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# First Molecular Characterisation of *Blastocystis* from Experimental Rats in Turkey and Comparison of the Frequencies Between Obese and Non-obese Groups

## *Türkiye'de İlk Kez Kemirgenlerde (Laboratuvar Ratlarında) Blastocystis'in Moleküler Karakterizasyonu ve Obez/Non-obez Ratlarda Yaygınlığının Karşılaştırılması*

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## ABSTRACT

**Objective:** *Blastocystis* is a zoonotic protozoan that infects a wide range of animals, including humans and rodents. This study aimed to determine the frequency and subtype distribution of *Blastocystis* in laboratory rats at a laboratory animal facility in Turkey.
**Methods:** This study included 54 male Sprague-Dawley rats from Aydın Adnan Menderes University Laboratory Animal Center. Among these rats, 30 were fed with high-fat diet (obese group) and the remaining 24 received standard chow (non-obese group). *Blastocystis* positivity was determined with amplification of small subunit 18S rRNA gene following their nucleic acid extraction from faecal samples. Subtypes were detected by submitting the partial 18S rRNA gene sequences to the database (pubmlst.org/blastocystis). The phylogenetic tree from evolutionary distance data was constructed using the neighbour-joining method.
**Results:** *Blastocystis* infection was detected in 33 (61.1%) of 54 laboratory rats. The frequency of *Blastocystis* was significantly different between obese and non-obese rats ($p<0.05$), with 43.3% and 83.3%, respectively. When referred to the database, exact matches were identified with *Blastocystis* subtype 4 (ST4) for all isolates. In the phylogenetic analysis of the partial 18S rDNA sequence, the sequence was closely clustered with reference ST4 subtypes from other countries, including China, Japan, United Kingdom and Czech Republic.
**Conclusion:** This study revealed the high rate of *Blastocystis* colonisation in laboratory rats, posing a risk for human transmission. The comparison of obese and non-obese groups supported the idea that *Blastocystis* might be an indicator of healthy gut flora. The detection of ST4 in all rats agreed with previous reports of the predominance of this subtype in rodents.
**Keywords:** *Blastocystis*, laboratory rats, obesity, Turkey

## ÖZ

**Amaç:** *Blastocystis*, insanlar ve kemirgenler dahil birçok canlıda görülen zoonotik karakterli bir protozoondur. Bu çalışmada, Türkiye'de ilk kez kemirgenlerde (laboratuvar ratlarında) *Blastocystis* görülme sıklığının ve alt tiplerinin belirlenmesi, ayrıca obezite ile ilişkisinin değerlendirilmesi amaçlanmıştır.
**Yöntemler:** Çalışmaya Aydın Adnan Menderes Üniversitesi Deney Hayvanları Merkezi'nde yer alan 54 erkek Sprague-Dawley rat dahil edilmiştir. Bunların 30'unu yüksek yağlı diyetle (obez) beslenmiş ratlar, kalan 24'ünü standart (normal) yem ile beslenen ratlar oluşturmuştur. *Blastocystis* pozitifliği, dışkı örneklerinden nükleik asit ekstraksiyonu sonrası küçük alt birim 18S rRNA geninin amplifikasyonu ile belirlenmiştir. *Blastocystis* alt tipleri, kısmi 18S rRNA gen sekanslarının veri tabanında (pubmlst.org/blastocystis) karşılaştırılması ile tespit edilmiştir. Ayrıca, neighbor-joining metodu kullanılarak izolatların evrimsel uzaklıklıklarını gösteren filogenetik ağaç oluşturulmuştur.
**Bulgular:** Çalışmamızda 54 laboratuvar ratının 33'ünde (%61,1) *Blastocystis* tespit edilmiştir. Ek olarak, *Blastocystis* görülme sıklığı obez ve normal ratlar arasında istatistiksel olarak anlamlı düzeyde farklılık göstermiştir ($p<0,05$, sırasıyla; %43,3 ve %83,3). Veri tabanı ile karşılaştırıldığında tüm izolatların alt tipi 4 (ST4) olarak tanımlanmıştır. *Blastocystis* 18S rDNA dizisinin filogenetik analizi, bu çalışmadaki sekansların Çin, Japonya, Birleşik Krallık ve Çek Cumhuriyeti dahil diğer ülkelerden alınan referans ST4 alt tipleriyle yakın olduğunu göstermiştir.

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Türkiye PARAZİTOLOJİ Dergisi

**Sonuç:** Çalışmamız, laboratuvar ratlarında *Blastocystis* kolonizasyonunun yüksek olduğunu ortaya koymakta olup, bu sonuç *in vivo* deney yapan araştırmacılar için bulaş riskinin yüksek olabileceğine dikkat çekmektedir. Obez rat grubunda normal ratlara göre *Blastocystis* görülme sıklığının daha düşük olması, *Blastocystis*'in sağlıklı bağırsak florasının bir göstergesi olabileceği fikrini desteklemektedir. Tüm sıçanlarda ST4'ün saptanması, bu alt tipin kemirgenlerde baskın olduğunu gösteren diğer araştırmalar ile uyum göstermektedir.
**Anahtar Kelimeler:** *Blastocystis*, deneysel rat, obezite, Türkiye

## INTRODUCTION

*Blastocystis* is one of the most frequent intestinal protozoon in the world, with an estimated one billion infected people. It also inhabits a variety of animal species and can be transmitted between humans and other animals (1). Phylogenetic analysis of the small subunit rRNA (SSU rRNA) gene from *Blastocystis* isolates showed the existence of 23 subtypes (STs), nine (ST1-9) of them were isolated from human fecal samples and host range was highly correlated with *Blastocystis* subtype (2,3). For example, ST3 was the most common subtype in humans and rodents were the main animal reservoir of ST4 (4).

Laboratory rats, the domesticated form of *Rattus norvegicus*, are most widely used animal model for biomedical and veterinary researches since 1850s. A variety of viral, bacterial, parasitic, and fungal agents was detected in laboratory rats. The frequency of these microorganisms declined considerably in recent years; however, they can be persistent and have potential effects to the progress of biomedical experimentation (5). In addition, some of these agents including *Blastocystis* have zoonotic potential and they were reported as a risk for researchers.

Despite its high prevalence, far too little attention has been paid to the potential role of *Blastocystis* in systemic diseases such as obesity. The high body mass index (BMI) is an important risk factor for non-communicable diseases such as: diabetes, cardiovascular diseases, and some cancers. Poor nutrition and hygiene in developing countries increase the risk of infectious diseases; however, in industrialized countries being overweight is a potential risk for infectious diseases (6). Intestinal microbiota or dysbiosis has important function in the development of obesity. The key factors for the development of obesity such as food absorption and low-grade inflammation are also directly related to the intestinal microbiota (7). Different rat models of *Blastocystis* have been developed with varying degree of success for a better understanding host and microbiota interactions (8). The aim of the present study was to investigate *Blastocystis* colonization in rats from a laboratory animal research center in Turkey and its relation with diet-induced obesity.

## METHODS

### Animals and Ethical Considerations

The present study investigated the frequency of *Blastocystis* among 54 male, 10-week old Sprague-Dawley (SD) albino rats in the Laboratory Animal Facility at Aydın Adnan Menderes University, Turkey.

The rats were housed in the following conditions: 12:12 h dark-light cycle, 21±1 °C, and humidity of 45-65% in polycarbonate transparent cages. There were two groups of randomly selected rats: 30 were fed with high-fat (obese) and 24 rats were fed with ad libitum supply of standard chow (normal). This type of diet included a fat content of 45% and supplied from a commercial facility. The animals fed with this diet for 24 weeks. The rats were weighted separately before taking fecal samples and Lee index (cube root of body weight/nose-anal length) was calculated (9). The rats that have Lee's index value above three were included in obese group.

All experiments were performed according to the Animal Care and Use Protocol of Aydın Adnan Menderes University. The study was reviewed and approved by the Local Ethical Committee for Laboratory Animals at Aydın Adnan Menderes University (no: 2018-109).

### Fecal Samples and DNA Isolation

The faecal samples were collected after high fat diet (HFD) diet and normal diet. Therefore, they were already obese when we took the samples. An individual rat was placed in an empty clean cage and allowed to defecate normally. The fecal samples were placed directly into a 1.5 mL tube and subjected to DNA isolation at the same day. A commercially available DNA extraction kit (Qıagen Stool Minikit, Germany) was used according to the manufacturer's instructions.

### Polymerase Chain Reaction (PCR) and Determination of Subtypes

*Blastocystis* "barcode region" was amplified in a single PCR reaction with RD5 (Forward) (5'-ATC TGG TTG ATC CTG CCA GT-3') and BhRDr (Reverse) (5'-GAG CTT TTT AAC TGC AAC AAC G-3') primers as previously reported (10). We used the isolate ADUBI201 (GenBank: KU361300) as positive control in the experiments. The amplicons were sequenced by a commercial facility with 377 applied biosystems DNA sequencer.

Subtypes were determined according to exact/closest matches at *Blastocystis* 18S rRNA database (http://pubmlst.org/blastocystis) (11). The present sequences and references were aligned by using ClustalW algorithm in molecular evolutionary genetics analysis (MEGA version 6.0). Neighbour-Joining method in the bootstrap test (1.000 replicates) was used to make a phylogenetic tree, the evolutionary distances were computed using the maximum composite likelihood (12,13). In the phylogenetic analysis, *Proteromonas lacertae* 18S ribosomal RNA gene partial sequence (AY224080) was included as the out-group.

### Statistical Analysis

The statistical analysis was performed with Statistical Package for the Social Sciences (SPSS version 13.0) program, (PASW Inc, Chicago. IL. USA). The frequency of *Blastocystis* in obese and normal groups were compared with chi-square test and weights were compared with Student t-test. The significance level was set at two-sided p-value <0.05.

## RESULTS

*Blastocystis* was observed in 33 (61.1%) of 54 laboratory rats, the expected size of PCR product was detected in agarose gel

electrophoresis with barcode region specific primers (Figure 1). In addition, *Blastocystis* had significantly high rate of frequency in normal rats than in the obese rats, the rates were 83.3% and 43.3% and respectively (Table 1). The mean body weight of obese rats was 427.8±42.5 and it was 271.9±21.1 in normal rats.

The barcode sequences of 33 *Blastocystis* isolates were identical and no polymorphism was detected. The sequence was deposited to GenBank® nucleotide database (accession number: MH285979). When referred to the "pubMLST database" it was detected as ST4. The evolutionary distance between the detected sequence and the common reference sequences was presented in Figure 2. This phylogenetic analysis also showed that the sequence was closed to the reference ST4 sequence.

The comparison of nucleotide sequence from the present study with available sequences from Genbank® with the basic local alignment search tool revealed 21 sequences that were identical to our sequence. These sequences had different country origins and isolated from different hosts (Table 2). In addition, 18 of them (85.7%) were reported as ST4 and the remaining three were ST5 according to the records in Genbank® database.

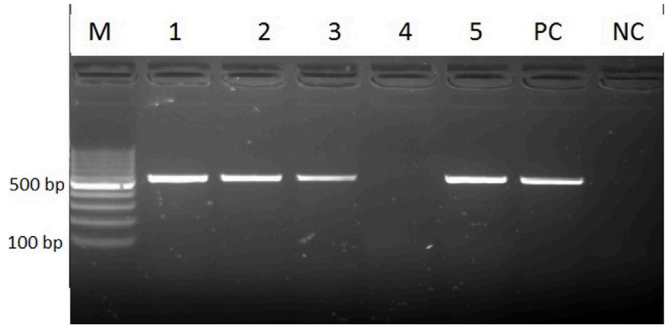


**Figure 1.** Agarose gel electrophoresis of some PCR products amplified with RD5 and BhRDr primers
M: Marker (100 bp, invitrogen™), Positive samples: Line 1-3, and 5, negative sample: Line 4, PC: Positive control, NC: Negative control, PCR: Polymerase chain reaction

**Table 1. The positivity of *Blastocystis* in obese and normal rats**

| | ***Blastocystis*** | | | |
|---|---|---|---|---|
| | **Positive n (%)** | **Negative n (%)** | **Total** | **Statistical analysis** |
| **Obese*** | 13 (43.3) | 17 (56.7) | 30 | |
| **Normal*** | 20 (83.3) | 4 (16.7) | 24 | $\chi^2$=8.97, p<0.05 |
| **Total** | 33 (61.1) | 21 (38.9) | 54 | |

*According to the Lee index

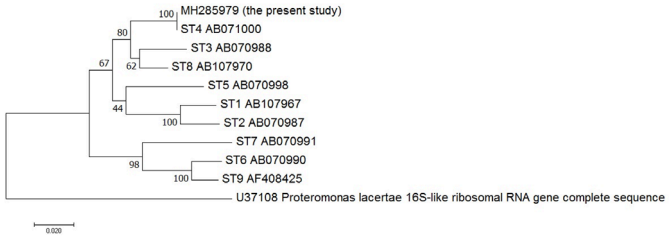


**Figure 2.** The evolutionary distance between barcode sequence from the present study (MH285979) and the reference sequences. The evolutionary history was inferred by using the maximum likelihood method based on the Tamura-Nei model

## DISCUSSION

*Blastocystis* is a frequent intestinal protozoon of humans with zoonotic transmission risk (14). Our study found a high frequency of *Blastocystis* (61.1%) in laboratory rats, indicating that these animals should be carefully monitored for the presence of *Blastocystis* and elimination strategies might be necessary before designing an experimental study, especially related with gastroenterology. A study from China reported that 8.2% of laboratory rat samples were positive for *Blastocystis*, in addition, SD rats had higher infection rates compared to Wistar rats and spontaneously hypertensive rats (15). The use of same areas for animal weighting, control and feeding might result in spread of *Blastocystis* among rats. Fecal-oral transmission way of *Blastocystis* cyst forms in rats was previously reported (16). Therefore, because of the zoonotic potential and possible effects to the findings of *in vivo* experiments researchers who study with laboratory animals should pay attention to avoid fecal contamination and transmission of *Blastocystis*.

In the current study, we defined the frequency and subtype distribution of *Blastocystis* isolates from rodents for the first time in Turkey. The analysis of thirty *Blastocystis* 18S *rRNA* genes revealed that all were identical and belonged to ST4 cluster. Several studies have investigated *Blastocystis* frequency or subtypes in rodents, they reported ST4 as the predominant subtype in those studies. Two *Blastocystis* subtypes (ST4 and ST7) were identified in a total of 355 fecal samples of experimental rats in China, the frequency of ST4 was 89.7% (15). In Peninsular Malaysia, wild rodents were examined for *Blastocystis* positivity, the frequency was 45.9% by *in vitro* culture (17). Additionally, the subtype distribution of 47 isolates was as follows: the most abundant (91.5%) subtype was ST4 that was followed by ST1 (4.3%), ST5 (2.1%) and ST7 (2.1%). It also highlighted the zoonotic potential of *Blastocystis* and considered as a source of infection for humans as in our study. Because, it was reported that ST4 is also found in human fecal samples especially in European countries including Turkey (4,18). In addition, *Blastocystis* ST4 was the only subtype that was detected in laboratory rats and Guinea pigs from Indonesia and Japan according to sequence data of the SSU rDNA (19). The authors examined totally 23 *Blastocystis* isolates from *R. exulans* and *R. novercious*, all were belonged to *Blastocystis* ST4. A study from southern Iran reported that 15.8% of rats were infected with *Blastocystis* and three subtypes were detected, including ST4, ST3, and ST1 (20). The distribution of *Blastocystis* subtypes was studied among symptomatic patients in Manisa and İzmir: ST3 (44.7%), ST1 (13.8%), ST2 (11.7%), ST6 (1%), ST7 (1.1%) and ST2+ST3 (2.1%) (21). It was reported that ST3 was the most common subtype followed by ST1, ST2 and ST7 subtypes in Aydın (22). A study determined the subtypes of *Blastocystis* isolates from faecal cultures of 350 patients with gastrointestinal complaints in Ankara. The researchers determined *Blastocystis* subtype distribution as: ST3 (28%), ST1 (13.9%), ST4 (11.6%), ST7 (11.6%), ST2 (7%), and ST6 (2.3%) (23). In addition, a recent study revealed an equal distribution of three *Blastocystis* STs including ST1, ST2 and ST3 in the same city (24). In Istanbul, it was reported that the most frequently detected *Blastocystis*

**Table 2. The list of partial *Blastocystis* 18S rRNA sequences that were identical to the sequence from our study**

| Genbank® accession | Number of sequences | Country | Host | Subtype |
|---|---|---|---|---|
| MH285979* | 1 | Turkey | Sprague-Dawley rats | ST4 |
| MK940492-3 | 2 | China | Patagonian mara | ST4 |
| MT039550 | 1 | Czech Republic | Human | ST4 |
| MN124100 | 1 | China | Chipmunk | ST5 |
| MN123809 | 1 | China | Guinea pig | ST4 |
| MN123799 | 1 | China | Guinea pig | ST5 |
| MN123530, 557 | 2 | China | Eurasian red squirrel | ST4 |
| MN123233 | 1 | China | Eurasian red squirrel | ST5 |
| MH127489,90,92-97,99,500 | 10 | Japan | Norway rat | ST4 |
| MF186667 | 1 | The UK | Eurasian red squirrel | ST4 |
| AB071000 | 1 | Japan | Norway rat | ST4 |

*The sequence from the present study

subtype was ST3, followed by ST1, ST5, ST2 and ST4, respectively (25). *Blastocystis* genetic diversity was investigated in 3-13 years aged children in Eskişehir, subtype distribution was as follows: ST3 (43.4%), ST1 (26.1%), ST4 (10.9%) and ST2 (8.7%) (26). *Blastocystis* subtype distribution in 28 samples from Çukurova region revealed that ST1 (35.7%), ST3 (25%), ST2 (17.8%), and ST4 (10.7%), ST5 (3.6%), ST6 (3.6%), and ST7 (3.6%) (27). In cancer patients, three *Blastocystis* subtypes were identified: ST3 (40%), ST2 (33%), and ST1 (20%), and one infected with ST1/ST2 (28). A study evaluated *Blastocystis* in animal hosts in Turkey in a large-scale. They reported that *Blastocystis* frequency was 19.4% in farm animals including sheep, water buffaloes, cattle, and chickens. No positivity was found in dogs, cats, and horses. The animal specific (ST10 and ST14) and zoonotic subtypes (ST7) were identified (29).

The role of *Blastocystis* in human health has long been a question of great interest in the recent literature. In the current study, the first time in the literature, comparing the obese rats with normal rats showed a lower colonization of *Blastocystis* in obese rats. The animals in our study shared a common environment during weight control and feeding, which facilitated the fecal-oral transmission of *Blastocystis*. Therefore, it may be assumed that all the animals have almost equally exposed to *Blastocystis* infection. These findings supported the evidence from a previous observation in humans that investigated the link between *Blastocystis* infection and BMI. They reported that *Blastocystis* was more common in lean individuals with a significant difference (30). A significant decrease in two dominant phyla (bacteroidetes and firmicutes) and some metabolic changes were reported after HFD induced obesity in rats. This type of diet also caused a lower colonization of *Lactobacillus*, a health-promoting bacteria genera (31). In our study, obese rats were fed with HFD, therefore, a similar dysbiosis of intestinal microbiota might be formed. The enrichment of certain bacteria was also observed in obese rats (32). Moreover, increased Firmicutes to Bacteroidetes ratio was associated with obesity in adult humans and in animal models (33). It was reported that individuals with *Bacteroides* dominant gut microbiota were less susceptible to *Blastocystis* infection than individuals with *Prevotella* and *Ruminococcus* dominant (34). In addition, *Blastocystis* colonization was significantly associated with high bacterial richness and *Bacteroides* enterotype and it was suggested that *Blastocystis* might be an indicator of a healthy intestinal flora rather than a dysbiosis (34,35). On the other hand, decreased *Blastocystis* colonization was reported in gastrointestinal disorders including inflammatory bowel diseases, ulcerative colitis, and irritable bowel syndrome (36-38).

### Study Limitations

A limitation of our study was that we did not have information about the initial positivity (prior to HFD diet feeding) of *Blastocystis* in experimental rats. This cross-sectional study was designed to compare *Blastocystis* positivity in obese and non-obese experimental rats. Therefore, we could not have data about the changes in *Blastocystis* positivity during 24 weeks of HFD and normal diet. We could study *Blastocystis* frequency only at the end of the both types of diets.

## CONCLUSION

The current study reported the frequency and subtypes of *Blastocystis* in rodents (laboratory rats) for the first time in Turkey. The study emphasized the importance of *Blastocystis* during *in vivo* studies and offered a possible negative association between *Blastocystis* and obesity. It was thought that unbalanced gut microbiota due to obesity might decrease *Blastocystis* colonization. However, there is a great need of experimental studies dealing with intestinal flora and *Blastocystis* colonization in obese rats. Further studies in obese individuals will also greatly contribute to the understanding of the relationship between *Blastocystis* and intestinal flora.

### * Ethics

**Ethics Committee Approval:** The study was reviewed and approved by the Local Ethical Committee for Laboratory Animals at Aydın Adnan Menderes University (no: 2018-109).

**Informed Consent:** All experiments were performed according to the Animal Care and Use Protocol of Aydın Adnan Menderes University. The study was reviewed and approved by the Local Ethical Committee for Laboratory Animals at the same university (no: 2018-109).

**Peer-review:** Externally and internally peer-reviewed.

## * Authorship Contributions

Surgical and Medical Practices: E.M., G.B., Concept: E.M., A.G., S.E., Design: E.M., A.G., H.E., Data Collection or Processing: E.M., G.B., Analysis or Interpretation: E.M., G.B., A.G., H.E., S.E., Literature Search: E.M., G.B., Writing: E.M., G.B.

**Conflict of Interest:** No conflict of interest was declared by the authors.

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