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The Investigation of *Blastocystis* Subtypes in Fecal Samples of Diabetes Mellitus Patients

Diabetes Mellitus Tanısı ile İzlenen Hastaların Gaita Örneklerinde *Blastocystis* Subtiplerinin Araştırılması

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Abstract

Objective: This study aimed to detect *Blastocystis* spp. To determine the incidence, genotypes, regulation of blood glucose level and its role in gastrointestinal symptoms.

Materials and Methods: The current study was conducted at Aydın Adnan Menderes University Medical Faculty Hospital, where stool samples of 100 patients diagnosed with type 2 diabetes mellitus (DM) and 100 healthy volunteers as a control group were examined. Stool samples were evaluated by direct microscopic examination and polymerase chain reaction (PCR). Statistical analysis of the data was performed using the SPSS 17.0 program, and a significance level of $p<0.05$ was determined.

Results: The presence of *Blastocystis* was investigated in a total of 200 samples. Of the cases, 81 (40.5%) were female and 119 (59.5%) were male. The mean age was 57.1±11.2. Most of the participants resided in the city center (52.5%). Eighteen samples that were *Blastocystis* positive by direct microscopy were also found to be positive by PCR. Additionally, two samples that were negative by direct microscopy in the control group were found to be positive by PCR. There was no significant difference between the groups in terms of *Blastocystis* positivity. The sensitivity and specificity of the direct microscopy method were calculated as 81.81% and 100%, respectively. Nine (45%) of the *Blastocystis* subtypes were ST3, seven (35%) were ST2, three (15%) were ST1, and one (5%) was ST6.

Conclusion: *Blastocystis* spp. is a pathogen that should be considered in patients with diabetes. Our study is one of the few studies investigating the prevalence and clinical features of *Blastocystis* spp. in patients with type 2 DM.

Keywords: *Blastocystis* spp., genotype, diabetes mellitus

Öz

Amaç: Bu çalışmanın amacı diyabetik hastalarda *Blastocystis* spp. insidansını, genotiplerini, kan glukoz düzeyinin regülasyonu ve gastrointestinal semptomlardaki rolünü belirlemektir.

Gereç ve Yöntemler: Aydın Adnan Menderes Üniversitesi Tıp Fakültesi Hastanesi'nde tip 2 diabetes mellitus (DM) tanısı almış 100 hasta ile kontrol grubu olarak 100 sağlıklı gönüllünün dışkı örnekleri incelenmiştir. Dışkı örnekleri direkt mikroskobik olarak ve polimeraz zincir reaksiyonu (PZR) ile değerlendirilmiştir. Verilerin istatistiksel analizi SPSS 17.0 programı ile yapılmış, anlamlılık değeri $p<0,05$ olarak belirlenmiştir.

Bulgular: Çalışma kapsamında toplam 200 örnekte *Blastocystis* varlığı araştırılmıştır. Olguların 81'ini (%40,5) kadınlar, 119'unu (%59,5) erkekler oluşturmaktadır. Yaş ortalaması 57,1±11,2 olarak saptanmıştır. Çalışmaya katılanlar büyük oranda şehir merkezinde 105 (%52,5) ikamet etmekte idi. Direkt mikroskopi ile *Blastocystis* saptanan toplam 18 örneğin tamamı PZR ile pozitif bulunmuştur. Ayrıca kontrol grubunda direkt mikroskopi ile negatif çıkan iki numune PZR ile pozitif bulundu. İstatistiksel olarak gruplar arasında

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anlamlı bir fark saptanmamıştır. Direkt mikroskopi yönteminin duyarlılığı %81,81, özgüllüğü ise %100 olarak hesaplandı. *Blastocystis* alt tiplerinin analizinde dokuzunun (%45) ST3, yedisinin (%35) ST2, üçünün (%15) ST1 ve birinin (%5) ST6 olduğu görülmüştür.

Sonuç: *Blastocystis* diyabetli hastalarda dikkate alınması gereken bir patojendir. Çalışmamız tip 2 DM hastalarında *Blastocystis* prevalansını ve klinik özelliklerini araştıran az sayıdaki çalışmadan biridir.

Anahtar Kelimeler: *Blastocystis* spp., genotip, diabetes mellitus

Introduction

Blastocystis spp., first described in 1911, is the most common intestinal protozoan found in human stool samples. The life cycle of *Blastocystis* has not been fully elucidated due to many intermediate forms that have not yet been identified (1). At least 30 subtypes of *Blastocystis* have been confirmed and 14 subtypes have been identified in humans. ST3 has been reported most frequently, followed by ST1 (2).

It has been reported that the high prevalence detected in developing countries is due to the risk factors for *Blastocystis* transmission, such as sanitation, contaminated food and drinking water, and contact with animals (3). In our country, the prevalence of *Blastocystis* has been reported to vary between 1% and 24% (2). Although it is often asymptomatic, it has been reported that non-specific symptoms such as diarrhea, abdominal pain, nausea, bloating, weight loss, fatigue, vomiting, constipation and excessive gas were observed in studies on the clinical findings of cases with *Blastocystis* (4).

The widespread use of molecular methods in diagnosis facilitates the detection of *Blastocystis*. Polymerase chain reaction (PCR) is one of the molecular methods accepted as the gold standard for detecting *Blastocystis* (5).

Blastocystis infections were studied in patients with colorectal cancer and acquired immunodeficiency syndrome (AIDS) (4). However, the number of studies investigating the presence of *Blastocystis* in patients with DM is relatively few. This study aimed to determine *Blastocystis* spp. incidence, genotypes and role in gastrointestinal symptoms in type 2 diabetes mellitus (DM) patients.

Materials and Methods

Stool samples were collected from 100 patients diagnosed with type 2 DM and 100 healthy volunteers as a control group in Aydın Adnan Menderes University Faculty of Medicine. Written informed consent was obtained from the volunteers before sample collection. A standardized questionnaire was designed presence of gastrointestinal symptoms. All procedures performed in this study were approved by the Ethics Committee of Aydın Adnan Menderes University Faculty of Medicine, with decision no 2017/1090 (date: 09.02.2017).

Stool samples were examined directly microscopically with Lugol's Iodine solution. DNA isolation was performed using a commercial kit (MagPure Stool DNA Kit) to determine *Blastocystis* by PCR. After DNA isolation, *Blastocystis* approximately 600 bp SSU-rDNA gene amplification performed using primers RD5 (forward) (5'-ATC TGG TTG ATC CTG CCA GT-3') and *Blastocystis* specific BhRDr (reverse) (5'-GAG CTT TTT AAC TGC AAC AAC G-3') (6).

Sequences were also submitted to phylogenetic analysis and published reference sequences from the GenBank database to confirm the subtype classification.

The samples were sequenced by a commercial Company with the Applied Biosystems 377 DNA Sequencer instrument. Sequence data were sequenced using the ClustalW software Bioedit for Windows (Tom Hall) with reference sequences downloaded from https://www.ncbi.nlm.nih.gov/genbank.

Reference sequences used for this purpose are: EU445486, KF848573, AB107967, EU445488, JF792497, EU445492, KF848534 for genbank samples ST1; AB070987, DQ232794, EU679345, JF792496 and JN003684 genbank sequences for ST2; sequences KF848561, AB070988 and JN587546 were included in the analysis for ST3.

Edited *Blastocystis* partial SSU rDNA sequences were compared with the sequences in the Genbank nucleotide database on a web-based basis using Basic Local Alignment Search Tool software.

After sequencing and editing, a genetic distance referenced tree was created by Neighbor-Joining method, 1,000 repetitive bootstrap test in Molecular Evolutionary Genetics Analysis program. The 18S ribosomal RNA gene of *Proteromonas lacertae* was used as the outgroup while plotting the tree based on genetic distance.

Statistical Analysis

The positivity of *Blastocystis* spp. was compared between diabetic patients and the control group using the chi-square test. Classified data were evaluated using the chi-square test while analytical data were assessed using the Student's t-test. Statistical data analysis was performed using the SPSS 17.0 Windows® program, and a significance level of $p<0.05$ was determined.

Results

The presence of *Blastocystis* was investigated in 200 samples, including 100 with DM and 100 controls. Of the cases, 81 (40.5%) were female, and 119 (59.5%) were male. The mean age was 57.1±11.2, with an age range of 21-86. Most of the participants resided in the city center (105,

52.5%), while 49 (24.5%) lived in districts, and 46 (23%) lived in villages.

Direct microscopic examination of stool samples revealed *Blastocystis* in nine individuals in the DM group and nine in the control group. There was no significant difference between the groups regarding *Blastocystis* positivity (p>0.05).

In the isolated genomic DNA samples, PCR detected amplification at the expected size (~600 bp) in 9 and 11 samples in the DM and control groups, respectively. All 18 *Blastocystis* samples positive by direct microscopy were also positive by PCR. Additionally, two samples that were negative by direct microscopy in the control group were found to be positive by PCR.

Identification of *Blastocystis* Subtypes

A total of 20 samples found positive by PCR were sent to the commercial company for sequencing, sorted with reference sequences, and evaluated by examining possible wrong readings. Partial SSU rDNA sequences of the edited *Blastocystis* were compared from the GenBank database, and it was determined that nine (45%) were ST3, seven (35%) were ST2, three (15%) were ST1, and one (5%) was ST6. Since the number of subtypes was low, no additional statistical analysis was performed (Table 1).

The samples were sequenced with the subtype reference sequences in GenBank and their evolutionary distances were determined using phylogenetic tools (Figure 1).

When *Blastocystis*-positive and negative cases were compared regarding age, gender, residence, and clinical findings, there was no statistically significant difference between the groups (p>0.05). A comparison of symptoms in *Blastocystis* positive and negative cases is shown in Table 2.

In our study, PCR found positivity in two samples that were not positive by the direct microscopy method. The total number of positives increased to 11 with PCR. As a result, when PCR was accepted as the gold standard, the sensitivity and specificity of the direct microscopy method were calculated as 81.81% and 100%, respectively.

Discussion

Intestinal parasitic infections are globally distributed and their prevalence is higher, especially in developing countries. It has been reported that they can cause serious clinical problems up to death in patient groups with impaired immunity, such as AIDS, organ transplant recipients, diabetics and oncology patients (7,8). In our study group, DM patients, the immune system is suppressed by the effect of hyperglycemia. In this situation, infections are more frequent and severe (9).

The few studies investigating the relationship between DM and parasitic infections suggest that DM is associated with an increased incidence of intestinal protozoa such as *Entamoeba histolytica* and *Giardia duodenalis*, and helminths such as *Ascaris lumbricoides*. Although *Blastocystis* spp. has been one of the most prevalent protozoa in human stool samples, there are many uncertainties, particularly regarding its pathogenic potential. On the other hand, the number of studies examining the relationship between the incidence of DM and *Blastocystis* spp. is quite insufficient (10,11). In a study investigating the prevalence of *Blastocystis* in diabetic patients in Thailand, it was reported that the parasite was detected in 12.3% of DM patients and 9% of the control group. However, the difference detected was insignificant (12). Poorkhosravani et al. (13) reported that *Blastocystis* spp. prevalence is 9.1% in the diabetes group and 10.1% in the control group. Similarly, in our study, 200 cases were evaluated and *Blastocystis* spp. was found in 9% of the patients with DM and 11% of the control group. There was no significant difference between the two groups regarding the

Table 1. Distribution of *Blastocystis* subtype

Group	ST3	ST2	ST1	ST6
DM (n=9)	4	3	1	1
Control (n=11)	5	4	2	-
Total (n=20)	9	7	3	1

DM: Diabetes mellitus

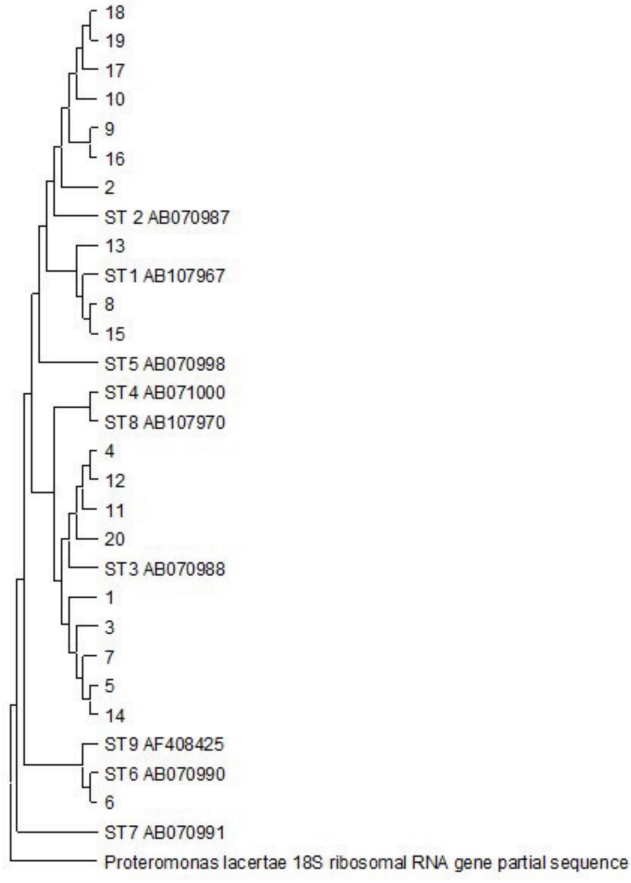


Figure 1. Evolutionary distances of partial SSU rDNA sequences

Table 2. Comparison of symptoms in *Blastocystis* positive and negative cases							
Blastocystis	**Pozitive (n=20)**		**Negative (n=180)**				
Symptom	**Number**	**%**	**Number**	**%**	**Total**	**χ^2**	**p-value**
Pruritus	1	5	21	11.7	22	0.817	0.700
Diarrhea	2	10	9	5	11	0.866	0.352
Abdominal pain	3	15	34	18.9	37	0.181	0.671
Nausea-vomiting	1	5	17	9.4	18	0.434	1.00
Constipation	1	5	4	2.2	5	0.570	0.413

presence of *Blastocystis* (p>0.05). In studies investigating the prevalence of *Blastocystis* spp., it is noteworthy that the incidence of the parasite shows significant differences even in close geographies and similar study groups. In various studies, the prevalence of *Blastocystis* spp. in diabetes patients has been reported to range from 2.7% to 65% (14,15). The positivity rates obtained also differed due to the use of different diagnostic methods, different study designs, the difference in the specificity/sensitivity of the diagnostic methods used, and the differences between the experiences of the laboratory personnel involved in the study. With the widespread use of molecular techniques in recent years, it has been shown that the prevalence of the parasite is much higher than expected (16). In our study, two samples that were negative by direct microscopy in the control group were also found to be positive by PCR.

Many studies comparing different diagnostic methods in the diagnosis of *Blastocystis* spp. Eida and Eida (17) showed that, when PCR was accepted as the gold standard, the sensitivity of direct microscopy was 62.2% and the specificity was 95.7% in the diagnosis of *Blastocystis*. In a similar study, Mohamed et al. (18) showed that 10% of 1330 stool samples had *Blastocystis* spp. by PCR, but only 6.5% were positive by direct microscopy. In a different study in our country, which also included molecular methods, the sensitivity of the direct microscopy method was reported as 12%, and the sensitivity of real-time PCR as 100%, and the high difference between them was remarkable (19). In our study sensitivity and specificity of PCR were found to be 100%. In comparison, the sensitivity of direct microscopy was 81.81% and the specificity was 100%, and these results were similar to other studies.

Blastocystis remains surrounded by many uncertainties, especially regarding its pathogenic potential. Nowadays, the most widely accepted opinion is that the clinical manifestations caused by *Blastocystis* are related to the parasite subtype, the host immune system, parasite density, intestinal microbiota and other accompanying pathogens. The evaluation should be made as a whole, guided by these data. It has been reported that it may be associated with abdominal pain, flatulence, diarrhea or constipation, nausea and anorexia in cases in which the parasite shows pathogenic features (4). In a study investigating symptoms in *Blastocystis* cases, the most common symptom was abdominal pain (40%), followed by bloating (36%) and vomiting (20%) (20). Dogan et al. (21) reported that no significant relationship exists between the presence and subtypes of *Blastocystis* and symptoms. In our study, abdominal pain was the most common (15%) in patients with *Blastocystis* spp., followed by diarrhea (10%). In addition, pruritus, nausea-vomiting and constipation were also seen in 5% of the cases. However, most patients were asymptomatic and no significant correlation was found between parasites and clinical findings (p>0.05).

In our study investigating the prevalence of *Blastocystis* and its role in gastrointestinal symptoms in diabetic patients, the difference between diabetic patients and the control group was not significant, and this result is similar to other studies. In addition, cases with *Blastocystis* and those that were not detected were statistically compared in terms of clinical findings, and no significant correlation was found with any of them. Since the number of subtypes was low, no statistical analysis was performed. The limitations of this study are that the sample size is small, the study period coincides with the COVID-19 pandemic, and a single stool sample can be obtained from the patients. However, our study is one of the few studies investigating the prevalence and clinical features of *Blastocystis* spp. in patients with type 2 DM.

Conclusion

In conclusion, *Blastocystis* spp. is a pathogen that should be considered in patients with diabetes. More comprehensive studies are needed in both diabetic and other immunocompromised patient groups.

Ethics

Ethics Committee Approval: All procedures performed in this study were approved by the Ethics Committee of Aydın Adnan Menderes University Faculty of Medicine, with decision no 2017/1090 (date: 09.02.2017).

Informed Consent: Written informed consent was obtained from the volunteers before sample collection.

Peer-review: Externally peer-reviewed.

Authorship Contributions

Surgical and Medical Practices: Ş.B.Ö., S.E., Concept: Ş.B.Ö., H.E., S.E., Design: Ş.B.Ö., E.M., H.E., Data Collection or Processing: Ş.B.Ö., E.M., İ.Y., E.T., Analysis or Interpretation: Ş.B.Ö., E.M., İ.Y., E.T., H.E., S.E., Literature Search: Ş.B.Ö., E.M., İ.Y., E.T., H.E., Writing: Ş.B.Ö., H.E., S.E.

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

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