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Original Article

Molecular Assessment of *Blastocystis hominis* in Patients with Diabetes Mellitus in Balad City, Saladin Province, Iraq

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Abstract

Background: *Blastocystis* sp. is the most prevalent intestinal protozoa found worldwide in both humans and animals. **Objectives:** This study aims to identify the subtypes of *Blastocystis* sp. parasite isolated from diabetes mellitus patients using molecular techniques such as polymerase chain reaction (PCR), DNA sequencing, and phylogenetic analysis. **Materials and Methods:** The current study was conducted on samples collected from a total of 300 patients (150 diabetic patients and 150 non-diabetic patients). The samples were taken between November 2022 and May 2023 at the laboratories of the General Balad Hospital in Balad City, Saladin, Iraq. Blood and stool samples were taken from each patient, and the investigation included microscopic examination and culturing of the samples on Modified Jones' Medium for parasitic identification; active infection was confirmed genetically using PCR, and subtyping was done via sequencing of the SSU rRNA gene. **Results:** Microscopically, only 80 diabetic patients (50 males and 30 females) were infected with *Blastocystis hominis,* and only 50 people (13 males and 37 females) among the healthy participants had *B. hominis* infection. Thirty samples were further studied using PCR, and only 16 showed clear bands on the gel, whereas only eight samples were successfully sequenced. **Conclusion:** This study demonstrated that diabetic mellitus patients (DM group) had a higher prevalence of *B. hominis* infection compared to the non-DM group. Furthermore, there is a link between the presence of *Blastocystis* and other risk factors, such as place of residence, water source, and keeping animals, which suggests that exposure to animal stools may result in the development of *Blastocystis* ST1 among the studied participants.

Keywords: *Blastocystis hominis*, diabetes mellitus (DM), SSU rRNA gene

Introduction

Blastocystis sp. is the most prevalent intestinal protozoa found worldwide in both humans and animals.[1] Morphologically, *Blastocystis* is a highly polymorphic organism that can take on a variety of forms throughout its life cycle, including vacuolar, cystic, ameboid, granular, multi-vacuolar, and avacuolar forms.[2] *Blastocystis* life cycle involves animal hosts, and eligible hosts can become infected with *Blastocystis* following ingestion of raw aquatic plants contaminated with cysts, or by drinking untreated water. Recent research has revealed that dirty hands can act as a vehicle for the direct contact transmission of cysts from infected people or contaminated soil.[3] The cysts only become vegetative after being ingested by the appropriate host; the cyst form is excysted in the large intestine to release the vacuolar form, which can then assume any of the other forms. The ameboid, avacuolar, and multi-vacuolar forms are frequently observed, raising the hypothesis that these forms may contribute to the pathophysiology of diarrhea.[4] The parasite, as per studies, is only transmitted in its cyst stage.[5]

Anorexia, diarrhea, abdominal pain, bloating, lethargy, and more gastrointestinal symptoms are only a few of the digestive symptoms related to *Blastocystis* parasite infection.[6] *Blastocystis* sp. pathogenicity appears to be significantly influenced by proteases, especially cysteine

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protease.[7] The risk factors for *Blastocystis* transmission include inadequate hygiene, contaminated food, contact with animals, and drinking of contaminated water.[8] These risk factors are likely responsible for the high prevalence of *Blastocystis* infection in underdeveloped nations. Infections with *B. hominis* are more common among immunocompromised people and people who have close exposure to animals, according to epidemiological studies.[9]

Immunosuppressed patients with persistent diarrhea frequently suffer from *Cryptosporidium parvum*, *Blastocystis hominis*, and *Cystoisospora belli* infection.[10] *B. hominis* has been identified in many cases of uncontrolled diabetes. There have been reports of parasite-induced immunosuppression in diabetes mellitus (DM) patients;[11] for instance, the prevalence of opportunistic parasites has been investigated in Greater Cairo among immunocompromised patients, of whom 30 were diabetic patients recruited from Al-Azhar University Hospitals. The findings revealed that 6% of the diabetic patients had opportunistic parasite infections.[12]

DM is a well-known metabolic disorder marked by high blood glucose levels because of relative or absolute insulin insufficiency, as well as other pathophysiological deficiencies.[13] The characteristic signs of polyuria, polydipsia, and polyphagia are associated with this elevated blood sugar level.[14] There are two forms of diabetes: type I DM or insulin-dependent diabetes, which occurs when the pancreas is unable to produce insulin, and type II DM characterized by insufficient insulin production.[15] The high occurrence of intestinal parasitic infections in many nations and its negative effects on nutrition has made it a significant public health concern on a global scale.[16] People with DM may have some parasite infections more frequently than non-diabetics. Diabetes is believed to be a long-lasting, chronic issue that gradually increases blood pressure, malfunctioning, and dysfunction in numerous organs; as a result, it increases a person's susceptibility to a variety of illnesses, particularly infectious diseases.[17]

Microscopic examination lacks sensitivity in diagnosing *Blastocystis sp.* compared to molecular methods such as polymerase chain reaction (PCR), which are utilized to identify the parasite. The benefits of PCR methods go beyond just parasite identification; since some genetic studies have shown that some subtypes (STs) of parasites *Blastocystis* are nonpathogenic, like ST2, while others are pathogenic, such as ST1. Hence, it has been reported that the absence of symptoms in people infected with *Blastocystis* parasites does not indicate the non-pathogenicity of the parasites.[18]

Most symptomatic patients are infected by the pathogenic STI subtype; hence, there is a link between this subtype and intestinal symptoms.[19] ST1 was found to be significantly more common in patients with irritable bowel syndrome and chronic gastrointestinal illnesses compared to people without symptoms. Therefore, studies have suggested a possible correlation between ST1 and a parasite's pathogenic potential.[20] Also, a previous study found that the prevalence of ST1 was higher among diabetic patients, followed by ST3.[21] Metronidazole (MTZ) has been the drug of choice for treating infections caused by *Blastocystis*; however, the need for safer and more effective alternative therapies is driven by the dangerous side effects and potential development of drug resistance to the conventional medication, MTZ. Therefore, recent studies have examined the effectiveness of several plant and herbal extracts against this parasite.[1,22,23] Researchers have underlined the necessity for accurate *Blastocystis* testing to diagnose patients and to separate treatments that completely eradicate the organism from those that just temporarily relieve symptoms.[24] Thus, this work aims to identify subtypes of *Blastocystis sp.* parasite isolated from diabetic mellitus patients using molecular techniques such as PCR, DNA sequencing, and phylogenetic analysis.

Materials and Methods

Study population

The current study was conducted on samples collected from 300 Iraqi patients (4–70 years old) attending the clinic at the General Balad Hospital in Saladin Province, Iraq; the sample size consisted of 150 DM patients and 150 non-DM patients (control group). The study was conducted from November 2022 to May 2023.

Collection of blood and stool samples

Blood and stool samples were taken from each patient and stored appropriately for laboratory analysis. Disposable syringes were used for the blood collection. Two milliliters of venous blood was drawn and preserved in an EDTA container for further analysis. Stool samples from each participant were taken in a clean container for the detection of *Blastocystis sp.* in three steps (wet mounts, *in vitro* cultivation, and gnomic DNA isolation).[25]

DNA extraction and PCR procedure

The samples that were positive in culture and/or microscopic methods were subjected to PCR. Suspensions of *Blastocystis* were centrifuged at 50×*g* for 5 min. The pellet was re-suspended in phosphate buffer saline solution, and the process was repeated five times. The final pellet was stored at −20°C for DNA extraction and further molecular assays.[26] Genomic DNA was isolated from the stool samples following the protocol of the QIAamp® Fast DNA Stool Mini Extraction Kit [Tables 1 and 2].

After PCR amplification, agarose gel electrophoresis was used to confirm the presence of amplification. The agarose

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Table 1: Primers used in this study for the detection of *B. hominis*

Primer name	Sequence 5′–3′	Annealing temperature (ºC)	Product size (bp)
SSU rRNA-F	GGA GGT AGT GAC AAT AAATC	53	1100
SSU rRNA-R	ACT AGG AAT TCC TCG TTC ATG	53	1100

Table 2: PCR program and steps

Steps	Annealing temperature (°C)	*m:s*	Cycle
Initial Denaturation	95	05.00	1
Denaturation	95	00.30	30
Annealing	60	00.30	
Extension	72	01.00	
Final extension	72	07.00	1
Hold	10	10.00	

solution was poured into the gel tray after both the edges were sealed with cellophane tape; the agarose was left to solidify at room temperature for 30 min. The comb was carefully removed, and the gel was placed in the gel tray. The tray was filled with 1× TAE-electrophoresis buffer until the buffer reached 3–5 mm over the surface of the gel. PCR products were loaded directly; 10 µL of the PCR product was loaded directly into the well. Electrical power was turned on at 100 v/mAmp for 75 min for the extracted DNA to move from the cathode to the anode poles in the electric field. The ethidium bromide-stained bands in the gel were visualized using a gel imaging system.

Sequencing and phylogenetic analysis of isolated *Blastocystis* sp.

The 1100-bp segment of the *Blastocystis* SSU rRNA gene's positive PCR results was sequenced in one direction. The PCR products were sent for Sanger sequencing using ABI3730XL, an automated DNA sequencer, manufactured by Macrogen Corporation – Korea. STs and ST (SSU-rDNA) alleles were called using the sequence query facility in the *Blastocystis* Sequence Typing website available at http://pubmlst.org/ *Blastocystis*. Subtypes were identified by determining the exact match or closest similarity to all known *Blastocystis* subtypes according to the classification by Stensvold *et al.*[27]

Ethical approval

The study was carried out in conformity with the ethical standards outlined in the Helsinki Declaration. Sample collection was done with the patient's verbal and verbal consent. A local ethics committee examined and approved the study protocol, as well as the subject information and permission form, in accordance with document number (1933) on (October 31, 2022) to get approval.

Results

Stool samples from the test (150 diabetic patients) and control (150 non-diabetic patients) groups were examined for the presence of *Blastocystis* using microscopic examination following staining using direct lugol's iodine. Positive samples from the microscopy stage were cultured on Jones' medium, the preferred medium for the growth of *Blastocystis* sp.[25] A total of 80 samples from the DM group tested positive for *B. hominis* on both microscopy and culture tests, while only 50 samples were positive from the non-diabetics group. The vacuolar form of the parasite was the most seen in the investigations, followed by the granular form [Figure 1].

Detection of the SSU rRNA gene

From a total of 80 diabetic patient samples that had been recorded as microscopically positive for *B. hominis*, only 30 samples were tested genetically using PCR analysis due to the high cost and time implications of the PCR. The conserved 1.1 Kbp fragment was amplified for the identification of the *Blastocystis* isolates, and subsequently the SSU rRNA gene target was verified using the previously mentioned primer Blas-F: (GGA GGT AGT GAC AAT AAA TC) and Blas-R: (ACT AGG AAT TCC TCG TTC ATG).[28] Only 16 of the 30 samples showed clear bands on the gel that matched the estimated product size (1100 kbp), while the remaining 14 samples showed faint bands possibly due to insufficient quantity of isolated DNA by PCR [Figure 2].

Additionally, eight PCR products were sent to South Korea for sequencing to confirm the positivity of infection

Figure 1: Vacuolar forms in the direct lugol's iodine smear (black arrow) (×40)

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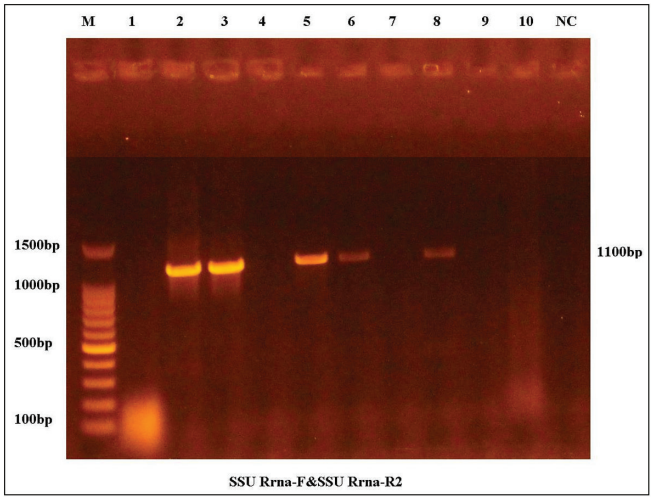

Figure 2: Results of the amplification of the SSU rRNA gene of stool samples were fractionated on 1.5% agarose gel electrophoresis stained with Eth.Br. M: 100-bp ladder marker. Lanes 1–10 resemble 1100-bp PCR products

and determine the genetic distance between the isolates and other isolates listed in the GenBank. Four out of the eight PCR products had clear bands, while the other four samples had faint bands on the gel. These eight samples were completed and proved to be the targeted parasite for this study. From the eight samples that were successfully sequenced, only two sequences shared a high degree of similarity (98.78%–100%) with *Blastocystis* sequences listed in the GenBank; they were almost compatible with *Blastocystis* ST1and ST3. However, the other six samples shared different degrees of similarity with *Blastocystis* sequences listed in the GenBank, possibly due to the occurrence of point mutation in some nucleotide bases. The nucleotides of the sequences produced in this investigation have been deposited in the GenBank database under the following accession numbers: OQ594924, OQ594881, OQ589913, OQ589912, OQ586443, and OQ581724. The results of the sequence analysis were also evaluated to determine the phylogenetic tree of the parasite species and the extent of their genetic heterogeneity with the subspecies.

Discussion

Blastocystis sp. is an intestinal protozoan most commonly reported in humans, especially in children and adults in developing countries; it has a worldwide distribution, with a prevalence of 30%–60% in developing countries and 1.5%–20% in developed countries.[26] Diabetic patients should have regular checks for parasite infections owing to the strong link between diabetes and gastroenteritis caused by intestinal parasites.[29] Diabetic patients have a weakened immune system and are more likely to be develop an intestinal parasite infection.[30] Based on the outcomes in Table 3, it is observed that the outcome of this work concurred with those of earlier studies,[31,32] where the incidence of parasitic infection was higher in diabetic patients compared to the control non-diabetic group. Conversely, another study[33] found that people with DM (T1DM + T2DM) have a lower likelihood of developing intestinal parasitic infections. The results of this study showed significant differences between genders, residence, source of drinking water, and keeping animals ($P \leq 0.05$), but there was no statistically significant correlation between the infection and age or educational level ($P > 0.05$).

Direct genotyping of the isolates was performed using the BLAST software to compare representative sequences of the various *Blastocystis* subtypes.[27] Numerous investigations have been conducted in recent years to determine the subtypes of *Blastocystis sp*. that were circulating in the human population,[34] with the most prevalent human subtypes in the world shown to be ST1–ST4.[35] The nucleotide sequences in the present study were recognized as belonging or almost compatible with two separate subtypes ST1 and ST3. *Blastocystis* ST1 was the most common subtype and was identified in almost all people who had pets at home, followed by ST3. *Blastocystis ST1* has been linked to zoonotic transmission to humans according to some research;[7] this is almost consistent with the observation of a study conducted in the Midwest region, which concluded that the higher prevalence subtype of *Blastocystis* among diabetic patients was ST1.[24] Accordingly, *Blastocystis ST1* was the most common subtype found in this study, followed by ST2 and ST3.[7] However, a study conducted in 2020 among diabetic patients concluded that the most prevalent subtype was ST3, followed by ST1, and ST4.[36] The phylogenetic analysis of the nucleotide sequences of the six *Blastocystis*-positive samples was compared with the reference subtype sequences in the GenBank.

The phylogenetic tree in Figure 3 consists of two main groups; the first group consists of one original isolate for this parasite, which showed 100% homology. Ten isolates, including those identified in this study, with the accession number, OQ586443, are part of the second group. The tree showed that these sequences were near to a standard sample in the GenBank (Query). Additionally, the phylogenetic tree in Figure 4 showed that our isolate with the accession number OQ594881 was also close to the origin of the species of this parasite and was located on the same cluster close to ST1.

The phylogenetic tree in Figure 5 showed that our samples with the accession numbers OQ589912 and OQ581742 were close to ST3 and not far from the species of the parasite. This can be explained by the fact that despite the occurrence of mutation, the parasite still conserved its genetic material as the mutation did not lead to the emergence of a new subspecies or deterioration of the genetic material. According to Moghaddam *et al.,* more cases of treatment failure were being reported, particularly in patients with severe *B. hominis* infections, proving that different patients have varying responses to

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Table 3: Detection of *Blastocystis hominis* in patients with diabetes mellitus in Balad City, Saladin Province, Iraq

Risk factor	No. of diabetic patients positive for the parasite	%	*P* value	No. of non-diabetic patients positive for the parasite	%	*P* value
Gender						
Male	50	62.5	0.01*	13	26.0	0.01*
Female	30	37.5		37	74.0	
Age (years)						
4–14	10	12.5	0.548	5	10.0	0.01*
15–24	8	10.0		9	18.0	
25–34	9	11.3		6	12.0	
35–44	8	10.0		12	24.0	
45–54	16	20.0		10	20.0	
55–70	29	36.2		8	16.0	
Residence						
Urban	14	17.5	0.01*	7	14.0	0.01*
Rural	66	82.5		43	86.0	
Level of education						
Non-educated	46	57.5	0.446	31	62.0	0.01*
Primary	16	20.0		11	22.0	
High	18	22.5		8	16.0	
Source of drinking water						
Treated	28	35.0	0.01*	26	52.0	0.01*
Untreated	52	65.0		24	48.0	
Keeping animals						
Yes	58	72.5	0.01*	42	84.0	0.01*
No	22	27.5		8	16.0	

Note:
*P-value < 0.05 is considered statistically significant.

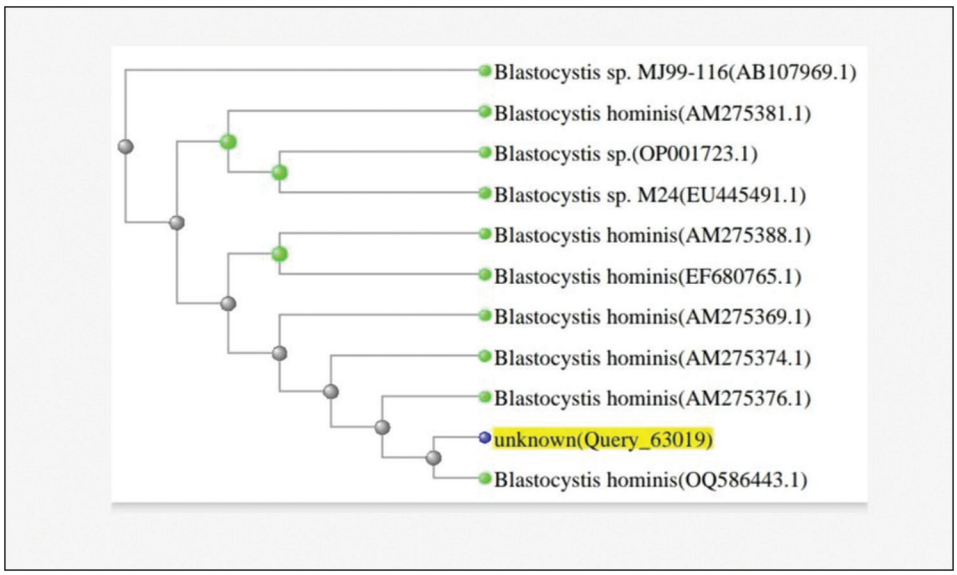

Figure 3: Phylogenetic tree of the SSU rRNA gene sequence of *Blastocystis* isolated from Iraqi patients with diabetes mellitus. The neighbor-joining method was used to build this tree. The Iraqi isolate has been assigned the GenBank accession number (OQ586443). Individual GenBank accession numbers come after species

various treatments.[37] Several factors, including mutations, infection density, MTZ resistance, and, most crucially, subtype or strain-to-strain variability in drug sensitivity have been reported to alter the efficiency of treatment.[21,38] Therefore, the outcomes of the phylogenetic analyses, as previously explained, indicated the presence of mutations in some nucleotide bases, but there was no evolution in the parasite as it remained in the same subtype cluster.

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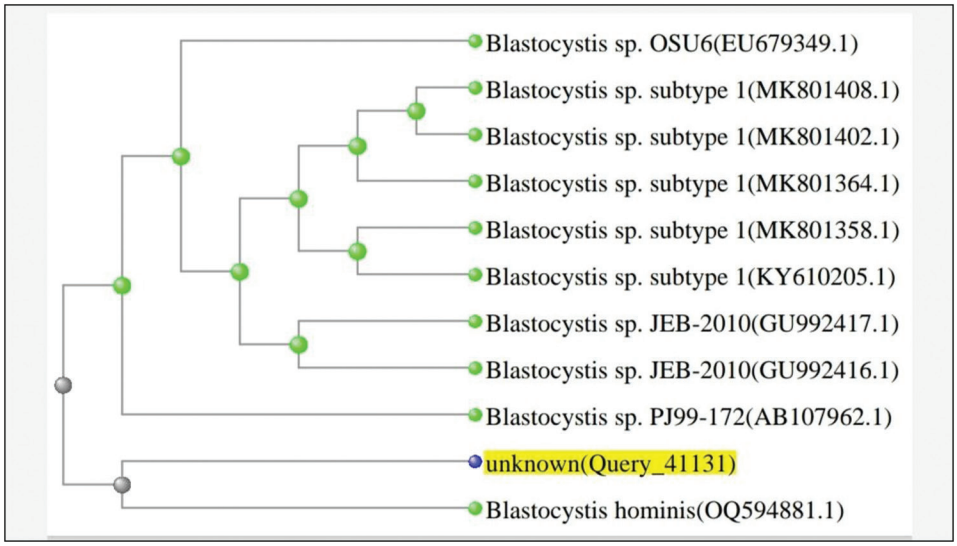

Figure 4: Phylogenetic tree of the SSU rRNA gene sequence of *Blastocystis* isolated from Iraqi patients with diabetes mellitus. The neighbor-joining method was used to build this tree. The Iraqi isolate has been assigned the GenBank accession number (OQ594881). Individual GenBank accession numbers come after species

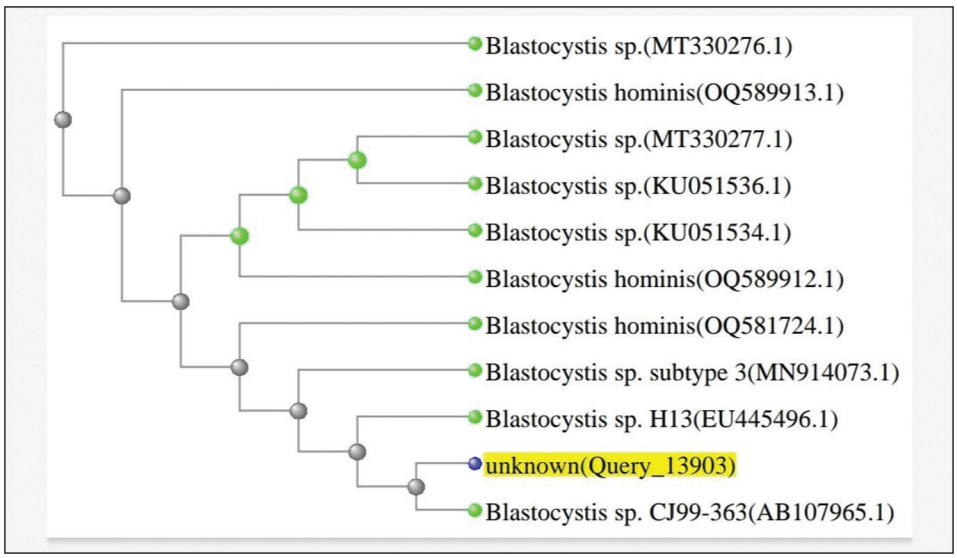

Figure 5: Phylogenetic tree of the SSU rRNA gene sequence of *Blastocystis* isolated from Iraqi patients with diabetes mellitus. The neighbor-joining method was used to build this tree. The Iraqi isolate has been assigned the GenBank accession numbers (OQ589912) and(OQ581742). Individual GenBank accession numbers come after species

Also, the genetic diversity of the samples was confirmed by detecting differences in the nucleotide sequence. Therefore, the treatment of the infection caused by this parasite is not affected by these mutations.

Conclusion

This report is the first on the prevalence of *B. hominis* among patients with DM in Saladin governorate, Iraq. This study demonstrated that the vacuolar form was the most often seen form of the parasite and was more prevalent in the DM than in the non-DM group. The study also noted an association between the presence of *Blastocystis* and other participant characteristics (risk factors), such as place of residence, water source, and maintenance of animals. Additionally, exposure to animal stools may result in the development of *Blastocystis ST1* in humans. Interestingly, the genetic diversity of the parasite in this study was confirmed by detecting differences in their nucleotide sequence, which showed the association of the parasite either directly with the species or indirectly with the subspecies.

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Conflicts of interest

There are no conflicts of interest.

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