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Molecular Detection of *Blastocystis* Subtypes in Domestic Pigeons and Their Owners in Tafresh City

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ABSTRACT

Backgrounds: *Blastocystis* is a common intestinal parasite among humans and various animals, including birds. The parasite has at least 28 known subtypes, of which nine subtypes have been reported in humans and livestock. The aim of this study was to determine the prevalence rate and common subtypes of *Blastocystis hominis* in pigeons and their owners in Tafresh city.

Materials & Methods: The present study was designed and conducted as a case control in Tafresh city (Markazi province) during 2020-2021. For this purpose, fecal samples were collected from pigeons (300 samples) and their owners (100 samples). Stool samples were studied by microscopic methods (direct and trichrome staining examinations). Then positive stool samples were examined by PCR method through amplification of 18 SrRNA gene and sequencing.

Findings: In direct stool examination, 39 (13%) out of 300 pigeon samples and 18 (18%) out of 100 human fecal samples were found to be positive for *Blastocystis*. In trichrome staining method, 18% of human samples and 15% of pigeon samples were positive, while in PCR test, only 2.5% of pigeon samples and 4.5% of human samples were *Blastocystis* positive. The alignment results showed that all *Blastocystis* strains isolated in this study (100%) were similar to subtype 3.

Conclusion: Due to the low prevalence rate of this parasite in pigeons in Tafresh city, their owners are less likely to be infected with this parasite. Therefore, the relative transmission risk of this parasite from pigeons to humans is low.

Keywords: *Blastocystis*, Human, Pigeon, Subtype, Tafresh.

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Introduction

Blastocystis is an anaerobic eukaryotic protozoan and one of the most common microorganisms found in fecal samples of various hosts, including humans. The prevalence of *Blastocystis* is global and varies from country to country and has been reported to be between 0.5 and 23% in developing countries [1]. However, in tropical and subtropical regions, its prevalence rate has been reported to be up to 60% [2]. The incidence of this infection may be due to poor hygiene, exposure to animals, or consumption of contaminated water and food. *Blastocystis* comprises at least 28 subtypes [3]. Among these 28 reported subtypes, subtypes 1 to 9 (ST1-ST9) have been reported in humans, with subtypes 1 to 4 (especially subtype 3) being the most common. Subtypes 10 to 17 have been isolated from animals, some of which have also been reported in humans [4, 5]. The parasite could be detected in various forms of fecal samples using a light microscope with the ability to differentiate it from other leukocytes and intestinal protozoa. Common stool examination methods such as concentration technique and permanent staining of stool smears with trichrome and hematoxylin are used to detect different forms of the parasite.

Numerous studies have been performed to determine the prevalence of this parasite in humans in different parts of the world and Iran. However, studies on birds, especially pigeons, are very limited. In this regard, to determine *Blastocystis* subtypes, some studies have been conducted by Iguchi et al. (2007) on chickens [6], Chandrasekaran et al. (2014) on ostriches [7], Roberts et al. (2018) on chickens [8], Wang et al. (2018) on birds such as chickens, pigeons, and fish-eating chickens [9], Maloney et al. (2020) on captive wild birds [10], and Asghari et al. (2019) on Iranian crows and pigeons [11].

Objectives: Considering that so far, no study has been conducted to determine the prevalence of this parasite in humans and animals in Tafresh city (Markazi province), this study aimed to determine the prevalence and common subtypes of *Blastocystis* in domestic pigeons and their owners in Tafresh city.

Materials and Methods

The present study was designed and conducted as a case-control study during the years 2020-2021 in Tafresh city. In this study, the prevalence rate of *Blastocystis* infection was investigated among pigeons (300 fecal samples) and humans (100 fecal samples).

Sampling location: Tafresh is a small town located in the west of Markazi province in Iran. According to the 2011 census, its population was 25,912 people (including 12,884 men and 13028 women). Tafresh is located 222 km southwest of Tehran among high mountains. The average altitude of Tafresh is 1912 meters above sea level, and it has a continental and semi-arid climate with an annual rainfall of 270 mm (Figure 1).

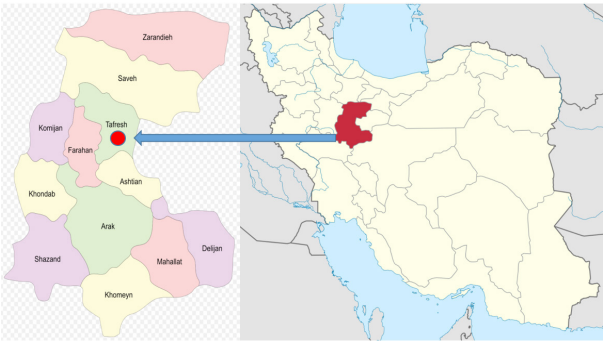

Figure 1) Map of the geographical location of Tafresh city in Markazi province

Sample collection: Written consent was obtained from all participants in this study. To conduct this research, stool samples of pigeons and their owners were first collected by referring to the homes of people who kept pigeons. The collected samples were kept in special containers containing fixatives such as 80% alcohol for molecular testing, 10% formalin for stool examination, and PVA

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(polyvinyl alcohol) for trichrome staining.

Stool examination: At first, examination of stool samples was done using direct stool examination and Lugol's staining. Then stool smear was prepared on a slide and stained with trichrome technique and examined microscopically. The diagnosis of the parasite was made based on the microscopic observation of the vacuolar and cystic form of the parasite on the slide.

Molecular study: After identifying *Blastocystis*-positive samples, DNA purification was performed by CTAB method [12]. Briefly, 50 mg of fecal sample and 1 mL of 1% NaOH were poured into a microtube and incubated at 60 °C for 30 min. After rinsing three times with saline, per-lysis buffer was added and incubated at 60 °C for 20 min. After washing three times with normal saline, the main lysis buffer was added, followed by 400 µL of TE buffer and 20 µL of proteinase K, and then kept overnight at 60 °C. CTAB isolation buffer (300 µL) was added and mixed for 5 min. The sample was incubated at 60 °C for 15 min with occasional mixing, and then 600 µL of chloroform/isoamyl alcohol (24:1 v/v) was added. The sample was vortexed briefly and then centrifuged at 11,000 rpm for 8 min. The supernatant was transferred into a new tube, 300 µL of ice-cold isopropanol was added to the tube, and the tube was inverted five times to precipitate the nucleic acid. The sample was centrifuged at 13,000 rpm for 5 min, and the supernatant was discarded. The pellet was air-dried for 2 hrs and then resuspended in 100 µL of hot distilled water. The extracted DNA samples were then stored in a freezer at -20 °C.

PCR test was used to amplify the 18s rRNA gene. RD5 and BhRDr primers were used with the following nucleotide sequences: F (RD5): ATCTGGTTGATCCTGCCAGT and R (BhRDr): GAGCTTTTTAACTGCAACAACG.

PCR reaction was performed in a final volume of 20 µL consisting of 5.5 µL of DNA template, 2 µL of (20 picomols) primers, 7.5 µL of master mix (Sinaclon, Iran), and 5 µL of distilled water. The denaturation step was started at 94 °C for 5 min, followed by 40 cycles of denaturation at 94 °C for 30 s, annealing at 55 °C for 30 s, and extension at 72 °C for 30 s and a final elongation step at 72 °C for 5 min. Then 6 µL of the PCR product was electrophoresed by 1% agarose gel impregnated with 50 µL of safe stain (Sinaclon Company, Iran) and photographed under short UV light. The PCR product size was evaluated with the commercial DNA-ladder (Sinaclon, Iran).

Finally, a total of four PCR products (two from humans and two from pigeons) were sent to Niagen Company (Iran) for sequencing. Then the sequences of the samples were aligned by ClustalX software. The sequenc-

Table 1) Direct examination of stool samples

Type of Sample	Gender	Number of Samples Studied	Positive Samples	
			No.	%
Humans	Male	86	13	15.11
	Female	14	5	35.71
	Total	100	18	18
Pigeons	Male	178	29	16.29
	Female	122	10	8.19
	Total	300	39	13

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es were then compared with the sequences registered in GenBank using MEGA7 software, and a phylogenetic tree was drawn.

Statistical analysis

For statistical analysis, descriptive statistics were performed using SPSS software Ver.15.1.

Findings

Direct stool examination: In the direct stool examination, 18 (18%) out of 100 human samples and 39 (13%) out of 300 pigeon samples were *Blastocystis*-positive. The results are presented in Table 1.

Trichrome staining results: At this step, stool smear samples stained with trichrome were examined microscopically, the results are shown in Table 2. In this method, a total of 18 (18%) positive samples were detected in human fecal samples, and 48 (15%) positive samples were detected in pigeon fecal samples.

Table 2) Number of *Blastocystis*-positive human and pigeon fecal samples based on microscopic examination of trichrome-stained stool smears

Type of Sample	Number of Samples Studied	Positive Samples	
		No.	%
Humans	100	18	18
Pigeons	300	45	15
Total	400	63	15.75

Molecular results: After DNA extraction, the relevant gene region was amplified by PCR. PCR was performed for all positive samples. Then after performing the PCR process for positive samples, finally four samples with clear bands were obtained, of which three samples were related to people whose pigeon fecal samples were also positive, and one of the samples was related to pigeon fecal samples. The image of the bands on 1% electrophoresis agarose gel is shown in Figure 2.

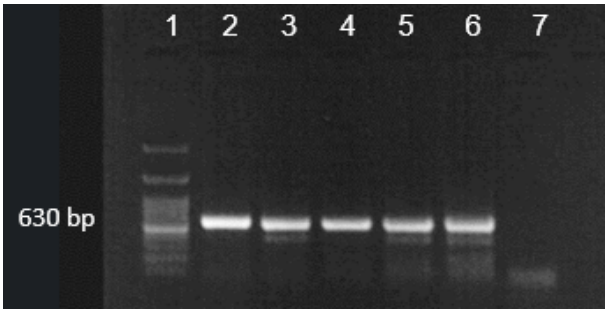

Figure 2) Electrophoresis results of PCR products of positive human and pigeon samples. From left to right: Lane 1: a 100 bp ladder, Lanes 2 to 5: human samples, and Lane 6: pigeon samples

Sequencing files were edited with the help of Sequencer software. Then using the BLAST method, the alignment was performed with the isolates registered in GenBank, and all the samples obtained in this study were 100% similar to subtype 3.

A phylogenetic tree was drawn using ST1 – ST28 sequences registered in GenBank. According to the drawn phylogenetic tree, the isolates of this study were grouped with subtype 3 isolates registered in GenBank (Figure 3).

Discussion

In recent years, *Blastocystis* has emerged as an opportunistic and emerging protozoan due to the extensive and varied studies performed on this parasite. The high prevalence of this parasite in all parts of the world, including developing and developed countries and tropical and subtropical regions, has made it the most common gastrointestinal parasite. The incidence of this infection is usually associated with poor hygiene, contact with animals, and consumption of contaminated food and water. In a study conducted by Zanetti et al. (2020), it was found that the parasite had the highest prevalence rates among birds and mammals, and that domestic animals were probably the most

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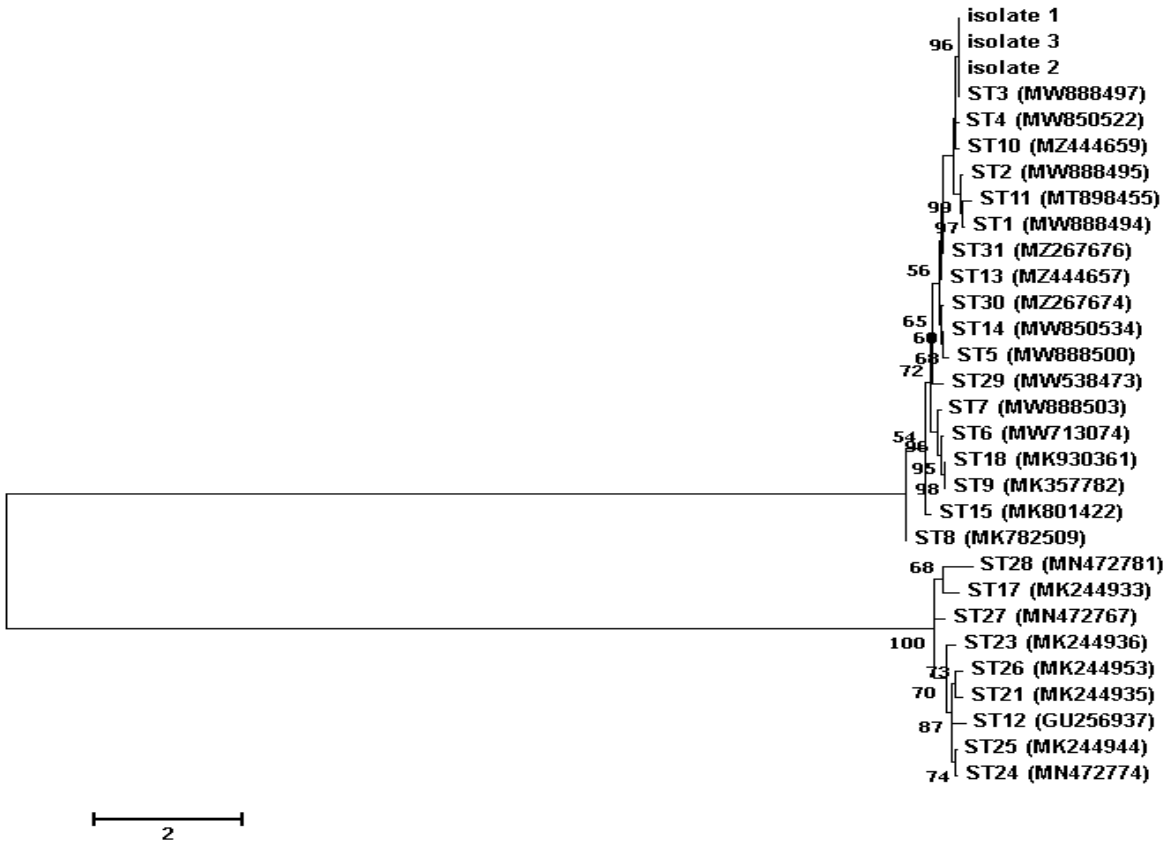

Figure 3) Phylogenetic tree drawn based on the 18s rRNA gene sequence of different *Blastocystis* subtypes using MEGA software, maximum likelihood algorithm, and bootstrap 50.

common cause of transmission of the parasite. Therefore, eco-epidemiological studies on *Blastocystis* parasite are considered to be very important [13].

In direct stool examination in this study, 39 (13%) out of 300 pigeon samples and 18 (18%) out of 100 human fecal samples were positive for *Blastocystis*, while in PCR test, out of 39 pigeon samples and 18 human samples, which were positive in direct method, only one (2.5%) pigeon sample and four (4.5%) human samples were *Blastocystis* positive.

The prevalence of this parasite in humans has been reported to be different in different parts of our country. For example, the prevalence rate of this infection has been reported to be 0.7% in Qazvin, 0.8% in Karaj, 2.2% in Zahedan, 4.2% in Khuzestan, 4.25% in Shiraz, 6.24% in Bandar Abbas, 7.15% in Yazd, and 12.8% in Tehran [14-21].

In the present study, the alignment results showed that all *Blastocystis* strains isolated from human and pigeon fecal samples (100%) were similar to subtype 3. In addition to humans, subtype 3 strains have been isolated from monkeys, cattle, horses, pigs, dogs, sheep and goats, pandas, cockroaches, and birds in Asia and Australia [22]. In other studies, conducted in Iran and the world, different results have been reported. In this regard, Iguchi et al. (2007) in their study identified subtypes 4 and 7 in rabbits and subtypes 2, 7, and 4 in chickens [6]. Chandrasekaran et al. (2014) identified subtype 6 in ostriches [7]. In another study conducted in Brazil, Maloney et al. (2020) identified subtypes 5, 6, 7, 10, 14, and 24 as well as two new subtypes (27 and 28) in captured wild birds [10].

Furthermore, Roberts et al. (2013) detected subtypes 6 and 7 in chickens, subtypes 1 and

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2 in hospitalized patients, and subtype 6 in humans and chickens [8]. In 2018, Wang et al. conducted a study on eight subtypes isolated from birds such as chickens, pigeons, and fish-eating birds and reported that 2.1% of pigeons were positive for *Blastocystis* [9].

In addition to the research mentioned above, several studies have been conducted in Iran to investigate the prevalence of *Blastocystis* subtypes. The prevalence of this parasite in humans is estimated to be 27% based on molecular epidemiological studies. So far, six known subgroups, including ST1 (32.01%), ST2 (21.9%), ST3 (36.7%), ST5 (0.3%), ST6 (2.43%), and ST7 (5.03%), and some unknown subgroups (1.52%) have been reported in humans [23-37]. According to these reports, the most common subtypes isolated from humans are subtypes 1 to 3 [38]. The prevalence of the above-mentioned subtypes is different in different regions of Iran. The potential sources of *Blastocystis* infection are also very different.

Very few studies have been performed on *Blastocystis* infection in Iranian animals. Badparva and colleagues (2015) reported a prevalence of 9.6% for this infection in cattle using STS primers [39]. Asghari et al. (2019) investigated *Blastocystis* subtypes in crows and pigeons in Tehran using nested-PCR RFLP and sequencing. In their study, 44.4% of crows and 42.5% of pigeons were positive for *Blastocystis,* and subtype 13 was identified in 100% of pigeons and 71.8% of crows [11].

In addition to humans and animals, Javanmard et al. (2019) reported the presence of ST2, ST6, and ST8 subtypes in wastewater samples. The presence of ST5-ST7 in humans, animals, and environmental specimens indicates the possibility of *Blastocystis* transmission through humans, animals, as well as consumption of contaminated water and food [40].

Although many molecular techniques have been proposed for the detection of *Blastocystis*, there are no specific standard primers for detecting the parasite. According to some studies results, about 13.9-45.8% of culture-positive parasite samples are negative in PCR [41-43]. These negative PCR results are usually due to the use of nonspecific primers to detect all subtypes, as well as differences in the SSU rRNA gene sequence of different *Blastocystis* subtypes. A particular pair of primers may have limited use and may not be able to identify all subtypes. For this reason, in the present study, only a limited number of positive samples in direct and trichrome staining methods were positive in PCR. In this study, out of 39 pigeon samples and 18 human samples, which were positive in direct method, only one (2.5%) pigeon sample and four (4.5%) human samples were found to be *Blastocystis* positive in PCR test.

Conclusion

In this study, human and pigeon fecal samples were examined for *Blastocystis* infection by direct stool examination, stool smear staining, and molecular analysis. In direct stool examination, 13% out of 300 pigeon samples and 18% out of 100 human fecal samples were found to be positive for *Blastocystis*. In trichrome staining method, 18% of human samples and 15% of pigeon samples were positive, while in PCR test, only 2.5% of pigeon samples and 4.5% of human stool samples were detected as positive. The alignment results showed that all *Blastocystis* strains isolated in this study (100%) were similar to subtype 3. Due to the very low prevalence of this parasite in pigeons in Tafresh city, their owners are less likely to be infected with this parasite. Therefore, the relative transmission risk of this parasite from pigeons to humans is low.

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Ethical permissions: This study was authorized by the Ethics Committee of Tarbiat Modares University of Medical Sciences, Tehran, Iran with code number: IR.MODARES.REC.1399.196. We conducted this study in accordance with the guidelines proposed in the Helsinki Declaration.

Conflicts of interests: The authors declare that they have no potential conflicts of interest with respect to the research, authorship, and/or publication of this paper.

Authors' contributions: Conceptualization: DA; data curation: DA, AS, and PM; formal analysis: DA and PM; funding acquisition: DA; investigation: AS; methodology: DA and AS; project administration: DA; resources: DA; supervision: DA and PM; writing of the manuscript original draft: DA; writing, reviewing, and editing: DA.

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