Molecular characterization and subtyping of Blastocystis in urticarial patients in Turkey

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ABSTRACT

Objective: To investigate Blastocystis’ etiologic role and association with gastrointestinal symptomatology in acute and chronic urticaria patients and to identify Blastocystis subtypes responsible for urticaria.

Methods: The study included urticaria patients and healthy individuals that presented to our polyclinic between June 2015 and May 2017. The participants were assigned into Group I (137 patients), subdivided into acute (72) and chronic urticaria patients (65), and Group II (129 control individuals). Blastocystis presence was investigated by native-Lugol examination, trichrome staining, PCR using sequence tagged site primers, and DNA sequencing analysis. The phylogenetic tree was constructed.

Results: The native-Lugol and trichrome staining methods revealed that 16 patients (16/133, 12.0%) had Blastocystis-positive stool samples, of which seven samples (7/133, 5.3%) belonged acute and nine (9/133, 6.8%) to chronic urticaria patients. Concerning Blastocystis subtypes, of the acute urticaria patients, three had subtype 1 (ST1), one had ST2, and three had ST3. Of the chronic urticaria patients, one had ST1 and eight had ST3. Blastocystis positivity was detected in two control individuals (2/123, 1.6%), both being ST3. All subtypes identified by PCR were confirmed by the sequencing analysis. The acute and chronic urticaria groups showed no statistically significant differences for Blastocystis positivity (P=0.60) and subtype distribution (P=0.15). A statistically significant difference was found between the urticaria patients and the controls for Blastocystis positivity (P<0.01), but not for subtype distribution (P=0.67) or for Blastocystis presence and gastrointestinal complaints.

Conclusions: This study on Blastocystis subtype distribution among Turkish urticaria patients showed results consistent with the literature. It was concluded that Blastocystis should be kept in mind in patients with urticaria.

1. Introduction

Blastocystis is defined as one of the most commonly found single-celled eukaryotes in human stool specimens and affects humans and a wide range of animals worldwide[1,2]. Blastocystis has a complex taxonomic history. In the past 20 years, it was considered as a
fungus, a sporozoan, or even a cyst of another organism at different points of its history[2,3]. Although molecular phylogenetic approaches have been applied for a long time, Blastocystis has just recently been clearly classified in the complex evolutionary combination of heterotrophic and photosynthetic protozoa, Stramenopiles[4]. The prevalence of Blastocystis varies from one country to another and even in different communities within the same country, reaching 20% in industrialized countries and exceeding 50% in developing countries[5-7]. In a recent study, the prevalence of Blastocystis in a group of children living in the rural parts of Senegal was reported to be 100%[7]. These differences may be associated with hygiene standards, waste disposal, close animal contact, and contaminated food or water consumption since it is thought that transmission is through the fecal-oral route, and cyst is the only form[5,6]. However, the extent to which human-human, human-animal and animal-human transmission occurrence remains controversial[6,7]. Based on the comparison of small subunit (SSU) rRNA gene sequences of the genus Blastocystis, at least 17 different ribosomal strains were classified as undisputed distinct species, also called subtypes (ST)[7,8]. Nine of these 17 subtypes (ST1 to ST9) are found in humans. In 90% of human epidemiological studies, one of the four subtypes (ST1-ST4) is observed, with ST3 being the dominant subtype[7,9]. In contrast, the less frequent subtypes for humans (ST5-ST8) are more common in other hosts, with ST5 being found in hoofed animals, and ST6, ST7 and ST8 in primates[3,7,9]. Rare subtypes detected in humans may be of zoonotic origin, and there are some evidences to support this: ST8 has been reported in zookeepers working with non-human primates, and ST5 in those dealing with pigs. ST9 has not been seen in any host other than humans[8].

The debate on the pathogenesis of Blastocystis remains controversial. Since Blastocystis is common both in healthy individuals and in patients suffering from gastrointestinal symptoms. Currently, it is not possible to distinguish colonization from infection[10,11]. The clinical characteristics of the disease attributed to this pathogen are not specific and include nausea, abdominal pain, gas, acute or chronic diarrhea, irritable bowel syndrome, angioedema, and urticaria[12,13]. One of the current hypotheses is that the variations in the clinical outcomes may be due to the differences in the subtypes[14].

Urticaria (hives) is a common disease that occurs in 15%-25% of individuals at any stage of life[15,16]. Urticaria is usually classified as acute, chronic or physical depending on the duration of symptoms and the presence or absence of stimuli. Acute urticaria is characterized by recurrent wheals for a maximum of six weeks with or without angioedema, whereas chronic urticaria refers to the recurrence of lesions for longer than six weeks[16,17]. The most common causes of urticaria (with or without angioedema) are drugs, food, viral infections, pesticides, and contact allergens, especially latex hypersensitivity. In 50% of patients with urticaria, the cause of the disease cannot be determined (idiopathic urticaria)[15,17]. Studies investigating the etiologic role of Blastocystis in urticaria are limited, with most presenting case reports. The current study aimed to investigate the etiologic role of Blastocystis in acute and chronic urticaria patients and determine the responsible subtypes and their relation with gastro-intestinal symptomatology.

2. Materials and methods

2.1. Patients and methods

This prospective study was carried out in the Dermatology Polyclinic of Erzincan University Training and Research Hospital in Turkey between June 2015 and May 2017. A total of 266 participants were included in the study and assigned into two groups as Group I (137 patients) and Group II (129 control individuals). Group I was further divided into two sub-groups as Group I A consisting of 72 patients with acute urticaria (wheals of less than six weeks’ duration) and Group I B comprising 65 patients with chronic urticaria (wheals of more than six weeks’ duration). Group II was consisted of 129 healthy individuals that presented to our hospital for routine health control without any particular complaint. Patients satisfying the following conditions were considered eligible for inclusion in the study: (1) the occurrence of angioedema with spontaneous wheals for six weeks or longer; (2) volunteering to participate in this study and providing written informed consent. The exclusion criteria were as follows: (1) pregnancy and breastfeeding; (2) presence of personal and family history of asthma, allergy or nasal allergy, or hypersensitivity to particular foods and drugs; (3) diagnosis of any systemic disease and other types of urticaria, such as physical urticaria, hereditary angioedema, and cholinergic urticaria; (4) complications with other skin disorders that interfere with efficacy evaluations; (5) having taken corticosteroids or immunomodulators within the past four weeks or antihistamines within the past three days; (6) presence of fever, arthralgia, or insect bites prior to the onset of symptoms.

2.2. Ethical approval and questionnaire

This study protocol was approved by the Ethics Committee of Erzincan University, Erzincan, Turkey (Approval No: 01/03, Date: 06.02.2015). Prior to data collection, the objectives, possible advantages and disadvantages of the study were explained to all participants. Written informed consent was obtained from each participant and the parents of children under 18 years of age. The participants were asked to complete a standard questionnaire to reveal information on their age, gender, type of water supply,
presence of domestic animals, and presence of gastrointestinal complaints (gas, nausea, vomiting, abdominal pain, diarrhea, constipation, bloating, and weight loss). The face-to-face interview method was used for the completion of the questionnaire.

2.3. Stool samples and microscopy

Stool samples taken from the patients and the control group were sent to the microbiology laboratory to be examined for the presence of parasites. The samples were aliquoted for DNA extraction alongside conventional stool examination by microscopy. The stool samples were analyzed immediately using native-Lugol and modified froumol-ether concentration methods, and in parallel with these examinations, trichrome and kinyoun acid fast staining was performed. *Blastocystis*-positive samples were cultured in Jones’s medium and checked by light microscopy to record morphologic features on day 3 and day 5 as described previously[18]. All of the patients that were found to have *Blastocystis* were treated with 750 mg metronidazole (10 mg/kg/dose in children) orally, three times over a period of 10 d. The stool samples were collected once more after treatment and the patients’ response to treatment was recorded.

2.4. DNA isolation and subtyping

The genomic DNA was extracted from all stool samples with *Blastocystis* positivity using a QIAamp DNA stool mini kit (Qiagen, Hilden, Germany) according to the manufacturer’s recommendations. The DNA concentrations were determined using a NanoDrop ND-1000 spectrophotometer (ThermoScientific, Wilmington, DE, USA) and stored at -20 °C for further experiment.

The PCR was performed using seven subtype-specific sequence tagged site primers [SB83 (351 bp) for ST1, SB340 (704 bp) for ST2, SB227 (526 bp) for ST3, SB337 (487 bp) for ST4, SB336 (317 bp) for ST5, SB332 (338 bp) for ST6, and SB155 (650 bp) for ST7] for the subtyping of *Blastocystis* species[18]. *Blastocystis hominis* Brumpt (ST1, ATCC®50752™) was used as the reference strain. Amplification reactions were performed in a total volume of 25 μL containing 12.5 μL of DreamTaq PCR Master Mix (2×) (Thermo Scientific, Waltham, MA, USA), 1.5 μL of each primer (10 pmol), and 2.5 μL of DNA. The PCR was carried out using the following conditions: initial denaturation at 94 °C for 1 min, followed by 40 cycles of denaturation at 94 °C for 1 min, annealing at 59 °C for 1 min, elongation at 72 °C for 1 min, and a final extension at 72 °C for 10 min. To verify the presence of a single band and the size of the amplified products (approximately 600 bp), the PCR products and 100 bp DNA marker (GeneRuler 100 bp DNA ladder, Thermo Fisher Scientific, USA) were electrophoresed in 2% agarose gels in a Tris-borate-EDTA (TBE) buffer, stained with ethidium bromide, and photographed under a UV transilluminator.

The PCR products were purified using the Agencourt AMPure XP Beads PCR purification kit (Beckman Coulter, CA, USA) and sequenced in both directions using the PCR primers (RD5 and BhRDr). DNA sequencing reactions were performed using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) and analyzed using the ABI PRISM 3130 Genetic Analyzer (Applied Biosystems, Foster City, CA, USA).

2.6. Phylogenetic analysis

The SSU-rRNA sequences were compared to those available in the GenBank nucleotide database using BLAST tool obtained from the website of the National Center for Biotechnology Information (NCBI). The results of two-directional sequencing were edited by the BioEdit software and aligned with the previously published data of the SSU-rRNA gene of *Blastocystis* isolates using the ClustalW program. Then, a phylogenetic tree was constructed with the neighbor-joining method using the MEGA software version 7.0, and the molecular distances were estimated by the number of differences model[20]. All the gaps were excluded from the analysis, and branch support was ascertained using 1 000 bootstrap replicates. *Proteromonas lacerate* (AY224080) was used as the out-group.

2.7. Statistical analysis

The statistical software SPSS (IBM SPSS Statistics for Windows, Version 20.0. Armonk, NY: IBM Corp. Released 2011) was used to analyze the data. For discrete and continuous variables, descriptive statistics (mean, standard deviation, median, minimum value, maximum value, and percentile) were obtained. In addition, homogeneity of variance, which is one of the prerequisites of parametric tests, was checked through Levene’s test. The assumption
of normality was tested via the Shapiro-Wilk test. To compare the differences between the two groups, the Student’s t-test was used when the parametric test prerequisites were fulfilled, and the Mann Whitney-U test was conducted when such prerequisites were not satisfied. The relationships between the categorical variables were analyzed by Fisher’s exact and chi-square tests. In cases where the expected frequencies were less than 20%, the Monte-Carlo simulation method was employed to include these frequencies in the evaluation. P<0.05 were considered to be statistically significant.

3. Results

3.1. Demographic and baseline characteristics of the study population

Of the 308 patients initially screened, 266 were included in the study. The Consolidated Standards of Reporting Trials (CONSORT) flowchart diagram is shown in Figure 1. A total of 10 samples, two in the acute urticaria group, two in the chronic urticaria group and six in the control group, were excluded from analysis due to either insufficient sample of stool or destroyed samples. The urticaria group comprised 50 (37.6%) male and 83 (62.4%) female patients. The age of the urticaria group ranged from 2 to 78 years, with the mean age being calculated as (41.6±17.1) years. Of the control group, 52 (42.3%) were male and 71 (57.7%) were female. The age range of the control group was 17 to 86 years, and the mean age was (41.6±17.1) years. Of the patients with chronic urticaria, 21 (21/63, 33.3%) were male and 42 (42/63, 66.7%) were female with a mean age of (44.7±16.2) years.

3.2. Prevalence and subtype distribution of Blastocystis among the groups

In the patient group, Blastocystis was found in 16 of stool samples (16/133, 12.0%) using native-Lugol and trichrome staining methods. Using the culture technique, 15 samples were identified as vacuolar (15/16, 93.8%) and one as the granular (1/16, 6.2%) form of the parasite. The Blastocystis-positive stool sample of one patient also had Giardia positivity. Except Blastocystis and Giardia, no other parasite was detected. Of the Blastocystis-positive patients, seven were in the acute (7/133, 5.3%) and nine were in the chronic (9/133, 6.8%) urticaria subgroups.

Of the seven acute urticaria patients with Blastocystis positivity, three had ST1, one had ST2, and three had ST3. Of the nine chronic urticaria patients with Blastocystis positivity, one had ST1 and eight had ST3. There was no statistically significant difference in Blastocystis positivity (P=0.60) nor in the distribution of subtypes (P=0.15), between the acute and chronic urticaria subgroups. In the control group, Blastocystis positivity was found in two of the 123 healthy individuals (2/123, 1.6%), and both isolates were identified as ST3. Both isolates (100%) were detected to be the vacuolar form of the parasite using the culture technique. A statistically significant difference was observed between the urticaria patients and the control group in terms of Blastocystis positivity (P<0.01), but there

![Figure 1](https://example.com/flowchart.png)  
**Figure 1.** Consolidated Standards of Reporting Trials (CONSORT) flowchart diagram of the study.
was no such difference in the distribution of subtypes, \((P=0.66)\) (Table 1). All subtypes identified by PCR were confirmed by the sequence analysis (Figure 2).

Table 1. Comparison of demographic and epidemiological data between the urticaria and control groups.

| Factors                  | Urticaria \((n=133)\) | Control \((n=123)\) | \(P\) value |
|--------------------------|-------------------------|----------------------|-------------|
| Age (Mean±SD)            | 41.6±17.1               | 37.3±19.5            | 0.84        |
| Gender                   | Male 50/37.6            | 52/42.3              | 0.45        |
| Blastocystis Positive    | 16/12.0                 | 2/1.6                | <0.01 \(\ast\) |
| Animal contact           | Yes 22/16.5             | 10/8.1               | 0.04 \(\ast\) |
| Drinking water           | Mains 117/88.0          | 112/91.1             | 0.42        |
|                          | Spring 16/12.0          | 11/8.9               |             |

\(\ast\) Chi Square test; \(\ast\) Mann Whitney-U test; \(\ast\) means there is significant difference.

Table 2. Comparison of Blastocystis positivity and demographic, clinical and epidemiological data in the urticaria group \((n=133)\).

| Factors                  | Patients with urticaria \((n=133)\) | Blastocystis positivity \((n=16)\) | \(P\) value \(\ast\) |
|--------------------------|--------------------------------------|------------------------------------|-----------------------|
| Gender                   | Male 50/37.6                         | 9/18.0                             | 0.10                  |
| Itching                  | Yes 133/100                          | 16/100                             | 0.54                  |
| Gas                      | Yes 69/51.9                          | 11/15.9                            | 0.15                  |
| Bloating                 | Yes 70/52.6                          | 9/12.9                             | 0.76                  |
| Nausea                   | Yes 33/24.8                          | 6/18.2                             | 0.21                  |
| Abdominal pain           | Yes 45/33.8                          | 5/11.1                             | 0.82                  |
| Constipation             | Yes 62/46.6                          | 5/8.1                              | 0.19                  |
| Diarrhea                 | Yes 23/17.3                          | 3/13.0                             | 0.87                  |
| Vomiting                 | Yes 11/8.3                           | 1/9.1                              | 0.75                  |
| Weight loss              | Yes 19/14.3                          | 1/5.3                              | 0.33                  |
| Animal contact           | Yes 22/16.5                          | 2/9.1                              | 0.64                  |
| Drinking water           | Mains 117/88.0                      | 14/12.0                           | 0.95                  |
|                          | Spring 16/12.0                      | 2/12.5                             |           |

\(\ast\) Chi Square test.

3.3. Relationship between Blastocystis and demographic, clinical and epidemiological factors

Of the patients with Blastocystis-positive stool samples, nine were male and seven were female. In the control group, the two having Blastocystis positivity were male. No statistically significant difference was found between Blastocystis positivity and gender in the patient and control groups \((P=0.10, P=0.10)\) (Table 2).

Of the 133 patients, 88.0\% \((n=117)\) reported using mains water and 12.0\% \((n=16)\) spring water as drinking water. Fourteen of the 16 patients that had Blastocystis positivity were found to use mains water for drinking. In the control group, 112 of 123 individuals (91.1\%) used mains water and 11 (8.9\%) spring water. Both healthy volunteers with Blastocystis positivity were reported to use mains water as drinking water. However, there was no statistically significant difference in the source of drinking water between cases with and without Blastocystis positivity in the patient and control groups \((P=0.95, P=0.66)\) (Table 2).

Animal contact was found in 22 of 133 patients and 10 of 123 healthy controls. While two of the 16 patients with Blastocystis positivity had animal contact, neither of the two healthy controls reported such contact. There was no statistical difference between the presence of Blastocystis and animal contact \((P=0.64, P=0.67)\) (Table 2).

In the patient group, the symptoms of individuals with Blastocystis positivity were as follows: itching (16/16), gas (11/16), bloating (9/16), nausea (6/16), abdominal pain (5/16), constipation (5/16), diarrhea (3/16), vomiting (1/16), and weight loss (1/16). There was no statistically significant difference between the presence of Blastocystis and any of these symptoms (Table 2). Sixteen
patients with Blastocystis-positive stool samples and two control individuals were treated with 750 mg of metronidazole orally for 10 d. No parasites were detected in the stool samples of any of these 18 individuals after two weeks. The primary outcome of this study was that in Blastocystis-positive patients, urticaria lesions disappeared after metronidazole treatment, revealing the possibility of a relationship between urticaria and Blastocystis, independent of gastrointestinal symptomatology and subtype.

4. Discussion

In this study, of the seven acute urticaria patients with Blastocystis positivity, three had ST1, one had ST2, and three had ST3, and among the nine Blastocystis-positive patients with chronic urticaria, one ST1 and eight ST3 were identified. There was no statistically significant difference between the patients with acute and chronic urticaria in terms of Blastocystis positivity or subtype distribution (P=0.60, P=0.15). However, a statistically significant difference was found between the urticaria patients and the control group in terms of Blastocystis positivity while subtype distribution did not significantly differ between the two groups. In the control group, Blastocystis positivity was detected in two of the 123 individuals, and both isolates were identified as ST3. Research on Blastocystis subtypes in urticaria cases has been undertaken in different parts of the world, and the predominant subtype has been shown to be ST3, and some of the studies in the literature have considered urticaria as a complication of Blastocystis[21-23]. In a study conducted with 54 urticaria cases, 18 acute (33.3%) and 36 chronic (66.7%), Abdel Hameed et al. reported that Blastocystis positivity was present in 33 (61.1%) patients. Of these 33 patients, 21 were symptomatic and 12 were asymptomatic. The authors subtyped all Blastocystis isolates in both groups as ST3[21]. In addition, in 2015, Casero et al. detected Blastocystis positivity in 67 of 270 patients from Argentina. The symptomatic group consisted of 39 patients, comprising 18 urticaria cases, 18 individuals with non-specific gastrointestinal symptoms, and 3 with both urticaria and non-specific gastrointestinal symptoms. The asymptomatic group contained 28 individuals. Morphological analysis, DNA extraction, 18S PCR, and DNA sequence analysis were performed on the Blastocystis isolates. Of the 67 Blastocystis isolates, 49 were identified as ST3 (71.6%), distributed as 71.4% (n=35) in the symptomatic group and 28.6% (n=14) in the asymptomatic group. Furthermore, 10 isolates (14.9%) were defined as ST1, 10 (7.5%) as ST6, and 4 (6.0%) as ST2. Two of 10 ST1 isolates were found in the symptomatic group and eight in the asymptomatic group. Five ST6 isolates were detected in the asymptomatic group. Two of the four ST2 isolates were observed in the symptomatic group and the other two in the asymptomatic group. The authors reported a high rate of ST3 in patients with urticaria and non-specific gastrointestinal symptoms, but there was no statistically significant difference between the symptomatic and asymptomatic groups in terms of subtype distribution[22].

Although only three subtypes (ST1, ST2 and ST3) were obtained in the current study, ST3 was dominant, especially in cases of chronic urticaria. In addition, the absence of animal transitional subtypes, such as ST5 or ST7, indicates that these isolates are not of zoonotic origin and have an anthropotonic profile.

In recent years, different Blastocystis subtypes have been discussed as having varying pathogenic potential. This possibility was first raised by Clark in 1997[24]. Later in 2001, Kaneda et al. reported that ST1, ST2 and ST4 might be responsible for gastrointestinal symptoms[25]. In 2012, Poirier et al. suggested that ST7 was correlated with irritable bowel syndrome[4]. Puthia et al. stated that in rat epithelial cells, ST4 might induce apoptosis independent of contact, thus increasing epithelial permeability[26]. In 2006, Yan et al. found the presence of only ST1 in a group of symptomatic patients. This finding was confirmed in 2013 by El Safadi et al., who showed that ST1 was associated with high pathogenicity[27,28]. The pathogenicity of ST4 was presented in a short report by Stensvold et al[29].

Urticaria is a heterogeneous group of diseases with a highly variable clinical manifestation and can be caused by a variety of factors. Parasitic diseases, especially Blastocystis, have long been considered to be the cause of potential urticaria. It is argued that Blastocystis may be a causal factor in the unclarified etiology of urticaria[30]. It has been suggested that aspirin or non-steroidal anti-inflammatory drugs may be a co-factor in the process of Blastocystis causing urticaria through a pathogenic route similar to the food-or exercise-induced anaphylactic reaction. To date, very little is known about why some subtypes cause urticaria only in certain patient groups[30,31]. In 2008, Katsarou-Katsari et al. detected Blastocystis in the stool sample of a patient with acute urticarial lesions and minor gastrointestinal symptoms and identified the isolate as ST3. Although antihistaminic drugs were given, the symptoms of the patient did not regress; thus, an oral metronidazole treatment was started and urticaria lesions were observed to regress after one week. The patient’s gastrointestinal symptoms disappeared, and reexamination of his stool showed no Blastocystis.

Similarly, in the current study, all the Blastocystis-positive patients responded to the metronidazole treatment and showed no symptoms of urticaria after the treatment[32]. Therefore, our study confirms that Blastocystis is a source of infection that must be kept in mind in the etiology of urticaria. In addition, the patients with acute and chronic urticaria were followed up clinically and parasitologically for a period of one year, during which the symptoms disappeared and did not recur. This is an evidence that Blastocystis infection may be one of the underlying causes of resistance to urticaria treatment.

In conclusion, our results are consistent with the international literature, demonstrating the relationship between Blastocystis presence and urticaria. Considering the limited number of studies on the relationship between Blastocystis subtypes and pathogenicity in patients with urticaria, there is a need for comprehensive studies to explore the efficacy of treatment in this patient group in different countries and populations.
Conflict of interest statement

The authors declare that they have no conflicts of interest.

Authors’ contributions

M.A., M.Y., M.D. and I.S.K. designed the study, M.A., M.Y., A.C., B.G., A.Y. and M.K. collected samples. M.A., M.D. and T.D. analyzed and interpreted data. M.A., I.S.K. and T.D. contributed to critical revision of the manuscript for important intellectual content and final approval of the manuscript.

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