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**RESEARCH ARTICLE**

# Molecular Prevalence and Phylogenetic Characterization of *Blastocystis* in Cattle in Kayseri Province, Turkey

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## ABSTRACT

*Blastocystis* is one of the most common emerging zoonotic parasites in humans and animals. This study aimed to determine the molecular prevalence and subtype of *Blastocystis* in cattle (*Bos taurus*). A total of 150 fresh fecal samples were collected from slaughtered cattle from various slaughterhouses in Kayseri Province, Central Anatolia. Genomic DNA was extracted from all samples and used in PCR analyses of the small subunit ribosomal RNA (SSU rRNA) gene of *Blastocystis*. *Blastocystis* positive samples were sequenced for identify subtypes. Obtained sequences were assembled with suitable genetic software, then phylogenetic relationships were revealed. According to PCR analyses, overall prevalence of *Blastocystis* was determined as 58.7%. The sequence analyses of the PCR product gene revealed the presence of one known livestock-specific subtype, ST10. Phylogenetic analysis revealed that ST10 isolates the characterized in the study were clustered with isolates identified previously from cattle. Molecular characterization and subtype of *Blastocystis* sp. in slaughtered cattle in slaughterhouses were obtained data with this study.
**Keywords:** *Blastocystis*, Cattle, Molecular Prevalence, Phylogenetic Characterization, Subtype

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## Kayseri Yöresinde Sığırlarda *Blastocystis*'in Moleküler Prevalansı ve Filogenetik Karakterizasyonu

## ÖZ

*Blastocystis* insanlarda ve hayvanlarda en yaygın bulunan zoonotik parazitlerden biridir. Bu çalışmada, sığırlarda (*Bos taurus*) *Blastocystis*'in moleküler prevalansı ve alt tiplerinin belirlenmesi amaçlanmıştır. Kayseri yöresinde bulunan çeşitli kesimhanelerde kesilen toplam 150 sığırdan taze dışkı örnekleri toplanmıştır. Dışkı örneklerinden genomik DNA ekstraksiyonu yapılmış ve DNA örnekleri *Blastocystis*'in small subunit ribosomal RNA (SSU rRNA) geninin amplifikasyonunda kullanılmıştır. Tüm PCR pozitif ürünler alt tiplerin belirlenmesi için sekanslanmıştır. Elde edilen sekanslar uygun genetik yazılımlarla işlenerek genotipik yapıları ve sonrasında da filogenetik ilişkileri ortaya çıkarılmıştır. İncelemesi yapılan örneklerin SSU rRNA geninin PCR analizleri ile *Blastocystis*'in ortalama prevalansı %58,7 olarak belirlenmiştir. *Blastocystis* SSU rRNA geninin PCR ürünlerinin sekans analizleri sonucunda izolatların ruminant spesifik alt tip, ST10, içerisinde olduğu tespit edilmiştir. Filogenetik analizler çalışmada karakterize edilen ST10 izolatlarının daha önce sığırlarda identifiye edilen izolatlarla birlikte kümelendiğini ortaya koymuştur. Bu çalışma ile mezbahada kesilen sığırlarda *Blastocystis* sp.'nin moleküler karakterizasyonu ve alt tipi üzerine veriler elde edilmiştir.
**Anahtar Kelimeler:** Alt tip, *Blastocystis*, Filogenetik Karakterizasyon, Moleküler Prevalans, Sığır

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## INTRODUCTION

*Blastocystis* are one of the most common anaerobic unicellular protozoan parasites that infect humans and many animals, including companion, wild, and domestic animals (Lee et al. 2019; Ren et al. 2019). Generally, *Blastocystis* sp. is transmitted by the fecal-oral route, especially via the consumption of infectious cysts from contaminated water or food (Ramírez et al. 2016; Lee et al. 2018).

The species are morphologically and genetically polymorphic protozoon (Yoshikawa et al. 2016; Maloney et al. 2019). Therefore, PCR-based molecular diagnostic tools have been widely used to identify the genetic diversity of *Blastocystis* in the different host species. To date, based on sequences analyses of the full length small subunit (SSU) rRNA gene in humans and different animal species, 22 subtypes (STs, ST1-ST17, ST21, and ST23-26) have been identified (Alfellani et al. 2013; Zhao et al. 2017; Maloney et al. 2019; Stensvold and Clark, 2020). Among them, ST-1 to ST-8 and ST-12 have been identified in both humans and animals, however, ST9 has been determined only in humans (Clark et al. 2013; Ramírez et al. 2016; Stensvold and Clark, 2016). ST10,11,13-17, 21, and 23-26 were only found in animals (Stensvold et al. 2009; Parkar et al. 2010; Valença-Barbosa et al. 2019; Stensvold and Clark, 2020). Recently, various studies have shown that *Blastocystis* is detected among people who have close contact with animals such as animal handlers, namely zoo-keepers, research institutions, and abattoir workers (Salim et al. 1999; Abe et al. 2002; Parkar et al. 2010; Li et al. 2018). Thus, there is reported that close contact with infected animals may be important for zoonotic transmission of *Blastocystis* infection (Lee et al. 2018).

*Blastocystis* infection is one of the important and common parasitic diseases causing acute or chronic gastrointestinal symptoms in humans in Turkey (Beyhan et al. 2015). The epidemiological status and molecular characterization of *Blastocystis* in cattle are somewhat limited. Only two studies have been done on a molecular survey of this parasite in cattle in Turkey (Aynur et al. 2019; Onder et al. 2021). In these studies, ST10 and ST14 subtypes were reported in cattle (Aynur et al. 2019; Onder et al. 2021). However, no information is present on the molecular characterization of *Blastocystis* in slaughtered cattle in slaughterhouses. Therefore, this study aimed to determine the genetic characterization and subtype distribution of *Blastocystis* in cattle that were brought for slaughter from different regions of Turkey.

## MATERIAL AND METHODS

### Study area and collection of fecal samples

For present study, Ethics Committees of Animal Experiments dated 05.06.2009 and "Research scope made to animals for diagnosis and treatment purposes other than, clinical applications, studies with dead animal or dead animal tissue, slaughterhouse materials, waste fetuses, milking, collection of feces or litter samples, blood collection, sampling with swap etc. ethics committee approval was not obtained based on the decision no 12.

This study was conducted on various cattle abattoirs located in Kayseri province. These abattoirs provide the beef requirements of the inhabitants of Turkey. The cattle were brought for slaughter from different regions of Turkey. The daily cattle slaughter in the slaughterhouse is 100-150 head/day. The slaughterhouses were visited several times between February and April 2019.

In total, 150 fresh fecal samples were collected from slaughtered cattle. Fecal samples were taken from the intestines of the slaughtered cattle around 7:00-9:00 am following the slaughtering process. Each fecal sample was placed in separate plastic containers, labeled with the age and breed, and date of collection. All samples were transported to the laboratory on ice packs and stored at 4°C until DNA extraction.

### DNA extraction and PCR amplification

Total DNA was extracted from each fecal sample by using the QIAamp® Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany) implementing the manufacturer's protocol. All extracted genomic DNA (gDNA) was eluted in 50 µL of AE elution buffer and stored at -20°C until further PCR analyses.

All gDNA samples were examined with PCR analyses to determine of the *Blastocystis* by amplifying a ~600 bp fragment of the SSU rRNA gene using the primers RD5 (5-ATCTGGTTGATCCTGGCCAGT-3) and BhRDr (5-GAGCTTTTTAACTGCAACAACG-3) (Ramírez et al. 2014). Each PCR reaction solution was carried out in a 25 µl total volume, a mix containing 12.5 µl Dream Taq Hot Start PCR Master Mix (Thermo Fisher Scientific, Waltham, MA, USA), 2 µl of genomic DNA (10-30 ng/µL), 8 µl distilled water, and 1.25 µl forward and reverse primers (10 pmol/µl). The PCR amplifications were performed an initial denaturation at 95°C for 3 min, followed by 35 cycles at 95°C for 30 sec, 56°C for 30 sec, and 72°C for 30 sec, and a final extension at 72°C for 5 min in a C1000 Thermal Cycler (Bio-Rad, Hercules, CA, USA). For each PCR amplification, *B. hominis* positive DNA samples and distilled water were used as a positive and negative control, respectively. The PCR product (10µl) was analyzed by 1.5% agarose gels electrophoresis with ethidium bromide-stained for 30 min at 135 V. Bands was visualized by Fusion FX Gel Documentation System (Vilber Lourmat, Collégien, France).

**Nucleotide sequencing and phylogenetic analysis**
All PCR positive amplicons were purified from agarose gel using a High Pure PCR Product Purification Kit (Roche, Mannheim, Germany). The gel-purified products were sequenced bidirectionally with the same PCR primers (Macrogen, the Netherlands). The obtained chromatograms were analyzed to obtain the consensus sequence with the Geneious Prime 2020.0.3 software (https://www.geneious.com). These consensus sequences were assembled and edited using the Geneious Prime 2020.0.3 software. The resulting SSU rRNA gene sequences were compared with the reference sequences available in the GenBank database using BLASTn, to determine the subtypes of the *Blastocystis* sp. The haplotype and nucleotide diversity were calculated using DnaSP software version 5.10.01 (Librado and Rozas, 2009).

A phylogenetic analysis of all nucleotide sequences obtained in this study was carried out together with reference sequences obtained from GenBank. Phylogenetic reconstruction based on the SSU rRNA dataset of *Blastocystis* was utilized by the Maximum Likelihood method (ML) with 1000 replicates bootstrap values, using the Mega 7 software (Kumar et al. 2016). The best-fit DNA-substitution model for ML based on the lowest Akaike information criterion (AIC) algorithm was selected as HKY+G + I using jModeltest v.0.1.1 (Posada, 2008). The nucleotide sequences obtained in the study were deposited in the GenBank database under accession numbers MK966392-94.

**Statistical Analysis**
The statistical differences in the prevalence of *Blastocystis* between age group and breed were analyzed by a Chi-square test with the software SPSS 21. The differences were considered statistically significant when $p<0.001$.

## RESULTS

**Molecular prevalence of *Blastocystis* sp. in cattle**
Of 150 cattle, 88 (58.7%) were *Blastocystis*-positive according to PCR analyses of the SSU rRNA gene. The prevalence of *Blastocystis* infection related to age and breed of cattle is presented in Table 1.

**Table 1.** Distribution and statistical analysis of cattle with *Blastocystis* positivity according to age and breed groups

| Parameters | No. of samples examined | No. of positive (%) | *p-value* |
|---|---|---|---|
| **Age groups** | | | |
| 1-3 years | 72 | 31 (43.0) | 0.000[a] |
| >3 years | 78 | 57 (73.0) | |
| **Breed group** | | | |
| Holstein | 35 | 19 (54.3) | 0.921 |
| Brown Swiss | 53 | 34 (64.2) | |
| Simmental | 12 | 7 (58.3) | |
| Crossbreed | 24 | 13 (54.2) | |
| Aberdeen-Angus | 18 | 11 (61.1) | |
| Limousin | 8 | 4 (50.0) | |
| **Total** | 150 | 88 (58.7) | |

a: statistically significant ($p<0.001$)

The highest prevalence rate (73.0%) was detected in adult cattle 3 years old and older. However, the cattle between 1 and 3 years old were observed low infection prevalence (43.0%). Statistically, significant difference ($p<0.001$) was determined between infection with *Blastocystis* and >3 years adult cattle. In addition, the lower infection was detected in Limousin and Simmental breeds, respectively, than in other breeds. There was no significant difference ($p>0.001$) between breeds of cattle and *Blastocystis* infection. Due to all slaughtered cattle were males, the analysis did not provide any results in terms of the gender of the cattle.

**Nucleotide sequence and phylogenetic analyses**

The final length of the contig nucleotide sequences of the SSU rRNA gene region were 568 bp. No intraspecific nucleotide differences were detected in the sequence analyses of partial SSU rRNA gene region. Nucleotide sequence analyses of the 88 SSU rRNA-positive samples revealed the presence of subtype ST10. Our sequences showed 99.47-100% identity to that of the SSU rRNA sequences of *Blastocystis* recorded previously from the cattle (Fig.1).

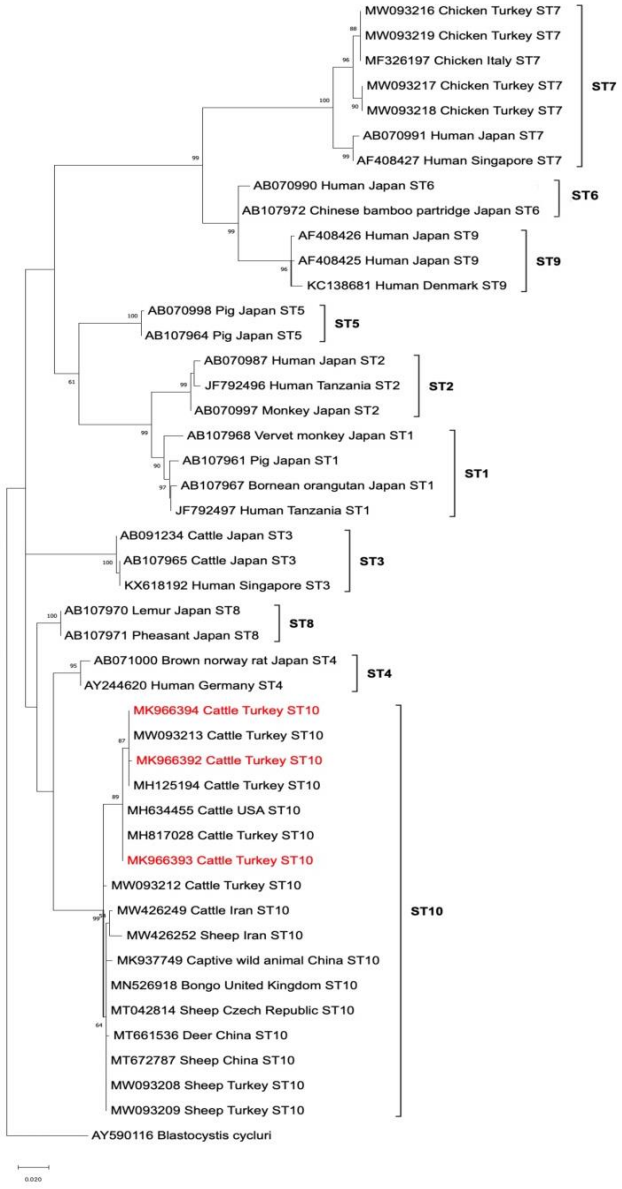


**Fig. 1.** Phylogenetic relationships between our *Blastocystis* (MK966392-94) isolates and other *Blastocystis* isolates as inferred by maximum likelihood obtained from SSU rRNA gene. Numbers at the nodes represent the Bootstrap values (1000 replicates). The sequences are given as GenBank accession number, host, country, and subtype. Nucleotide sequences determined in this study are indicated in red. The scale bar represents 0.02 substitutions per nucleotide position.

## DISCUSSION

*Blastocystis* is an important pathogen commonly detected in humans and animals (Clark et al. 2013; Maloney et al. 2019). Most studies on *Blastocystis* in Turkey are performed on humans, farms and pet animals, laboratory rats, and environmental samples (Koltas and Eroglu 2016; Dogan et al. 2017; Koloren et al. 2018; Malatyali et al. 2019, Malatyali et al. 2021; Onder et al. 2021). In the present study, we analyzed the commonly used SSU rRNA gene to molecularly characterize and genotyping of *Blastocystis*. The previous study conducted by Aynur et al. (2019) in the southwestern part of Turkey had resulted in 11.25% (9/80) of examined cows and cattle samples were *Blastocystis*-positive. Notably, recent surveys of *Blastocystis* infection in cattle from Central Anatolia and the Middle Black Sea Region of Turkey revealed lower prevalence rates, at 16.0% (32/200). We determined *Blastocystis* infection with an overall prevalence of 58.7% in the cattle in various slaughterhouses, which is higher than the rate reported in the previous studies (Aynur et al. 2019; Onder et al. 2021). *Blastocystis* sp. has been determined as the most prevalent species in calves and cattle in various countries such as Colombia (80%), Japan (37.5%, 26%, and 100%) USA (2.9% and 19%), Nepal (16.7%), China (27.6%, 10.3%, and 9.5%), Libya (41.7%), UK (22.6%), and Iran (9.6%) (Abe et al. 2002; Yoshikawa et al. 2003; Yoshikawa et al. 2004; Santin et al. 2011; Fayer et al. 2012; Lee et al. 2012; Alfellani et al. 2013; Ramírez et al. 2014; Badparva et al. 2015; Zhu et al. 2017; Wang et al. 2018; Maloney et al. 2019; Ren et al. 2019). It has been reported that these differences in the prevalence of *Blastocystis* infection may be related to some factors such as sample size, animal age, sampling season, farm management, geographical location, and feeding conditions (Zhu et al. 2017; Lee et al. 2018; Suwanti et al. 2020).

*Blastocystis* infection has been reported with a high prevalence in weaned, yearling, and adult cattle than pre-weaned calves (Zhu et al. 2017; Maloney et al. 2019). Higher infection rate was determined in >3 years adult cattle in this study. Data for older age groups are consistent with reports published previously, documenting higher rates of *Blastocystis* infection among older cattle from China (Zhu et al. 2017), the USA (Maloney et al. 2019), and Korea (Lee et al. 2018). It has been reported that lower prevalence observed in the age group between 1 and 3 years could be related to being the immune-protective effect of maternal antibodies and provided their food directly from the breast or bottle (Zhu et al. 2017; Maloney et al. 2019).

In the present study, ST10 was found to be more prevalent in slaughtered cattle. In contrast, two subtypes, ST10 and ST14, have been detected in cattle in the southwestern part of Turkey, with a larger proportion of ST14 (Aynur et al. 2019). Similarly, in another study conducted in Central Anatolia and the Middle Black Sea Region of Turkey, *Blastocystis* ST10

was reported to be more prevalent in farm animals including cattle and sheep (Onder et al. 2021).

The findings in the present study were highly similar to results of research by Santin et al. (2011) that reported that their cattle samples were only infected with ST10. *Blastocystis* ST10 is the predominant ST reported subtypes in cattle in the USA (Fayer et al. 2012), Denmark (Stensvold et al. 2009), China (Zhu et al. 2017; Wang et al. 2018), UK, Libya (Alfellani et al. 2013), Thailand (Santín et al. 2011), and Lebanon (Greige et al. 2019). Therefore, these data confirmed the hypothesis of Cian et al. (2017) that cattle may be natural hosts of *Blastocystis* sp. ST10. Interestingly, *Blastocystis* ST10 was also reported in dogs in France (Osman et al. 2015), cats in the USA (Ruaux and Stang, 2014), and dogs and birds in Malaysia (Noradilah et al. 2017).

The three sequences in our study were located in the same clade with *Blastocystis* ST10 sequences reported from cattle in the USA and Turkey. Our sequences within ST10 were highly similar (99.8-100%) to the sequences reported from cattle in the USA (MH634455) and Turkey (MH817028, MW093213, MH125194). However, this clade was a different clade of *Blastocystis* ST10 from other ruminants within ST10.

The present study contributes to molecular characterization and subtype distribution of *Blastocystis* in cattle in various slaughterhouses in Kayseri province, Turkey. The detection of this parasite in cattle slaughtered in slaughterhouses is a warning for the slaughterhouses, and it is highlighted that this pathogen should not be neglected. Therefore, preventive measures should be taken in slaughterhouses to prevent the risk of transmission of zoonotic *Blastocystis* subtypes to slaughterhouse workers who are in close contact with animals.

**Conflict of Interest:** The authors declared that there is no conflict of interest.

**Ethical Approval:** This study is not subject to the permission of HADYEK in accordance with Article 8 (k) of the "Regulation on Working Procedures and Principles of Animal Experiments Ethics.

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