blastocystis spp. has many genetically different subtypes although they cannot be distinguished morphologically from each other (Tan 2008). To determine the subtype differences between human and animal species, the most commonly used method today is the detection of gene regions on small-subunit rRNA (SSU rRNA) using the PCR method with STS (Sequence-Tagged Site) primers (Yoshikawa et al. 2016).

Genetic studies on *Blastocystis* spp. isolated from cattle in Turkey are very limited. This study, therefore, aimed to determine the molecular prevalence and subtypes of *Blastocystis* spp., in calves in the Siirt province of Turkey.

Materials and Methods

The study area and sample collection

This study was carried out in the Kurtalan and Şirvan districts of Siirt province in the Southeastern Anatolia Region of Turkey (37°55'38" N, 41°56'37" E). Fecal samples were collected from a total of 100 calves (<15 months), distributed as 43 males and 57 females. Fecal samples were placed in individual specimen containers and gender, age, fecal type, and location information for the samples were recorded. All samples were brought to the laboratory following the cold chain rules.

DNA extraction

DNA extraction was performed in all stool samples using the Gene Matrix Stool DNA Purification Kit (EURx, Poland), following the manufacturer's protocol. The DNA isolates obtained were stored at -20°C until further use.

PCR amplification

All extracted DNA samples were analyzed by PCR amplification of 500 bp of the SSU rRNA gene of *Blastocystis* spp. with the primers Forward Blast (5' -GGAGGTAGTGACAATAAA TC-3') and Reverse Blast (5' -TGCTTTTCGCACTTGTTTCATC-3') (Santín et al. 2011). The PCR mixture (25 µl) contained 1.25 units of HOT FIREPol DNA polymerase (Solis BioDyne, Estonia), 1X PCR buffer (Solis BioDyne, Tartu, Estonia), 10 pmol Forward Reverse primers, 2 µl genomic DNA, 1.5 mM MgCl₂, and nuclease-free water up to the desired volume. The PCR was initiated at 95°C for 15 min, followed by 35 cycles of 95°C for 30 s, 54°C for 30 s, and 72°C for 30 s, with a final extension at 72°C for 5 min. PCR products were subjected to 1.5% agarose gel (BioShop, Canada) electrophoresis and visualized by staining with RedSafe™ Nucleic Acid Staining Solution (iNtRON Biotechnology, Inc., Korea).

Sequence and phylogenetic analysis

The bidirectional sequence was applied to eight of the positive PCR samples (BM Labosis, Ankara, Turkey). The nucleotide sequences obtained were compared with the reference sequences in GenBank by BLAST search to determine the *Blastocystis* subtypes. The nucleotide sequences obtained in this study have been deposited in GenBank under accession numbers OM920832 - OM920839. Phylogenetic reconstruction based on the SSU rRNA gene of *Blastocystis* spp. was performed using the Neighbor-Joining method (NJ) (Saitou and Nei 1987) with 1000 replicate bootstrap values. The evolutionary distances were computed using the Maximum Composite Likelihood method (Tamura et al. 2004). Evolutionary analyses were conducted in MEGA11 software (Tamura et al. 2021).

Statistical analysis

The data obtained in the study were analyzed using the SPSS V16.0 (IBM, Chicago, IL, USA) program. The relationship between grouped variables was calculated using the chi-square test. The difference was considered statistically significant when $p < 0.05$.

Table 1. *Blastocystis* spp. subtypes determined from cattle around the world.

Country	Determined Subtypes	References
Malaysia	ST1, ST3, ST4, ST5, ST10, ST14	(Kamaruddin et al. 2020)
Denmark	ST5, ST10	(Stensvold et al. 2009)
Libya	ST5, ST10, ST14, Mixed	(Alfellani et al. 2013)
Japan	ST1, ST3, ST6	(Yoshikawa et al. 2004)
Arabia	ST10	(Abuodeh et al. 2019)
China	ST4, ST5, ST10, ST14	(Zhu et al. 2017)
Korea	ST1, ST5, ST10, ST14	(Lee et al. 2018)
Spain	ST5, ST10	(Abarca et al. 2021)
England	ST1, ST5, ST10, Mixed	(Alfellani et al. 2013)
Colombia	ST1, ST3	(Ramírez et al. 2014)
USA	ST3, ST4, ST5, ST10, ST14, ST17, ST21, ST23, ST24, ST25, ST26	(Maloney et al. 2019)
Turkey	ST10, ST14	(Aynur et al. 2019, Önder et al. 2021, Tavour and Önder 2022)
Turkey	ST10, ST14, ST25	This study

Table 2. Prevalence and distribution of *Blastocystis* spp. according to age, gender, stool status, and location of calves.

Categories	(n)	Positive		p value	
		(n)	(%)		
Age (month)					
≤3	49	8	16.33	0.935	NS
4-12	36	5	13.89		
>12	15	2	13.33		
Gender					
Female	57	8	14.04	0.756	NS
Male	43	7	16.28		
Fecal type					
Diarrheic	50	6	12.00	0.401	NS
Non-diarrheic	50	9	18.00		
Location					
Kurtalan	46	6	13.04	0.613	NS
Şirvan	54	9	16.67		
Overall	100	15	15		

NS: Non-significant

Ethical approval

This study was approved by the Animal Experiments Local Ethics Committee of Siirt University (Decision number: 20220102).

Results

Prevalence of *Blastocystis* spp. in Animal Hosts

Of the 100 fecal samples, 15 (15%) were determined to be *Blastocystis*-positive by PCR analysis

of the SSU rRNA gene (Table 1). The prevalence of *Blastocystis* spp. infection related to age, gender, fecal type, and location of calves is presented in Table 2. The highest positivity was found in the under three months of age group (16.33%), in the male group (16.28%), in the non-diarrheic group (18.00%), and the Şirvan district group (16.67%). There was no statistically significant difference between the groups ($p > 0.05$).

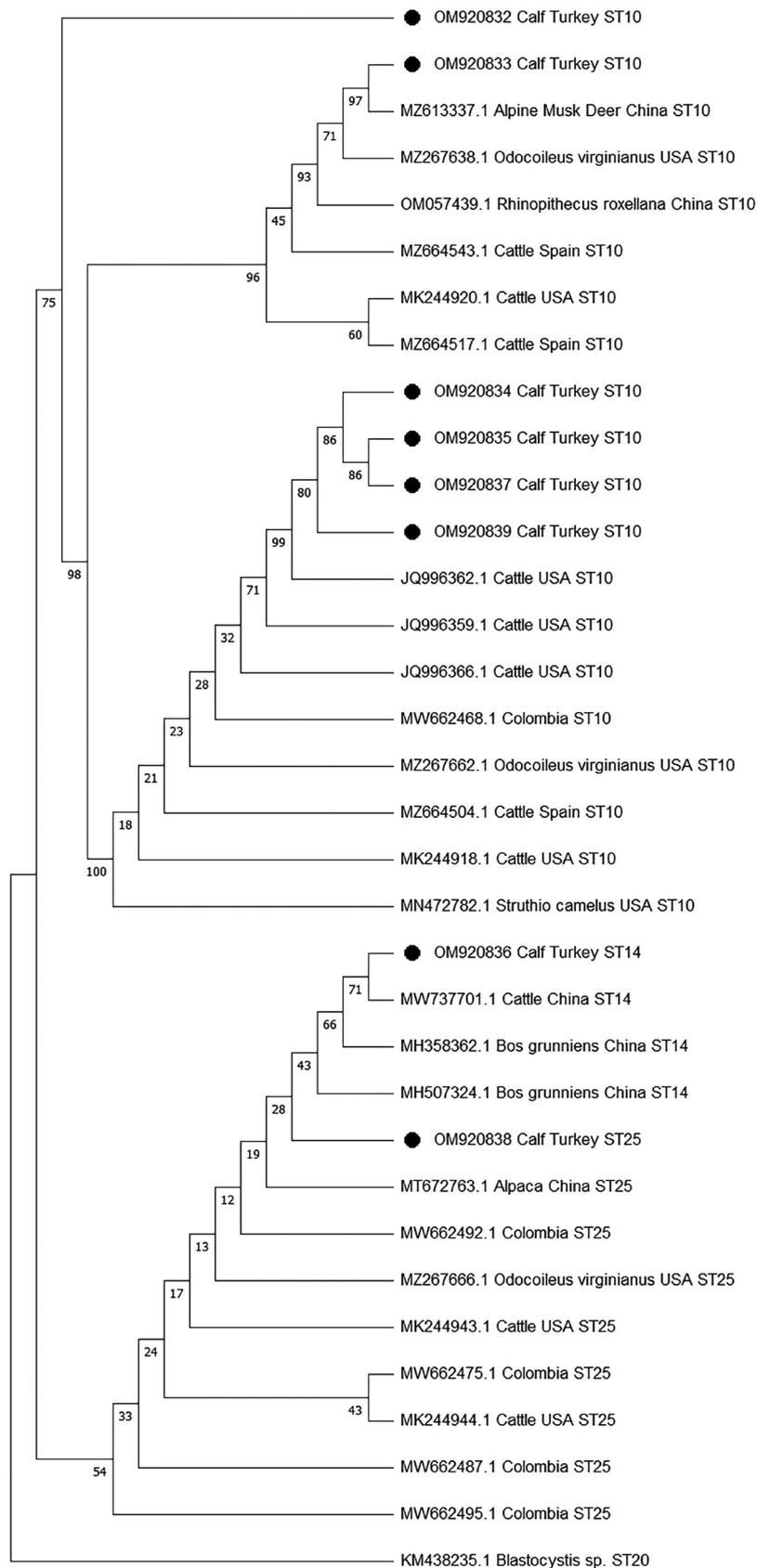


Fig. 1. Phylogenetic relationships of *Blastocystis* spp. isolates, using Neighbor-Joining method (NJ) analysis based on SSU rRNA gene region. Numbers at the nodes represent the Bootstrap values (1000 replicates). *Blastocystis* spp. ST20 (KM438235) was used as an outgroup.

Subtypes of *Blastocystis* isolates

Eight of the positive samples were successfully subtyped using sequence analyses of the SSU rRNA gene. Three subtypes were identified using BLAST search and sequence analysis of the SSU rRNA gene, including ST10 (OM920832-35,37,39), ST14 (OM920836), and a novel subtype, ST25 (OM920838).

Phylogenetic results

The isolates obtained as a result of the sequencing were determined to be ST10, ST14, and ST25. Phylogenetic analysis revealed that all sequences from calves were closely associated with animal-derived sequences in GenBank, and subtype-based clustering was observed in the NJ tree (Fig. 1).

It was determined that the sequences of ST10 from this study (OM920834, OM920837, OM920839) were 100% identical to the sequences of cattle isolates from the USA (MK244918.1, JQ996366.1, JQ996362.1, JQ996359.1) and Spain (MZ664504.1). The sequence with accession number OM920835 showed 99.79% homology to cattle isolate (MK244918.1, MZ664504.1) from the USA, and the sequence with accession number OM920833 showed 99.07% homology to the Alpine Musk Deer isolate (MZ613337.1) and *Rhinopithecus roxellana* isolate (OM057439.1) from China. In addition, the sequence with accession number OM920832 was 92.24% identical to cattle isolate (MZ664543.1, MZ664517.1) from Spain.

It was found that the ST14 (OM920836) sequence had 99.39% similarity to cattle isolate (MW737701.1), 99.35% similarity to *Bos grunniens* isolate (MH507324.1), and 98.54% similarity to *Bos grunniens* isolate (MH358362.1) from China.

The ST25 (OM920838) sequence was found to be 100% identical with sequences from cattle (MK244943.1), *Odocoileus virginianus* (MZ267666.1) from the USA, and Colombia (MW662492.1). This sequence was also found to have 99% similarity with cattle isolate from the USA (MK244944) and Alpaca isolate from China (MT672763).

Discussion

Blastocystis spp. is a common intestinal protozoan with uncertain pathogenicity (Deng et al. 2021) and is common all over the world (Popruk et al. 2013). This parasite is estimated to colonize between one and two billion people worldwide according to epidemiological research (Deng et al. 2021). It is suggested that the organism is zoonotic, but there is insufficient evidence to support or disprove this claim (Daryani et al. 2008).

The prevalence of *Blastocystis* varies significantly depending on geographical location and host type (Ruau and Stang 2014). In studies conducted on cattle, the prevalence was reported as 21.4% in Brazil (Moura et al. 2018), 22.7% in Arabia (Abuodeh et al. 2019), 32.1% in Spain (Abarca et al. 2021), 10.3% in China (Zhu et al. 2017), 6.7% in Korea (Lee et al. 2018), 80% in Colombia (Ramírez et al. 2014), 34.5% and 43.8% in Malaysia (Hemalatha et al. 2014, Kamaruddin et al. 2020), 71% in Japan (Abe et al. 2002) and 33% in calves in Italy (Gabrielli et al. 2020).

Genetic studies on *Blastocystis* spp. isolated from cattle in Turkey are very limited. In the studies conducted, varying ratios of prevalence such as 16% (Onder et al. 2021), 11.25% (Aynur et al. 2019) and 58.7% (Tavur and Önder 2022) were reported.

Microscopic methods such as native-lugol and trichrome staining (Ertuğ et al. 2015), culture and molecular methods (PCR) are used in the diagnosis of *Blastocystis* spp. (Stensvold et al. 2012, Popruk et al. 2013). It is reported that the PCR protocol is more sensitive and specific compared to the microscopic examination and culture method (Malatyali and Özçelik 2011, Stensvold et al. 2012, Popruk et al. 2013), and PCR-based molecular diagnostic tools are widely used to determine the genetic diversity of *Blastocystis* spp. in different host species (Tavur and Önder 2022).

In this study, 15 (15%) of 100 calf stool samples were found to be *Blastocystis* positive according to PCR analysis of the SSU rRNA gene. The results of this study are similar to the results reported by other researchers (Zhu et al. 2017, Aynur et al. 2019, Onder et al. 2021).

The reasons for the differences between the studies include geographical location, sampling season, animal species, number of animals, animal age, immune status, care, nutritional conditions, stress and methods used (Zhu et al. 2017, Lee et al. 2018).

Although the prevalence of the disease in humans varies according to geographical region, it is reported that it is nonetheless higher in developing countries than in developed countries. This difference is attributed to differences in hygiene standards, waste disposal methods, exposure to infected animals, and consumption of contaminated food or water (Popruk et al. 2013, Eroğlu 2015, Abuodeh et al. 2019, Nemati et al. 2021).

Blastocystis spp. shows a wide genetic diversity. It has been reported that 26 subtypes (ST) have been defined in animals so far and ST10 is the most common subtype in cattle in the world (Aynur et al. 2019, Gabrielli et al. 2020). It was reported that two subtypes, ST10 and ST14, were detected in cattle in the southwest of Turkey and ST14 was more common (Aynur et al. 2019), ST10 was detected in cattle and sheep in the

Central Anatolia and Central Black Sea Region (Onder et al. 2021), and in a different study conducted also in the Central Anatolia Region, the ST10 subtype was determined in cattle (Tavur and Önder 2022).

ST1, ST2, ST3, and ST4 cause more than 90% of *Blastocystis* spp. infections in humans (Alfellani et al. 2013). ST1, ST2, and ST4 have low host specificity among these subtypes and are probably zoonotic infections (Parkar et al. 2010). Studies conducted in Turkey reported that ST1, ST2, ST3, ST4, ST6, and ST7 subtypes were detected in humans (Özyurt et al. 2008, Dagci et al. 2014, Sankur et al. 2017).

ST10 and ST14 subtypes, as well as ST25, were detected in this study, similar to the studies conducted on cattle in Turkey (Aynur et al. 2019, Önder et al. 2021, Tavur and Önder 2022). As far as our literature survey reveals, the ST25 subtype is reported for the first time in Turkey. In this study, as a result of the sequence of 8 PCR positive samples, it was determined that 6 (75%) samples were ST10, 1 (12.5%) sample was ST14, and 1 (12.5%) sample was ST25. The results of this study showed the presence of three non-zoonotic subtypes (ST10, ST14, and ST25) among cattle in Turkey.

Different prevalences were detected between the genders in the studies. Some researchers report that the rate is higher in males (Daryani et al. 2008, Lee et al. 2018), while others report that it is higher in females (Duda et al. 1998, Kamaruddin et al. 2020). In this study, the findings of the researchers (Daryani et al. 2008, Lee et al. 2018) were supported by the finding that the prevalence was higher in males (16.28%) than in females (14.04%). There was no statistical significance between the groups, however.

In studies conducted on cattle, Kamaruddin et al. (2020) reported a higher prevalence in those younger than 3 months of age, while Lee et al. (2018) reported a higher prevalence in the 3–12-month age group. Both researchers reported that there was a statistically significant difference between the age groups. It was reported that the prevalence was higher in adults compared to juveniles in a study conducted on dogs by Daryani et al. (2008). In a study conducted by Duda et al. (1998), it was reported that there was no statistically significant difference between cat and dog age groups. In this study, the highest prevalence was detected in animals younger than 3 months of age, but no statistical significance was found. These results are similar to the data of the researchers Duda et al. (1998), Daryani et al. (2008), Lee et al. (2018), and Kamaruddin et al. (2020) ($p > 0.05$).

In the studies conducted by Lee et al. (2018) and Kamaruddin et al. (2020), a higher prevalence was detected in the groups without diarrhea compared to the

groups with diarrhea, and the difference was found to be statistically significant ($p < 0.01$, $p < 0.05$). In this study, similar to the results reported by Lee et al. (2018) and Kamaruddin et al. (2020), a higher prevalence was found in the diarrhea-free group (18.00%) but here the difference was not statistically significant ($p > 0.05$).

Conclusion

This study sheds light on the molecular characterization and subtype distribution of *Blastocystis* spp., in calves in Siirt province. The three subtypes identified as part of this study do not have zoonotic properties. However, the obtained results reveal the potential risk of zoonotic transmission of *Blastocystis* spp. for calves and indicate that it can serve as an invasion reservoir for humans and other animals. Such animals may be a source of invasion for human *Blastocystis* spp. through direct contact or contamination of the water sources. Further studies are needed to determine the zoonotic subtype potential of the agent in the region.

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