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Original article

Molecular prevalence and subtyping of *Blastocystis* sp. isolates in stray cats of İzmir, Turkey: First report of "ST4 allele 42" in cats

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Abstract

Blastocystis sp. is one of the most frequently detected intestinal parasites in humans and can inhabit a wide range of animals. Close contact with animals is one of the transmission factors of *Blastocystis* sp. infection in humans. In this study, we aimed to investigate the molecular prevalence and subtypes of *Blastocystis* sp. in stray cats living in İzmir, Turkey. The PCR targeting the barcode region in the SSU rRNA gene was performed with DNA samples isolated from feces (n:465) to investigate the presence of *Blastocystis* sp. PCR positive samples were sequenced for subtyping analysis. Among the samples analyzed, *Blastocystis* sp. DNA was detected in 17 (3.65%) of them and sequence data were obtained from only seven isolates. Phylogenetic analysis showed that seven *Blastocystis* sp. isolates clustered with the reference *Blastocystis* ST4 isolates. Similarity rates were between 83.22% and 99.25%. In addition, *Blastocystis* database results confirmed that all of these were "allele 42" corresponding to ST4. As a result, the present study shows for the first time the presence of "ST4 allele 42", the prevalent subtype in humans, in stray cats in İzmir, Turkey. This finding supports the notion that stray cats can be a source of *Blastocystis* sp. infection in humans.

Key words: *Blastocystis* sp., subtyping, ST4, allele 42, stray cats

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Introduction

The enteric parasitic protist *Blastocystis* sp. is one of the most frequently detected intestinal parasites in humans and can inhabit a wide range of animals including non-human primates, birds, and amphibians. Although *Blastocystis* sp. has previously been considered as a fungus and a sporozoan, it has been placed among the Stramenopiles based on recent molecular studies addressing the small subunit rDNA (SSU rDNA) gene (Stensvold and Clark 2016). In the life cycle of *Blastocystis* sp., cyst, vacuolar, granular, and amoeboid forms exist, and transmission caused by the cyst form is believed to occur through the fecal-oral route (Tan 2008). In humans, *Blastocystis* sp. infection has been reported to be generally asymptomatic although gastrointestinal disorders such as abdominal pain, diarrhea, and vomiting as well as cutaneous lesions (urticarial) and irritable bowel syndrome (IBS) can occur (Cian et al. 2017). In addition, it has been stated that some patient groups including HIV/AIDS and cancer patients are more susceptible to *Blastocystis* sp. infection (Lepczyńska et al. 2017).

Many studies have been conducted in various countries including Turkey, to investigate the prevalence of *Blastocystis* sp. in fecal samples from humans and animals as well as in drinking water samples (Dogruman-Al et al. 2009, Lee et al. 2012, Mohammadpour et al. 2020). It has been subsequently reported that the prevalence rates in developing countries are higher than in developed countries (Tan 2008). For example, in a study conducted in Senegal, the prevalence of *Blastocystis* sp. was found dramatically high (100%) in stool samples collected from children (El Safadi et al. 2014). These high prevalence rates are associated with poor hygiene, exposure to animals, and consumption of contaminated food or water (Tan 2008).

Traditionally, Blastocystis isolates detected in humans were identified as *Blastocystis hominis* but since significant heterogeneity was found among these isolates by serology, isoenzyme and karyotyping studies and then DNA sequence analysis, its scientific name was changed to *Blastocystis* sp. subtype n (where n is the subtype number) (Coyle et al. 2012, Stensvold and Clark 2016). According to the sequencing results of SSU rDNA, 17 subtypes of *Blastocystis* sp. (known as ST1, ST2, ST3, ST4, ST5, ST6, ST7, ST8, ST9, ST10, ST11, ST12, ST13, ST14, ST15, ST16, and ST17) have been detected in samples isolated from humans and animals as well as in environmental samples. Among these subtypes, prevalent sub-types in humans are ST1, ST2, ST3, and ST4, whereas subtypes from ST1 to ST8 and ST12 are known to be zoonotic (Rudzińska et al. 2020). In addition, Stensvold and Clark have reported five novel subtypes named as ST21, ST23, ST24, ST25 and ST26 (Stensvold and Clark 2020).

In Turkey, although there have been many studies investigating the presence and the sub-types of *Blastocystis* sp. in fecal samples collected from humans (Dogruman-Al et al. 2008, Dogruman-Al et al. 2009, Dagci et al. 2014, Ertug et al. 2015, Coskun et al. 2016, Dogan et al. 2016, Koltas and Eroglu 2016, Yersal et al. 2016), the presence of *Blastocystis* sp. in animals that the humans have close contact with such as stray animals and livestock, is unknown. In relation to this issue, the zoonotic subtypes among humans and animals are also uncertain.

İzmir is the third largest city in Turkey and harbors many stray animals such as cats and dogs. For example, the Izmir Chamber of Veterinarians reported that more than 500 thousand stray dogs live in İzmir. The number of cats is unknown but it is believed that the number of stray cats is equal to dogs or may be even higher. In addition, these stray animals do not have good living conditions because of inadequate veterinary care. Insufficient veterinary care can facilitate the transmission of zoonotic parasites including *Blastocystis* sp. Therefore, the present study aimed to investigate the molecular prevalence of *Blastocystis* sp. in stray cats living in İzmir, Turkey. We also aimed to subtype the *Blastocystis* sp. isolates in order to show the potential zoonotic subtypes.

Materials and Methods

Collection of feces samples

A total of 465 fecal samples were collected from Veterinary Clinics located in 5 different districts [Çiğli (n:74), Karabağlar (n:25), Karşıyaka (n:38), Konak (n:183) and Narlıdere (n:145)] of İzmir. The protocol for collecting fecal samples from stray cats was declared as exempt according to the instructions and approval of the Institutional Animal Care and Use Committee (IACUC) of Ege University for animal ethical norms.

PCR

DNA isolation was performed using a commercial stool DNA isolation kit (RTA Labs, Turkey) according to the manufacturer's instructions. A PCR targeting the barcode region in the SSU rRNA gene was performed using RD5 5-ATCTGGTTGATCCTGCCAGT-3 and BhRDr 5-GAGCTTTTTAACTGCAACAACG-3 primers (Scicluna et al. 2006). During the PCR, 25 µl reactions contained 1 µl template DNA, 5 µl PCR enzyme mix (GeneMark), 1 µl primers (10 µM each), and 17 µl dis-

Fig. 1. Distribution of *Blastocystis* sp. positive cats in İzmir.

tilled water. The amplification reaction was performed as follows: 1 min initial denaturation step at 94°C, followed by 35 cycles of 1 min at 94°C, 1 min at 59°C, and 1 min at 68°C with a final extension for 5 min at 68°C. PCR products were separated using 1% agarose gel electrophoresis and visualized.

Subtyping

For detecting the subtypes of *Blastocystis* sp. isolates, PCR products of the expected size were sequenced using ABI3730XL. Generated sequences were aligned with known *Blastocystis* sp. subtypes in MEGA 7.0 Software. The phylogenetic tree containing different subtypes was constructed using MEGA 7.0 software based on the Maximum Likelihood method using the Kimura 2 Gamma distribution (K2+G) model with 500 Bootstrap replications. For constructing the phylogenetic tree, a total of 41 *Blastocystis* sp. isolates with known subtypes from ST1 to ST17 as well as *Proteromonas lacertae* as an out-group were used, and according to clustering results, subtypes were detected. A Blastocystis typing database (https://pubmlst.org/blastocystis/) was also used to confirm the subtype results obtained using the phylogenetic analysis.

Results

The presence of *Blastocystis* sp. was investigated by PCR in 465 stool samples collected from stray cats, and subtypes were determined using the sequence data belonging to *Blastocystis* sp. isolates. Subsequently, 17 (17/465; 3.65%) samples were found to be *Blastocystis* sp. positive. Stray cats that are *Blastocystis* sp. positive were detected in Çiğli, Karabağlar, Karşıyaka and Narlıdere districts (Fig. 1). Among the 17 *Blastocystis* sp. positive samples, seven of them could be sequenced (Fig. 2).

The phylogenetic analysis indicated that all *Blastocystis* sp. isolates clustered together reference *Blastocystis* ST4 isolates with accession numbers

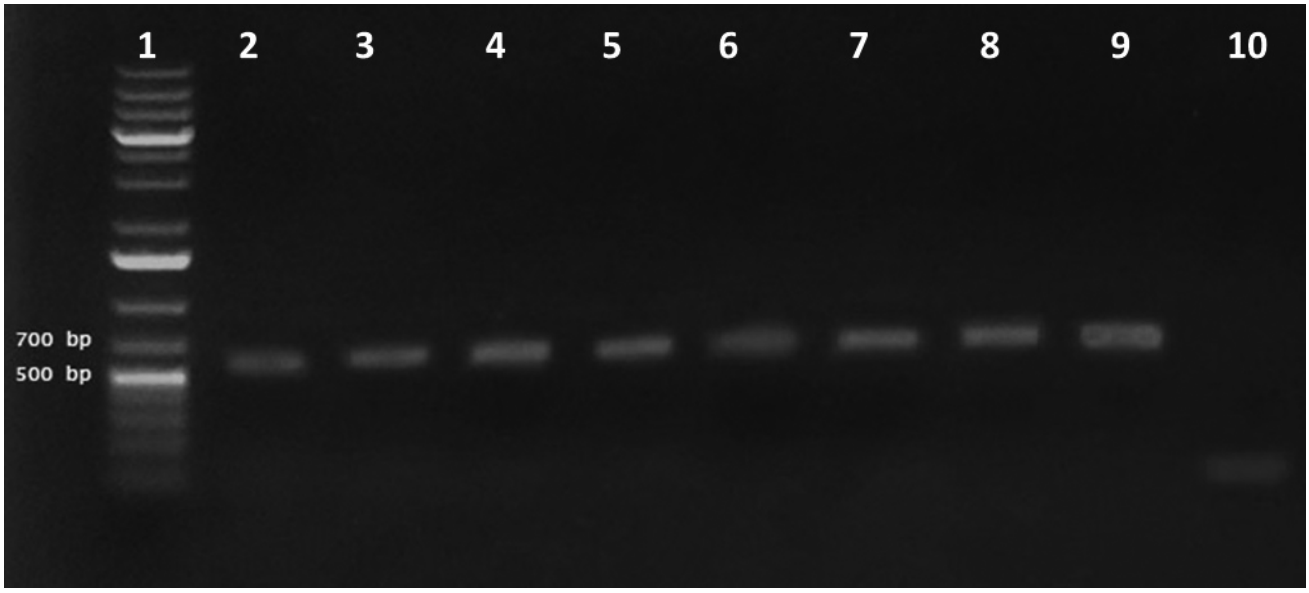

Fig. 2. Sequenced *Blastocystis* sp. isolates. **Line 1:** DNA ladder, **Lines 2-8:** *Blastocystis* sp. positive samples, **Line 9:** Positive control (Blastocystis ST7 isolate), **Line 10:** Negative control.

MK782501.1, MK782521.1 and MK782512.1 (Fig. 3). Similarity rates varied from 83.22% to 99.25% among reference ST4 isolates and *Blastocystis* sp. isolates of stray cats (Table 1). The isolate named as "*Blastocystis* sp. Izmir stray cat isolate 7" showed a very high similarity (99.25%) with MK782501.1 and MK782521.1 strains. The other isolate "*Blastocystis* sp. Izmir stray cat isolate 4" had the lowest similarity rates (varying 83.22% to 86.36) with MK782501.1, MK782521.1 and, MK782512.1 strains.

The *Blastocystis* database results were compatible with the phylogenetic analysis and it was confirmed that all *Blastocystis* sp. isolates were ST4. Also, all *Blastocystis* sp. isolates were found to show the closest matches with allele 42 corresponding to ST4 using the *Blastocystis* database.

Sequenced *Blastocystis* sp. isolates were submitted to GenBank under the accession numbers of MW305246, MW305477, MW306043, MW309143, MW309141, MW309387 and MW309422.

Discussion

Recent studies show that there is increasing research interest in *Blastocystis* sp. because it is one of the most common parasites in the human gastrointestinal system and is also a pathogen. In İzmir, Turkey, results obtained from routine patient controls showed that the prevalence of *Blastocystis* sp. in humans gradually increased over time. For example, the prevalence of *Blastocystis* sp. was 4.96% in humans in 2005 and increased to 32.33% in 2012 and 39.8% in 2019 (Değirmenci et al. 2007, Turgay et al. 2012, Ulusan et al. 2019). This increase may depend on the number of samples tested, increased personal experience as well as the number of stray animals living in İzmir.

It has been shown that close contact with animals is one of the most significant factors affecting the prevalence of this parasite (Tan 2008). İzmir, our study area, is a rich province in terms of stray animals such as cats and dogs. Therefore, it has been necessary to reveal the potential role of stray animals in the transmission of *Blastocystis* sp. to humans by a molecular prevalence study and by subtyping analysis for the determination of zoonotic subtypes circulating between humans and animals. For these reasons, in this study, *Blastocystis* sp. was investigated in fecal samples of stray cats by PCR targeting the barcode region in the SSU rRNA gene and the molecular prevalence was detected as 3.65%. Although the prevalence rate is low, this is the first study showing the presence of *Blastocystis* sp. in stray cats living in Turkey.

In cats, the prevalence rates of *Blastocystis* sp. vary according to the methods used, the number of samples tested and the region or country. For example, in Australia, a study reported that *Blastocystis* sp. positivity was found as 67.3% by microscopic examination among the 52 fecal samples collected from cats (Duda et al. 1998). In another study conducted in the US Pacific Northwest, the positivity of *Blastocystis* sp. was reported to be 11.65% in 105 fecal samples from shelter-resident cats using nested PCR targeting the SSU rDNA fragment (Ruaux and Stang 2014). In a different study performed in Iran, nested PCR targeting the 18S rRNA gene fragment was applied to 119 fecal samples from cats and 21 (17.7%) of them were found to be *Blastocystis* sp. positive (Mohammadpour et al. 2020). Our results, like previous results, indicate that

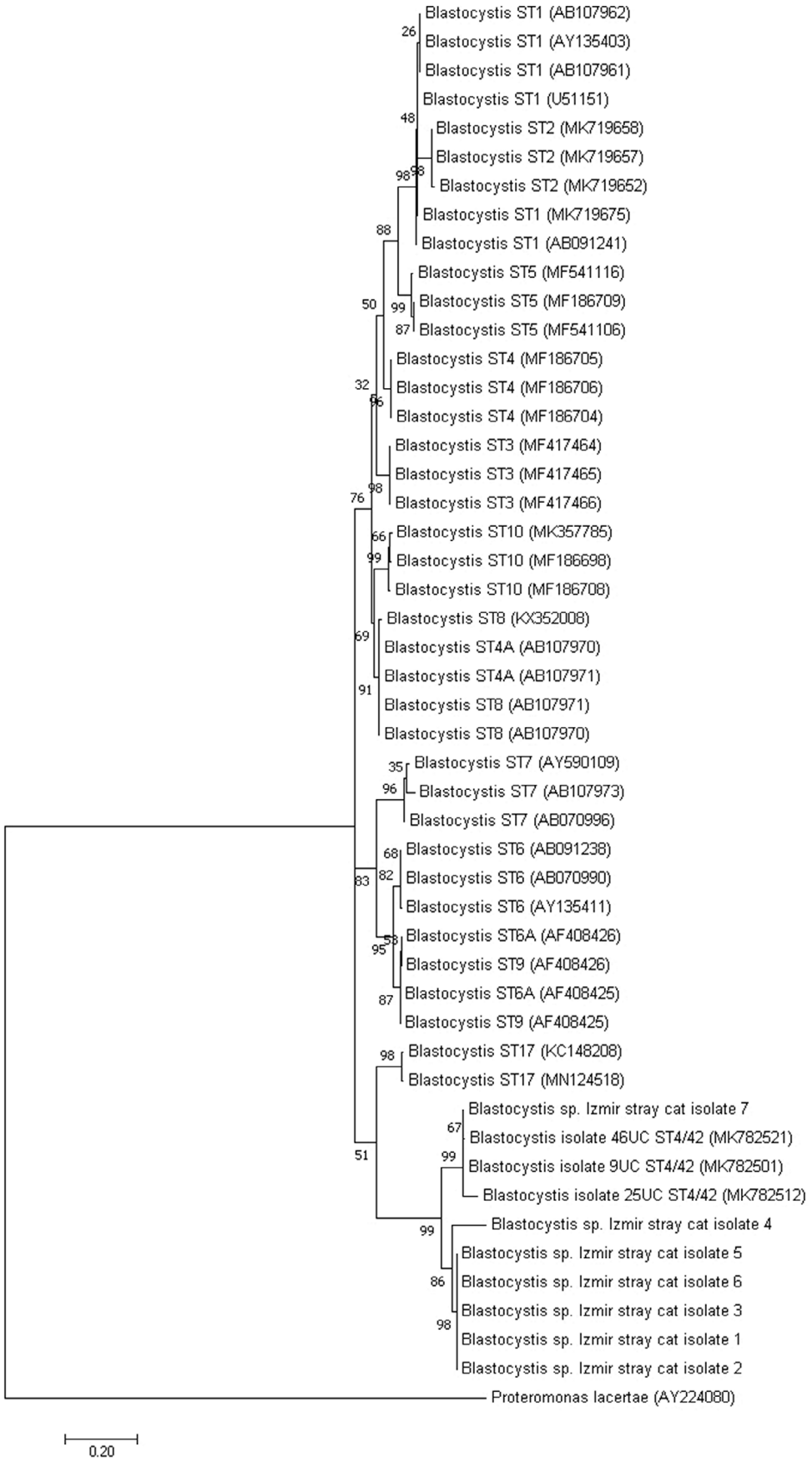

Fig. 3. Phylogenetic tree showing that *Blastocystis* sp. isolates clustered with ST4/42 isolates.

Table 1. Similarity rates of *Blastocystis* sp. isolates with reference ST4/42 isolates.

Isolate name	Blastocystis ST4/42 isolate 9UC (MK782501.1)	Blastocystis ST4/42 isolate 46UC (MK782521.1)	Blastocystis ST4/42 isolate 25UC (MK782512.1)
Blastocystis sp. Izmir stray cat isolate 1	93.60%	93.33%	90.13%
Blastocystis sp. Izmir stray cat isolate 2	93.33%	93.07%	89.87%
Blastocystis sp. Izmir stray cat isolate 3	93.60%	93.33%	90.13%
Blastocystis sp. Izmir stray cat isolate 4	86.36%	86.36%	83.22%
Blastocystis sp. Izmir stray cat isolate 5	93.09%	92.82%	89.63%
Blastocystis sp. Izmir stray cat isolate 6	92.82%	92.55%	89.63%
Blastocystis sp. Izmir stray cat isolate 7	99.25%	99.25%	95.02%

cats can play a role as reservoir hosts or source of infection in the transmission of *Blastocystis* sp. to humans and other animals.

In our study, sequence data could be obtained from only seven of 17 *Blastocystis* sp. isolates and, according to the phylogenetic results, all of them clustered with *Blastocystis* ST4 allele 42 isolates detected in human patients with urticaria from Brazil (Melo et al. 2019) (Fig. 3). One (*Blastocystis* sp. Izmir stray cat isolate 7) of the seven *Blastocystis* sp. isolates had a very high similarity rate (99.25%) to two isolates (MK782501.1 and MK782521.1). In contrast to this, although one Blastocystis isolate (*Blastocystis* sp. Izmir stray cat isolate 4) clustered with our other isolates and reference "ST4 allele 42" isolates, it had a lower similarity rate (83.22%) to "*Blastocystis* ST4 allele 42" isolates (Fig. 3 and Table 1). Also, *Blastocystis* database confirmed our *Blastocystis* isolates to be "ST4 allele 42". These findings demonstrate that the region identifying the ST4 isolates can be highly polymorphic.

Although *Blastocystis* ST4 has been detected in humans, it has not been reported in cats before, except for our study as well as a previous study detecting "ST4 allele 94" in fecal samples from cats (Mohammadpour et al. 2020). On the other hand, in a study conducted by Ruaux and Stang (2014), despite different *Blastocystis* subtypes such as ST1, ST3, ST10 and ST14-related isolate were found in fecal samples from felines, ST4 was not been detected (Ruaux and Stang 2014). Among animals, Blastocystis ST4 is prevalent in rodents (Katsumata et al. 2018, Li et al. 2020), and it has also been reported that ST4 is one of the major subtypes among humans in Europe (Domínguez-Márquez et al. 2009, Stensvold et al. 2011, Alfellani et al. 2013).

In Turkey, ST1, ST2, ST3, ST4, ST5, ST6 and ST7 were detected in human fecal material in previous studies (Dogruman-Al et al. 2008, Dogruman-Al et al. 2009, Dagci et al. 2014, Ertuğ et al. 2015, Coskun et al. 2016, Dogan et al. 2016, Koltas and Eroglu 2016, Yersal et al. 2016). Among these STs, ST4 was detected in two different studies and the prevalence rate of ST4 varied from 10.7% to 13.9% (Dogan et al. 2016, Koltas and Eroglu 2016). Detection of ST4 in our study and the presence of ST4 in humans indicate the possible circulation of *Blastocystis* ST4 isolates between stray cats and humans in Turkey.

In conclusion, our findings show that stray cats are one of the important hosts for the spreading of *Blastocystis* sp. Also, the detection of ST4 which is found as prevalent in humans indicates that stray cats may be responsible for the circulation of ST4 in humans. Moreover, the phylogenetic analysis demonstrates that *Blastocystis* ST4 isolates obtained from cats are highly polymorphic.

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