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ORIGINAL RESEARCH

Identification and Genetic Characterization of *Blastocystis* Species in Patients from Makkah, Saudi Arabia

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Background: *Blastocystis* species (sp.) are gastrointestinal protozoan parasites with high prevalence rates worldwide. *Blastocystis* sp. show extensive genetic diversity with 17 different subtypes (STs) described to date. A few studies have investigated the prevalence and STs of *Blastocystis* sp. in Makkah, Saudi Arabia. Therefore, we aimed in this study to identify and characterize subtypes of *Blastocystis* sp. in the City of Makkah, Saudi Arabia.

Methods: Stool samples were collected from 140 patients who presented to King Abdulaziz Hospital, Hera General Hospital and Modern Medical Center in Saudi Arabia. Different microscopic examination methods of patients' stools and molecular analyses (using primers targeting SSU rRNA gene) were performed to identify and characterize STs of *Blastocystis* sp.

Results: Our microscopic examination of stool samples showed that 96/140 patients (68.6%) had *Blastocystis* sp. infection. Clinical examination of infected patients revealed that 81 patients were symptomatic, whereas 15 were asymptomatic. Next, we isolated DNA from *Blastocystis* sp.-positive stool samples followed by PCR amplification of small-subunit ribosomal RNA (SSU rRNA) gene and sequence analysis. Our sequence analysis showed that subtype 3 (ST3) was the most prevalent (53.13%) followed by subtype 1 (ST1) (45.83%), whereas subtype 2 (ST2) was the least prevalent (1.04%). Moreover, our results showed that all three STs resulted in more symptomatic than asymptomatic cases. Finally, we identified novel haplotypes which comprised of 8 ST3, 6 ST1, and one ST2 haplotypes.

Conclusion: Our identification of several haplotypes in patients' stools confirms the genetic diversity of *Blastocystis* sp. and may explain the reported low host specificity and differential pathogenicity of *Blastocystis* sp. We believe that additional molecular epidemiological and genomic studies are needed to understand the prevalence and pathogenicity of different subtypes in humans and animal hosts.

Keywords: *Blastocystis* sp., genotyping, subtype, haplotype, ST1, ST2, ST3, Saudi Arabia

Background

Blastocystis species (sp.) are intestinal protozoan parasites that have several forms in lifecycle including vacuolar, granular, amoeboid, and cyst forms. Human to human transmission is common, through the fecal-oral route, which explains significantly higher rates of infection among food and animal handlers[1,2].

According to previous reports, developing countries have higher prevalence rates (55–70%) of *Blastocystis* sp. compared to developed countries (10–15%). This could be attributed to higher standards of hygiene, lesser contact with animals and lower consumption rates of contaminated food and drink in developed countries.[3,4] Recent studies have suggested that *Blastocystis* sp. exist as part of gut microbiota in healthy individuals (asymptomatic) and in patients presenting with gastrointestinal disease (symptomatic). However, the pathogenicity of the parasite, factors and

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mechanisms that trigger the progression to disease remain poorly understood.[5,6] Symptomatic patients may present with nonspecific gastrointestinal symptoms such as abdominal pain, vomiting, and diarrhea. However, irritable bowel syndrome and urticaria sensitization were also reported.[7,8] Several genomic, in vitro, and in vivo studies have identified multiple virulence factors of *Blastocystis* sp. and demonstrated the parasite's pathological effects on the intestine.[6,9]

By 2013, Blastocystis sp. have been classified into 17 subtypes (STs) (ST1-17) in birds and mammals (including humans) based on extensive genetic variations in small-subunit ribosomal RNA (SSU rRNA) gene sequence.[10] Eleven additional STs (ST18-28) have been described in literature since then; however, four of these STs (ST18-20, and ST22) are still being validated.[11] These different STs are believed to result in differential symptoms and distribution among animal hosts and humans.[12] Ten STs have been isolated from human stools (ST1-9 and ST12) and more than 90% of all human cases have been associated with ST1-4.[13,14] However, only eight subtypes (ST10-17) were isolated from animal hosts.[12,15–17] A study has shown that *Blastocystis* sp. ST3, isolated primarily from symptomatic patients, produces proteases that play a major role in host protein degradation and immune evasion.[4,18]

Several molecular methods have been used to identify *Blastocystis* sp. subtypes that are isolated from humans and animals. These methods include random amplified polymorphic DNA (RAPD) using four different arbitrary polymerase chain reaction (PCR) primers, and restriction fragment length polymorphism (RFLP) followed by PCR to detect SSU rRNA gene variations.[19,20] To date, a few studies investigated the prevalence and subtypes of *B. hominis* in Saudi Arabia. Therefore, the aim of this study was to identify *Blastocystis* sp. subtypes and their prevalence in stool samples of patients living in City of Makkah, Saudi Arabia.

Methods

Study Population and Sample Collection

The present study involved 140 patients who presented, in 2017–2018, to King Abdulaziz Hospital, Hera General Hospital, and Modern Medical Center in the City of Makkah, Saudi Arabia. Some of the participants were complaining of GIT symptoms such as diarrhea or abdominal pain, while others were asymptomatic. Stool samples were collected from each patient in a clean container and divided into 2 parts. The first part was examined microscopically for the presence of *Blastocystis* sp. using direct mount (saline and iodine) and concentration technique; as previously described.[21] The second part was stored at −20°C for further molecular analysis.

PCR Amplification and Sequencing

Stool samples that tested positive for *Blastocystis* sp. were subjected to molecular analysis. DNA was extracted from stool samples using QIAamp DNA Stool Mini Kit (QIAGEN) according to the manufacturer's instructions. The specific primers, forward primer Blast 505–532 (5′ GGA GGT AGT GAC AAT AAATC 3′) and reverse primer Blast 998–1017 (5′ TGC TTT CGC ACT TGT TCATC 3′),[22] were used to amplify the SSU rRNA gene using AccuPower® PCR PreMix (Bioneer). The PCR conditions were as follows: initial denaturation at 95°C for 4 min then 35 cycles of {denaturation at 95°C for 30 seconds, annealing at 54°C for 30 seconds, extension at 72°C for 30 seconds}, and a final extension step at 72°C for 5 minutes. Next, PCR products were separated by electrophoresis on 1.5% agarose gels and visualized by UV following ethidium bromide staining. Positive *Blastocystis* sp. samples generated PCR products of 500bp. For characterization of *Blastocystis* sp. genetic variations, all PCR products as well as positive and negative controls were sent to Macrogen company in South Korea for sequencing (https://dna.macrogen.com/).

Sequence Analysis

The DNA sequences of PCR products generated from *Blastocystis* sp.-positive samples were analyzed. The BLASTN software was used to compare the SSU rRNA sequences obtained with those in GenBank. MEGA X software was used to build a phylogenetic tree using the neighbor-joining method and the Kimura 2-parameter model.[23] The bootstrap method with 1000 replicates was used to determine the support of monophyletic communities. All sequences were aligned by MUSCLE software and phylogenetic analysis was performed using MEGA X the maximum likelihood (ML), and Neighbor Joining (NJ) method. The maximum parsimony (MP) methods with 1000 bootstrap replicates were then

used to construct the phylogenetic tree. The evolutionary history was concluded using the NJ method.[24] The Maximum Composite Likelihood method was used to calculate the evolutionary distances[25] and are in the units of the number of base substitutions per site. The analysis included 46 nucleotide sequences and any equivocal position was deleted for each sequence pair (pairwise deletion option). There was a total of 48,775 positions in the final dataset. MEGA X software was used for evolutionary analyses.[23]

Statistical Analysis

All collected data were entered into the SPSS program (version 22) and chi-square test was used to analyze categorical variables. *P*-values of <0.05 were considered statistically significant.

Results

Microscopic Examination of Patients' Stools Identified 96 *Blastocystis* sp.-Infected Patients with Significantly Higher Number of Symptomatic Than Asymptomatic Patients

The present study included 140 patients aging between 7 and 80 years (mean ± SD = 35.5±12.4) of which 105 (75%) were males (Table 1). Microscopic examination of stool samples of all patients showed that 96 patients (96/140; 68.6%) were infected with *Blastocystis* sp. Our results showed that there was a significantly higher percentage of males infected with *Blastocystis* sp. (N=77/105; 73.3%) compared to females (N=19/35; 54.3%) (Table 1; $p<0.05$). Interestingly, age group 21–40 years (yrs) had the highest percentage of infected patients (70/94; 74.5%) compared to age group <20 yrs (1/4; 25%), age group 41–60 yrs (23/37; 62.2%), and age group >60 yrs (2/5; 40%), Table 1. However, the differences were not statistically significant ($p=0.3$).

Physical examination of *Blastocystis* sp.-infected patients concluded that a significantly higher number were symptomatic (diarrhea) than asymptomatic (81 vs 15; $p< 0.05$).

Sequence Analysis of PCR Products Amplified from DNA of *Blastocystis* sp., Isolated from Stools of Infected Patients, Showed Prevalence of *Blastocystis* sp. Subtype 3 (ST3)

Our PCR on the DNA extracted from the 96 *Blastocystis* sp.-positive stool samples generated PCR products of the right size (500 bp) from all samples, which confirmed *Blastocystis* sp. infection.

Next, we sequenced PCR products to identify the genetic subtype of *Blastocystis* sp. in each sample. The obtained sequences were analyzed using BLAST software and aligned with *Blastocystis* sp. sequences deposited in the gene bank. Sequence analysis showed that ST3 existed in significantly higher percentage of stool samples compared to ST1 (53.13% vs 45.83%). ST2 was detected in only 1.04% of samples ($p<0.05$).

Our results showed that 100% of ST2-infected patients were symptomatic followed by ST1 (88.6%), and ST3 (80.4%).

Table 1 Gender and Age Group of *Blastocystis* sp.-Infected and Uninfected Patients

Age Group (N)	Females (N=35)		Males (N=105)	
	Infected	Uninfected	Infected	Uninfected
<20 yrs. (4)	0 (0%)	2 (5.7%)	1 (0.95%)	1 (0.95%)
21–40 yrs. (94)	16 (45.7%)	6 (17.1%)	54 (51.4%)	18 (17.1%)
41–60 yrs. (37)	2 (5.7%)	8 (22.9%)	21 (20%)	6 (5.7%)
>60 yrs. (5)	1 (2.9%)	0 (0%)	1 (0.95%)	3 (2.9%)
Total (140)	**19 (54.3%)**	**16 (45.7%)**	**77 (73.3%)**	**28 (26.7%)**

Notes: Stool samples from patients were examined microscopically for the presence of *Blastocystis* sp. The numbers (N) of infected and uninfected patients are presented according to gender and age group.

Novel *Blastocystis* sp. Haplotypes Were Identified in Subtypes with Inter- and Intra-Subtype Genetic Variations

Median joining network analysis, of sequences of PCR products, demonstrated genetic variations between the different subtypes, and identified intra-subtype genetic variations within ST1 and ST3 (Figure 1). The median joining network analysis identified 15 haplotypes; H1-15, in *Blastocystis* sp. subtypes. ST3 comprised 8 haplotypes, ST1 comprised 6 haplotypes, whereas ST2 comprised only one haplotype.

Further sequence analysis of PCR products revealed inter-subtype genetic variations with genetic differentiation values ranging from a minimum of JC = 0.0991, between ST1 and ST2 subtypes, to higher values between ST1 and ST3 (JC = 0.248) (Table 2). Moreover, high intra-subtype genetic variations were found between ST1 sequences (JC = 0.0834) followed by ST3 (JC = 0.0356) (Table 2).

Higher Genetic Variations Were Detected Between ST1 Haplotypes Compared to ST3 Haplotypes

Next, we performed an overall analysis of sequences of SSU rRNA gene PCR products, obtained from all *Blastocystis* sp.-positive stool samples. We identified 113 sites that were polymorphic and 91 sites that were parsimony informative, which led to the distinction of 15 haplotypes. The total number of non-InDel and InDel sites were 431 and 25, respectively. The 91 parsimony informative sites included 59 sites that had two variants, 24 sites that had three variants, and 8 sites with four variants. The average number of nucleotide differences was 52.076. We found that the haplotype and nucleotide diversity were 1.000 and 0.12083, respectively.

Consistent with the data shown in Table 2, the analysis of haplotypes of each subtype confirmed that ST1 has higher genetic variations than ST3, with higher nucleotide diversity, higher numbers of polymorphic sites and higher number of nucleotide differences (Table 3). All subtypes showed negative neutrality tests (Tajima's test and Fu's Fs) which means that DNA sequence is evolving naturally and not under directional selection.

Next, we constructed the phylogenetic tree and included all our identified haplotypes. We determined the relation of the identified haplotypes to other *Blastocystis* sp. isolates (Figure 2). We used thirty reference sequences of *Blastocystis* sp. subtypes ST1-ST17 (Table 4) and *Proteromonas lacertae* (U37108) as an outgroup sequence.

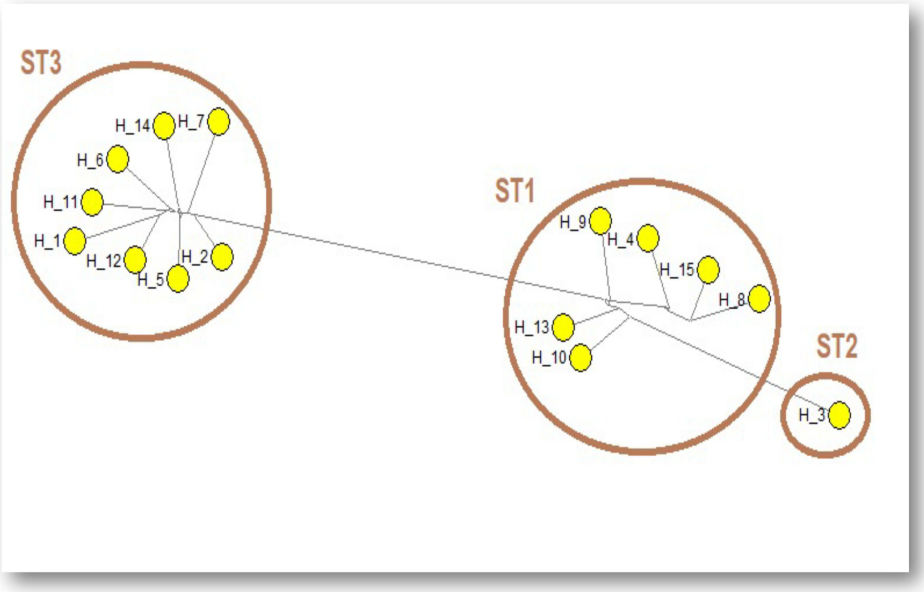

Figure 1 Median joining network sequence analysis of SSU rRNA gene-PCR products demonstrated different haplotypes (H1-15) within *Blastocystis* sp. subtypes. Maximum parsimony (MP) analysis was used as the optional post-processing calculation using Network 4.6.1.3 software. Each haplotype represents a distinct subtype. The haplotypes are numbered from H1 to H15.

Table 2 Inter- and Intra-Subtype Genetic Variations Characterize *Blastocystis* sp. Subtypes/Haplotypes

	ST2	ST3	ST3	ST3	ST3	ST3	ST3	ST3	ST3	ST1	ST1	ST1	ST1	ST1	ST1
ST2	0.000														
ST3	0.1950	0.000													
ST3	0.2133	0.0236	0.000												
ST3	0.2041	0.0212	0.0284	0.000											
ST3	0.1950	0.0164	0.0212	0.0236	0.000										
ST3	0.2133	0.0188	0.0260	0.0260	0.0332	0.000									
ST3	0.2163	0.0308	0.0356	0.0332	0.0332	0.0236	0.000								
ST3	0.2071	0.0117	0.0236	0.0308	0.0188	0.0260	0.0332	0.000							
ST3	0.2102	0.0260	0.0284	0.0308	0.0332	0.0117	0.0236	0.0308	0.000						
ST1	0.1315	0.2010	0.1920	0.2010	0.2041	0.2102	0.2257	0.2102	0.2071	0.000					
ST1	0.0991	0.1830	0.1950	0.1950	0.1890	0.1980	0.2102	0.1920	0.2010	0.0529	0.000				
ST1	0.1343	0.2163	0.2133	0.2194	0.2226	0.2288	0.2352	0.2226	0.2320	0.0405	0.0705	0.000			
ST1	0.1371	0.1950	0.2041	0.2102	0.2133	0.2102	0.2194	0.2071	0.2163	0.0554	0.0680	0.0284	0.000		
ST1	0.1483	0.2163	0.2288	0.2194	0.2352	0.2288	0.2480	0.2226	0.2352	0.0731	0.0834	0.0504	0.0332	0.000	
ST1	0.1151	0.1950	0.2071	0.2071	0.2071	0.2102	0.2163	0.2071	0.2133	0.0260	0.0454	0.0454	0.0529	0.0756	0.000

Notes: The JC model with Mega X software program was used for the analysis of sequences of PCR products. Sequence analysis identified inter-subtype genetic variations with a minimum JC=0.0991, between ST1 and ST2 subtypes, to a maximum JC=0.248 between ST1 and ST3. High intra-subtype genetic variations were also detected between ST1 sequences (JC=0.0834) followed by ST3 (JC=0.0356).

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Table 3 Genetic Variability of *Blastocystis* sp. Haplotypes

Parameters	ST3	ST1
Number of mutations	28	67
Parsimony informative sites	15	26
Nh (haplotype number)	8	6
Hd (haplotype diversity)	1.000	1.000
π (nucleotide diversity)	0.02243	0.05981
K (average number of nucleotide differences)	10.071	26.733
S (number of polymorphic sites)	21	54
Tajima's test	−0.35616	−0.57236
Fu's Fs	−2.160	−0.19732

Notes: Several parameters were used to identify genetic variations in ST1 and ST3 haplotypes. ST2 could not be analyzed as it was obtained in only one sample.

Discussion

Blastocystis sp. infections are prevalent in both developing and developed countries. Multiple studies have described several gastrointestinal manifestations following *Blastocystis* sp. infections.[26] However, several studies have shown that only a few subtypes of *Blastocystis* sp. are associated with diseases in humans, whereas other subtypes cause asymptomatic infections.

In the present study, we aimed to identify and characterize *Blastocystis* sp. subtypes in 140 patients who attended King Abdulaziz Hospital, Hera General Hospital and Modern Medical Center in City of Makkah, Saudi Arabia. We detected *Blastocystis* sp. infection in 68.6% (96/140) patients who presented with gastrointestinal symptoms (symptomatic) or remained asymptomatic. Other studies from different countries have shown variable prevalence rates of 0.54% in Iran,[27] 2.5% in Japan,[28] 3.3% in Singapore,[29] 19% in Lebanon,[17] 22.1% in Libya,[30] 33.3% in Egypt,[31] 40.7% in the Philippines,[32] and 46.9% in Venezuela.[33] This could be attributed to variations in sanitation standards in different countries. Our small number of patients could be the reason for the higher prevalence rates of *Blastocystis* sp. in our study compared to other studies.

We report higher prevalence rate of *Blastocystis* sp. in males compared to females, which is consistent with the findings of previous studies in Iran, Libya and Turkey.[34–36] The higher prevalence rate of *Blastocystis* sp. in males compared to females may be attributed to their more frequent outdoor activities. There are several contradictory studies regarding *Blastocystis sp*. pathogenicity.[37] In the present study, we report that the number of symptomatic *Blastocystis* sp. -infected patients were significantly higher than the number of asymptomatic patients (81 symptomatic patients vs 15 asymptomatic).

Nine subtypes of *Blastocystis* sp. (ST1 to ST9) were isolated from human stools[38] of which ST1, ST2, ST3, ST4, and ST5 were the most common. It has been shown that ST4 is the prevalent subtype in European countries, while ST1, ST2, ST3, and ST5 are the common subtypes in other countries all over the world.[39] However, there is still debate about the relationship between *Blastocystis* sp. subtypes and clinical symptoms.[40–42]

Our molecular analysis, of patients' stool samples, showed that ST3 was detected in significantly higher percentage of samples than ST1 (53.13% vs 45.83%). We identified ST2 in only one sample. This finding indicates that ST3 is the predominant subtype of *Blastocystis* sp. in our patients. Predominance of ST3, in our study, is consistent with a previous report[43] which characterized *Blastocystis* sp. subtypes in symptomatic (diarrhea) and asymptomatic patients in City of Makkah, Saudi Arabia. Moreover, ST3 was found to be the prevalent subtype (41–92%) in a comparative study between Japan, Bangladesh, Pakistan and Germany,[28] whereas the prevalence rates in other studies were reported at 78%, 75.9%, 54.5%, 53.5% and 33.3% in Singapore, Turkey, Egypt, France and Lebanon, respectively.[17,29,36,44,45]

We additionally showed that a significantly higher percentage of ST3-infected patients were symptomatic than asymptomatic (80.4% vs 19.6%). This finding is in agreement with findings of another study where most ST3 patients showed gastrointestinal symptoms.[5] Tan et al also showed similar results and reported that all symptomatic patients in his

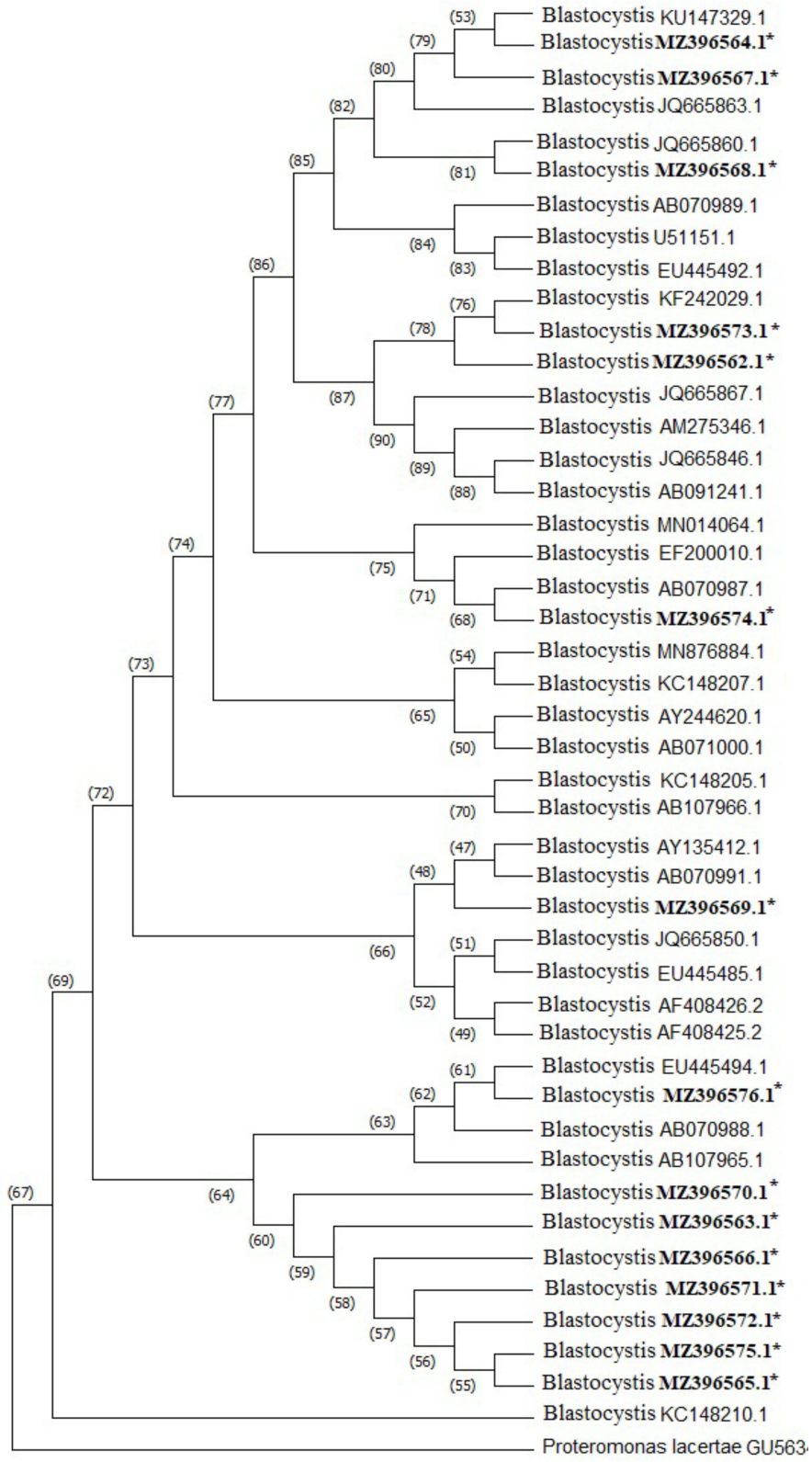

Figure 2 Phylogenetic relationship of *Blastocystis* sp. haplotypes identified in the current study to other *Blastocystis* sp. isolates, based on SSU rRNA gene sequences. The reference GenBank sequence accession numbers of all isolates are shown, including our haplotypes accession numbers in bold. *Proteromonas lacertae* served as the outgroup sequence. *Indicates the haplotypes identified in the current study.

Table 4 GenBank References for the *Blastocystis* sp. Subtypes Used to Construct the Phylogenetic Tree

No.	Accession no.	Host	Subtype	Reference
1.	U51151	Human	1	[54]
2.	AB070989	Human	1	[55]
3.	AB091241	Chicken	1	[55]
4.	EU445492	Human	1	[56]
5.	JQ665860	Human	1	[57]
6.	JQ665863	Human	1	[57]
7.	JQ665846	Human	1	[57]
8.	JQ665867	Human	1	[57]
9.	EF200010	Human	2	[58]
10.	AB070987	Human	2	[55]
11.	AB070988	Human	3	[55]
12.	AB107965	Cattle	3	[59]
13.	EU445494	Human	3	[56]
14.	AB071000	Rat	4	[55]
15.	AY244620	Human	4	[28]
16.	AB107966	Cattle	5	[59]
17.	EU445485	Chicken	6	[56]
18.	JQ665850	Human	6	[57]
19.	AB070991	Human	7	[55]
20.	AY135412	Duck	7	[60]
21.	AF408425	Human	9	[28]
22.	AF408426	Human	9	[28]
23.	KC148207	Camel	10	[12]
24.	KC148205	Cattle	14	[12]
25.	KC148210	Camel	15	[12]
26.	KU147329	Human	1	[61]
27.	AM275364	Human	7	[62]
28.	KF242029.1	Human	1	[63]
29.	MN876884.1	Cattle	10	[64]
30.	MN014064.1	Human	2	[64]

study were ST3-infected.[46] Symptoms other than gastrointestinal manifestations were also reported in *Blastocystis* sp. ST3-infected patients. A patient infected with ST3 presented with acute urticaria and gastrointestinal symptoms.[47] We also showed that most ST1-infected patients (88.6%) were symptomatic which is in agreement with other studies which

reported the ability of ST1 to cause symptomatic infections.[28,48] This indicates that *Blastocystis* sp. STs, that were identified in our study, tend to cause symptomatic than asymptomatic infections.

Our sequence analysis, of PCR products obtained from all samples, identified three parsimony informative sites and three mutations (not parsimony informative) across the entire obtained sequences, resulting in the identification of 15 *Blastocystis* sp. haplotypes. (8 different ST3 haplotypes, 6 ST1 haplotypes, and one ST2 haplotype). The sequence analysis of ST1 and ST3 samples revealed high genetic variations. This high level of genetic variability in ST1 and ST3 appears to support t's low host specificity.[49] Despite our findings, we believe that large-scale molecular epidemiological studies, both in humans and in animals, will be useful to further understand the transmission of *Blastocystis* sp. Moreover, immunological and pathogenicity studies should be performed for better understanding of the *Blastocystis* sp. pathogenicity and immune responses in humans.

Several studies have isolated ST1 and ST3 from a variety of species including monkeys, goats, pigs, dogs, and non-human primates. As a result, it has been proposed that these subtypes are zoonotic subtypes, capable of infecting humans at varying rates.[48,50–52] A study analyzed both the nuclear SSU rRNA and the MLST of the mitochondrion-like organelle genome and discovered significant intra-subtype genetic variation in ST3. The fact that ST3 has high intra-subtype variation means that this subtype may have co-evolved with human hosts over a long time.[53]

Conclusions

A few studies investigated the prevalence and subtypes of *Blastocystis* sp. STs in patients from Saudi Arabia. Our study showed that 81/96 *Blastocystis* sp.-infected patients from Saudi Arabia were symptomatic, whereas stool analysis identified 15 genetically distinct haplotypes that are distributed in three STs (8 ST3, 6 ST1, and 1 ST2). We believe that future genomic analysis could shed light on potential coevolutionary aspects, which could help researchers better understand *Blastocystis* sp. Biology, interaction with the host, and pathogenicity. Furthermore, proteomic studies of the various subtypes of *Blastocystis* sp. could enhance our understanding of the parasite's pathogenic mechanisms.

Institutional Review Board Statement

The study was conducted according to the guidelines of the Declaration of Helsinki, and approved by the ethics and research committee, Faculty of Applied Medical Sciences, King Abdulaziz University (Protocol number: FAMS-ERC2017-12). An informed consent was obtained from each patient involved in this study. The Saudi Ministry of Health, Saudi Arabia cooperated in this study.

Data Sharing Statement

All genetic sequences are available at [https://www.ncbi.nlm.nih.gov/genbank/]. GenBank accession numbers are: MZ396562, MZ396563, MZ396564, MZ396565, MZ396566, MZ396567, MZ396568, MZ396569, MZ396570, MZ396571, MZ396572, MZ396573, MZ396574, MZ396575, MZ396576.

Disclosure

The authors declare no competing interests in this work.

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