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Original Article

Investigation of *Blastocystis hominis* Frequency in Patients with Diabetes by Microscopy and Conventional PCR Methods

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Abstract

Background: We aimed to determine the frequency and subtype of *B. hominis* in diabetic patients.

Methods: One hundred and fifty diabetic patients and 100 healthy people without any chronic disease were included in the study. Stool samples were analyzed by native-Lugol, condensation, trichrome staining and PCR methods.

Results: İn 150 patients with diabetes; *B. hominis* was detected in 38 (25.3%) by PCR, in 34 (22.7%) by native-Lugol and trichrome staining. In the control group, 14 (14%) out of 100 subjects were positive by PCR, and 10 (10%) were positive by native-Lugol and trichrome staining. In the statistical evaluation, a significant difference was found between gender (P=0.023), age (P=0.045; ≤35 and >35 comparison), duration of diabetes (P=0.04), the HbA1c value (P=0.023; <8 and ≥8 comparison), and *B. hominis* positivity. ST1 was determined in 76.9% of patients with diabetes, and ST2 was determined in 23.07%. Considering the 3 methods, *B. hominis* positivity was detected in 40 patients (26.7%) in diabetic group and in 14 participants (14%) in the control group (P=0.011).

Conclusion: *B. hominis* is a factor to be considered in patients with diabetes. Herein, the most common subtype detected in the patients with diabetes mellitus was ST1, but this result was not considered sufficient to reveal the importance of the subtype factor in the pathogenicity of *B. hominis* in patients with diabetes. In this context, there is a need for more comprehensive studies in both diabetic and other immunocompromised patient groups.

Introduction

The taxonomy of the *Blastocystis* was described first by Brumpt in 1912 is still controversial (1). *Blastocystis* sp. has been reported as the most common intestinal protozoan in human stool samples in many studies. There are many unknown points regarding its life cycle, genetic diversity, pathogenicity, relationship with gastrointestinal symptoms, diagnosis, and treatment of the parasite (2).

Symptoms associated with *Blastocystis hominis* infection are nonspecific and usually include diarrhea, abdominal pain, cramping, and nausea. This parasite has been found in healthy individuals as well as in gastrointestinal symptoms, ulcerative colitis, and celiac and crohn's patients (3).

Diabetes is an endocrine system disease that occurs because of partial or absolute deficiency or peripheral ineffectiveness of the insulin hormone secreted from the beta cells of the pancreas. Hyperglycemia and neutrophil dysfunction seen in the course of infection in these patients cause suppression of humoral immunity (4). Although there is no conclusive evidence, it has been suggested that *B. hominis* may be an opportunistic pathogen in immunocompromised patients and that the symptoms associated with this parasitic infection are more severe than in healthy people (3). Symptoms are associated with subtypes of *B. hominis* (5).

In routine laboratories, the diagnosis of *Blastocystis* sp. is usually made with low-sensitivity microscopy-based methods such as native-Lugol and trichrome staining. Methods such as molecular and culture, which have high sensitivity, are mostly used in scientific studies (6-8).

The aim of this study was to determine the frequency of *B. hominis* in diabetic patients and determine which subtype is more common in these patients.

Materials and Methods

Ethics Approval was obtained from the Ethics Committee of Van Yüzüncü Yıl University Non-Interventional Clinical Trials for this study (Decision No: 2019/18-14 & Date: 27/12/2019).

This study was conducted between January 2020 and November 2021, which included 150 patients with diabetes and 100 healthy people without any chronic disease, examined in the Internal Medicine Polyclinics of Van Yüzüncü Yıl University Dursun Odabaş Medical Center or hospitalized in its wards.

Direct microscopy

Stool samples were evaluated by the native-Lugol and trichrome staining methods. Trichrome staining was performed with a commercially available trichrome dye kit (GBL) following the manufacturer's instructions. Trichrome staining preparations X1000, and native-Lugol preparations X100 and X400 were examined under light microscope with magnification.

Genomic DNA isolation from stool samples

DNA extraction was performed for the entire stool samples included in the study, using the stool DNA isolation kit (Norgen, Canada) in accordance with the manufacturer's instructions. The DNA samples were stored at –20 °C until PCR was performed.

Conventional PCR

F1- 50- (5' GGA GGT AGT GAC AAT AAATC 3') and R1-50-(5' CGT TCA TGA TGA ACA ATT AC 3') primers that amplifying the 1100 bp-long region of the 18S SSU rDNA gene were used to detect *B. hominis* (9).

The reaction was adjusted to a total volume of 25 µl containing 12,5 µl of Tag 2x master mix (12.5 mM MgCl2), 0.5 mM MgCl2, 0.2 µM from each primer, 2 µl MgCl2, 2 µl Q solution and 3 µl of sample DNA. The amplifica-

tion was programmed for 35 cycles of 30 s each at 95 °C, 40 s at 55 °C, and 45 s at 72 °C. In addition, a 4-min denaturation step at 95 °C was applied before the first cycle, in addition to an extension phase at 72 °C for 10 min after the last cycle. In order to display the results of the PCR process, 15 µl gel from the reaction products was subjected to electrophoresis and displayed in the UVP Gel documentation system.

Sequence analysis

The analysis of DNA sequencing was carried out on 13 positive PCR samples. The SSU rDNA sequences obtained were compared with the gene sequences available at the gene bank using the BLAST program (http://www.ncbi.nlm.nih.gov/BLAST/): ST-1/U51151, ST-1/AB107962, ST-1/AB070989, ST-2/AB070987, ST-3/AB091234, ST-3/AB070992, ST-4/AY244620, ST-5/AB070998, ST-5/AB107964, ST-5/AB070999, ST-6/AB070990, ST-7/AY590109, ST-7/AF408427. The distance-based analysis was conducted on the sequencing data by using molecular evolutionary genetic analysis version (What's New in SnapGene 3.3) and a phylogenetic tree was constructed using the neighbor-joining method with the Geneious Prime 2022.1 model. The support of monophyletic groups was assessed by the bootstrap method with 1,000 replicates.

Statistical analysis

Frequency distributions of strains and demographic information were analyzed by descriptive statistical methods, and qualitative variables were analyzed by the chi-square test.

Results

The ratio numbers of the females to males in patients and the control groups were 83/67 and 43/57 respectively. The mean age of the patients was 52.52±14.67 years while in the control group was 27.143±20.42 years.

B. hominis was detected by PCR in 38 (25.3%) of 150 diabetic patients, and by native-Lugol and trichrome staining in 34 (22.7%) patients. Of the 100 healthy individuals included in the control group, 14 (14%) were determined as positive by PCR, and 10 (10%) as positive by native-Lugol and trichrome staining. Considering the 3 methods, *B. hominis* positivity was detected in 40 of the patients with diabetes (P=0.011).

A total of 250 samples, taken from 150 patients and 100 healthy individuals (control group), were included. *B. hominis* positivity was detected by PCR in 52 (20.8%) samples and by native-Lugol staining in 44 (17.6%) samples. Two of the 44 samples (in the diabetic group) found by native-Lugol were negative by PCR. Three of the 44 samples (2 in the diabetic group and 1 in the control group) found by the trichrome staining method were negative by native-Lugol. Of the 52 samples that were positive by PCR, 10 (6 in the diabetic group and 4 in the control group) were negative by native-Lugol.

When PCR was accepted as the gold standard, the sensitivity of the microscopy was 80.76% and the specificity was 98.99%. When microscopy was accepted as the gold standard, the sensitivity and specificity of the PCR were calculated as 95.45% and 95.14%. Sensitivity and specificity calculations were made according to the results in Table 1.

Table 1: Sensitivity and specificity results according to the gold standard method

When PCR was taken as the gold standard					
Method		**PCR**		**Sensitivity**	**Specificity**
		Positive	**Negative**		
Native-Lugol	Positive	42	2	80.76%	98.99%
	Negative	10	196		
When native-Lugol was taken as the gold standard					
		Native-Lugol		Sensitivity	Specificity
		Positive	Negative		
PCR	Positive	42	10	95.45%	95.14%
	Negative	2	196		

In the statistical evaluation, a significant difference was found between gender (*P*=0.023), age (*P*=0.045; ≤35 and >35 comparison), duration of diabetes (*P*=0.04; <10 years and ≥10 years), HbA1c value (*P*=0.023; <8 and ≥8) and *B. hominis* positivity. There was no significant difference between the place of residence, diabetes type, cholesterol, fasting blood sugar, and LDL levels and the positivity of this parasite (Table 2).

Table 2: *B. hominis* positivity according to potential risk factors in patients with diabetes

Variable	***Patients with diabetes***			***B. hominis Positive***		***P value***
	(N: 150)			**(N: 40)**		
	Risk factor	**n**	**%**	**n**	**%**	
Gender	Male	67	44.6	24	35.82	**0.023**
	Female	83	55.3	16	19.27	
Age(yr)	≤35	16	10.66	8	50	**0.045**
	>35	134	89.33	32	23.88	
	≤20	7	4.6	4	57.14	0.082
	>20	143	95.33	36	25.17	
Place of residence	Rural	28	18.66	7	25	0.822
	Urban	122	81.33	33	27.04	
Duration of diabetes	<10	113	74.66	25	22.12	**0.04**
	≥10	37	25.33	15	40.54	
Diabetes type	Tip 1	11	7.33	6	54.54	0.051
	Tip 2	139	92.66	34	24.46	
HbA1c	≤6	19	12.66	5	26.31	0.955
	>6	130	87.24	35	26.92	
	<8	90	60.4	18	20	**0.023**
	≥8	59	39.59	22	37.28	
Cholesterol	≥200	65	43.33	17	26.15	0.912
	<200	75	50	19	25.33	
Fasting blood sugar	<300	137	92.56	35	25.54	0.481
	≥300	11	7.43	4	36.36	
	<200	109	73.64	26	23.85	0.269
	≥200	39	26.35	13	33.33	
	<126	55	37.16	13	23.63	0.558
	≥126	93	62.83	26	27.95	
LDL	<160	114	82.6	34	29.82	0.623
	≥160	24	17.39	6	25	

N: Total number of patients, n: Number of positive patients, LDL: low-density lipoprotein

ST1 was determined in 10 (76.9%) of 13 samples and ST2 was determined in three (23.07%) samples belonging to patients with diabetes. The 1100 bp band images of the *B. hominis*-positive samples amplified by PCR and visualized in gel electrophoresis are given in Fig. 1, the pedigree of the isolates subtyped because of sequence analysis is given in Fig. 2, and the GenBank numbers of the subtyping isolates are given in Table 3.

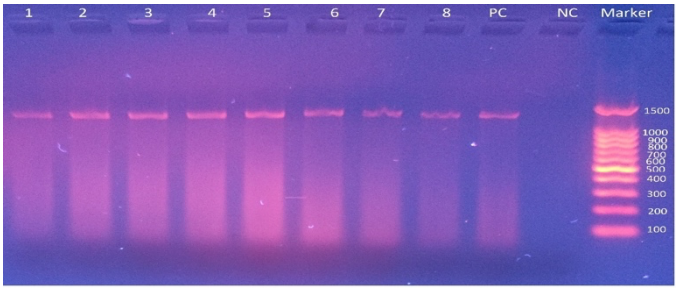

Fig. 1: Band images of the *B. hominis*-positive samples, which were amplified by PCR and visualized in gel electrophoresis (in the presence of 0.15% ethidium bromide and 1X tris boric acid-EDTA buffer) (Marker: 100 bp DNA marker (Grisp Mark), PK: Positive control, NK: Negative control)

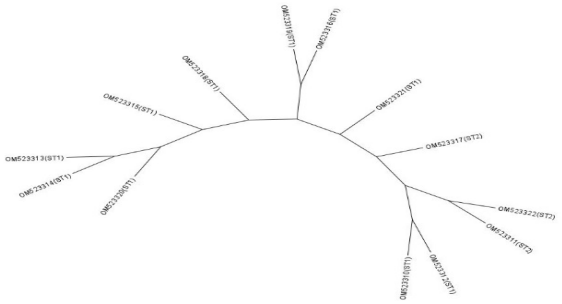

Fig. 2: Pedigree of the isolates subtyped because of the sequence analysis

Table 3: GenBank numbers of the subtyped isolates

Examined Isolate No.	*GenBank Acceptance No.*	*Subtype*
BLSVAN1	OM523310	ST1
BLSVAN3	OM523311	ST2
BLSVAN18	OM523312	ST1
BLSVAN35	OM523313	ST1
BLSVAN60	OM523314	ST1
BLSVAN63	OM523315	ST1
BLSVAN68	OM523316	ST1
BLSVAN80	OM523317	ST2
BLSVAN87	OM523318	ST1
BLSVAN110	OM523319	ST1
BLSVAN112	OM523320	ST1
BLSVAN126	OM523321	ST1
BLSVAN132	OM523322	ST2

Discussion

Some intestinal parasites known to be apathogenic, such as *B. hominis*, may become pathogenic in immunocompromised patient groups, such as those with AIDS, organ transplant recipients, diabetes, and oncology patients (10).

Studies were conducted to compare the frequency of *B. hominis* in diabetic patients and healthy control groups. In these studies, in patients with diabetes and those in the control groups (two groups) the parasite was determined, respectively, at rates of 9.1% and 10.1% by Poorkhosravani et al (11), 2.7% and 1.2% by Tangi et al (12), 9.3% and 2.5% ($P<0.05$) by Mohtashamipour et al (13), 42% and 4% ($P<0.05$) by Aourarh et al (14), 24.4% and 23.2% by Bafghi et al (15), 12.3% and 9% by Popruk et al (16), 65% and 25% by Ibrahim et al (17), as well as 12.1% and 7.9% by Melo et al (18).

In this study, *B. hominis* was detected in 26.6% of the patients with diabetes and 14% of those in the control group, and there was a statistically significant difference between the two groups in terms of the positivity of this parasite ($P=0.011$). The statistical results obtained in the above two studies (13, 14) were similar to the current study. This finding shows that this parasite should be considered as a potential risk factor in patients with diabetes.

In some studies that evaluated the effect of age on *B. hominis* positivity (19-22), no significant difference was found between the positivity of the parasite and age. In some studies (23-25), a statistically significant difference was found between age and the positivity of the parasite, similar to the finding obtained in the current study (≤35 and >35 comparison; $P=0.045$).

In this study, *B. hominis* was determined at a higher rate in men with diabetes than in women, and a statistically significant difference was found between gender and blastocystosis positivity. Similar findings were obtained in a study by Mohtashamipour et al (13). In some studies (19, 21, 25-27), no statistically significant difference was found between the positivity of this parasite and gender.

Few studies have evaluated the relationship between *B. hominis* positivity and the duration of diabetes. Popruk et al (16) reported that a duration of diabetes for more than 10 years affected the positivity of this infection and when statistically evaluated, there was a significant difference between the duration of blastocystosis and diabetes. The results of the study herein showed parallelism with their study. In two other studies (28, 29), unlike this study, there was no relationship between the duration of diabetes and this infection.

No study could be found comparing the HbA1c level, which is an important parameter in the control and treatment of diabetes, and *B. hominis* positivity. In the current study, a significant difference ($P=0.023$) was found between the HbA1c (<8 and ≥8 comparison) levels and *B. hominis* positivity in patients with diabetes. With the increase in the HbA1c level, immunity may also be impaired, which may lead to opportunistic parasitic infections such as *B. hominis*.

B. hominis has been diagnosed using different diagnostic methods in many studies, and the positivity rate obtained according to the method used also differed. When PCR was accepted as the gold standard, the sensitivity of the microscopic view was 36.2%-73.4%, and the specificity was in the range of 66.7%-99.4% (6, 30, 31). In this study, native-Lugol, trichrome staining, and PCR method were used to detect this parasite, and positivity was determined via native-Lugol and trichrome staining in 17.6% of 250 samples and via PCR in 20.8%. When PCR was accepted as the gold standard, the sensitivity of native-Lugol was 80.76% and the specificity was 98.99%; When native-Lugol was accepted as the gold stand-

ard, the sensitivity and specificity of the PCR were calculated as 95.45% and 95.14%. These results were based on the sensitivity and specificity of the microscopy detected in three studies (6, 30, 31); higher sensitivity and specificity than PCR detected in a study. The results determined herein confirmed that the sensitivity and specificity of PCR is higher than that of microscopy, as reported in the literature.

Two studies on subtypes of *B. hominis* were found on patients with diabetes, which were also evaluated in the current study. In Thailand, Popruk et al (16) identified ST1 (3%) and ST3 (9.3%) in the patient group, and ST1 (2%), ST3 (6%) and ST4 (1%) in the control group. In Brazil, Melo et al (18) identified ST1 (38.2%), ST2 (11.8%), ST3 (35.3%), ST6 (2.9%), and ST7 (2.9%) in the patient group, and ST1 (21.8%), ST2 (21.8%), ST3 (43.5%), and ST6 (4.4%) in the control group.

Mohamed et al (9) identified ST1 (38%), ST2 (40%), and ST5 (22%) in 50 patients with and without cancer. Adıyaman Korkmaz et al (8) studied 43 patients with and without diarrhea and identified ST1 (13.9%), ST2 (7%), ST3 (28%), ST4 (11.6%), ST6 (2.3%), and ST7 (11.6%).

In Diyarbakır, 345 individuals (264 immunosuppressed patients and 81 healthy individuals) were evaluated for *Blastocystis* and 69 were positive for this parasite. In their study, ST1 (17.4%), ST2 (17.4%), ST3 (60.9%), ST5 (1.4%), and ST6 (1.4%) were detected in the positive samples. Moreover, the rates of ST1 and ST2 were higher in the symptomatic patient group, as was that of ST3 in the control group (32).

ST1 was more common than ST3 in symptomatic patients (33). ST4 was the most common subtype in patients with acute diarrhea (34). ST1, ST2, and ST4 were associated with gastrointestinal symptoms and ST3, the most frequently isolated, was not responsible for any symptoms (35). Hussein et al (36) stated that ST1 was the most lethal, and ST3 and ST6 were composed of pathogenic and non-pathogenic strains. ST1 was associated with symptomatic infection and ST2 was associated with asymptomatic infection (37).

Hussein et al (36) found, in their study on symptomatic and asymptomatic humans and rats, that ST1 was clinically and statistically correlated with the pathogenicity of *B. hominis*, while ST2 was not. Stensvold and Clark (38) stated that a certain subtype of this parasite has not been associated with any disease thus far. ST4 was the only subtype identified in patients with acute diarrhea in Denmark, but the overall prevalence of the parasite was lower than in other studies conducted in this patient group in Denmark. They reported that ST4 was predominant in symptomatic patients in Spain.

It is seen that different results were obtained in the studies in which the subtypes of *B. hominis* were evaluated above. Four of the studies were on ST1 (18, 33, 35, 36), one was on ST2 (9), and ST3 (8, 16, 32) was dominant in four of them. In this study, ST1 was determined at a rate of 76.9% and ST2 23.07%, and this result was found in the four studies given above (18, 33, 35, 36), were consistent with the results. ST1 was high in asymptomatic diabetic patients in this study, and in symptomatic patients in three other studies (33, 35, 36).

Conclusion

B. hominis is a factor to be considered in patients with diabetes. Those who have had diabetes for more than 10 years are at greater risk for this parasite. PCR has high sensitivity and specificity in diagnosis and it would be appropriate to use it together with native-Lugol or trichrome staining if possible. Although ST1 was the most common subtype detected in asymptomatic diabetic patients in this study, this result was not considered sufficient to reveal the importance of the subtype factor in the pathogenicity of *B. hominis* in diabetic patients. In this context, there is a need for more comprehensive studies in both diabetic and other immunocompromised patient groups.

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Conflict of Interest

The authors declare that there is no conflict of interests.

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