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ORIGINAL PAPER

An Association Between *Blastocystis* Subtypes and Colorectal Cancer Patients: A Significant Different Profile from Non-cancer Individuals

Shaimaa H. Ali[1] · Mousa A. M. Ismail[2] · Ayman A. El-Badry[3] · Enas Y. Abu-Sarea[1] · Ahmad M. Dewidar[4] · Doaa A. Hamdy[1]

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Abstract

Purpose *Blastocystis* is a common enteric human parasite of non-conclusive pathogenicity which may be determined by subtype (ST) variation. Colorectal cancer (CRC) is considered one of the primary causes of cancer mortality. *Blastocystis* ST7 has been shown to reduce beneficial intestinal microbiota and may exacerbate CRC. This study assessed the possible association between *Blastocystis* STs and CRC in comparison to non-cancer patients.

Material and Methods A total of 200 fecal samples were obtained from CRC (100) and non-CRC (100) individuals attending Beni-Suef University Hospital, Egypt. *Blastocystis* was searched for in all samples using microscopy and culturing. Positive subculture samples were genetically sequenced and subtyped using conventional polymerase chain reaction (PCR). *Blastocystis* STs were determined by sequencing and a phylogenetic tree was created. Related patient characteristics and tumor stages were analyzed for association with presence of *Blastocystis*.

Results *Blastocystis* was identified in 52% and 42% of CRC and non-cancer individuals, respectively. ST1, 2, and 3 were isolated from both cancer and non-cancer individuals; however, for the first time, ST7 was only isolated from CRC stool samples with significant association. Associated patient characteristics were evaluated as predictors.

Conclusion Blastocystosis is highly prevalent in CRC patients, predominantly in the latest CRC grades and stages. To the best of our knowledge, this is the first study to report the identification of *Blastocystis* ST7 in CRC patients. To determine whether certain STs of *Blastocystis* are associated with CRC would require further research, including the role played by gut microbiota.

Keywords *Blastocystis* · Subtypes · Colorectal cancer · Egypt

✉ Doaa A. Hamdy
doaahamdypara@gmail.com

Shaimaa H. Ali
shaimaahussien88@yahoo.com

Mousa A. M. Ismail
dr.mousa.abdelgawad@gmail.com

Ayman A. El-Badry
aelbadry@kasralainy.edu.eg; aaelbadry@iau.edu.sa

Enas Y. Abu-Sarea
enasyahia71@yahoo.com

Ahmad M. Dewidar
ahmaddewidar@yahoo.com

[1] Department of Medical Parasitology, College of Medicine, Beni-Suef University, Beni Suef, Egypt

[2] Department of Medical Parasitology, College of Medicine, Cairo University, Giza, Egypt

[3] Department of Microbiology-Medical Parasitology Section, College of Medicine, Imam Abdulrahman Bin Faisal University, Dammam, Saudi Arabia

[4] Department of Oncology, College of Medicine, Beni-Suef University, Beni Suef, Egypt

Introduction

Blastocystis species (sp.) is one of the most common parasites inhabiting the human colon and encountered in human feaces which has aroused scientific attention due to great controversy about its biology. It is currently considered the most commonly [1, 2].

Blastocystis sp. has zoonotic potential with pandemic prevalence representing wide variations between different countries and even between multiple areas in the same country [3]. Generally, the prevalence in developing countries is higher (22.1–100%) than developed ones (0.5–23.1%) [4]. This difference might be explained by differences in level of hygiene standards, exposure to animals, consumption of contaminated food or drink, and unsanitary waste disposal [4].

The non-specific symptoms caused by blastocystosis include nausea, anorexia, vomiting, diarrhea, abdominal pain, flatulence, and weight loss. *Blastocystis* sp. was even reported to have an important role in enhancing carcinogenesis in *Blastocystis* sp. infected mice [5].

Many factors may affect *Blastocystis* sp. infection such as the immune status and age of hosts [6]. Among immunosuppressed individuals, blastocystosis was identified as a clinically relevant enteric infection that led to severe chronic diarrhea [3, 7].

CRC is the fourth most commonly and third most deadly diagnosed cancer in the world, and three-to-four times more prevalent in developed countries than in developing countries [8, 9]. Infectious agents might be responsible for 20% of CRC [7] and at least one sixth of all cancer cases worldwide [10]. Recent studies have highlighted the substantial role of *Blastocystis* in CRC, and routine screening for the parasite is recommended [7, 8].

Initiation and progression of CRC were evidenced by involvement of the intestinal microbiota. The role of intestinal microbiota outside the gut, as well as in gut physiology, is well documented. Intestinal microbiota has an essential impact on immune system maturation, most metabolic pathways, and different gastrointestinal diseases, such as inflammatory bowel diseases and CRC [11].

Worldwide distribution of *Blastocystis* sp. and its genetic diversity had been approved. Genotyping of *Blastocystis* isolates has received great attention nowadays in an attempt to correlate variance pathogenic behaviors of the parasite to its different STs [1]. Up to date, 17 *Blastocystis* STs, mostly with low host specificity, have been identified and ten of them were reported in humans, with variable prevalence [12–14].

The current study aimed to investigate and assess the frequency and potential role of *Blastocystis* infection among CRC patients in comparison to non-cancer individuals in Beni Suef Governorate, Egypt. As well as, to determine the STs of encountered *Blastocystis* and to evaluate their association potential with CRC.

Material and Methods

Study Design and Population

A cross-sectional, hospital-based study was performed on two hundred (200) patients, 100 patients diagnosed with CRC (cases) (cancer group), and 100 apparently healthy (sex- and age-matched) subjects (control) attending the out-patient clinics of Beni-Suef University Hospital for regular checkup or healthy individuals accompanying cancer patients assigned as control (non-cancer) group. Control group was free from any tumors or chronic diseases ($n=100$). The study was conducted between February 2019 and February 2021. Cases (cancer group) included patients of both sexes, of all ages having CRC ($n=100$). This group was subdivided into two groups; Group A: ($n=50$) diagnosed as CRC patients that had not started any anticancer treatment regime and had not received any anti-parasitic medication for the past 2 weeks. Group B: ($n=50$) patients with CRC receiving radio or chemotherapy. Inclusion criteria included cooperative individuals of all ages and both sexes who agreed to be engaged in the study and were able to provide adequate samples. Exclusion criteria included patients having any type of tumors other than CRC, and patients taking anti-parasitic medications 2 weeks before sample collection. A data collection sheet containing relevant clinical and demographic data was obtained from each participant. Colonoscopy grading of CRC was done according to the American Society of Clinical Oncology [15]. Colorectal Cancer, grading describes how the cancer cells look compared to normal, healthy cells.

Stool Sampling and Processing

Direct Microscopic Examination and Staining

Fresh fecal specimens (about 2 gm each) were collected in clean, dry, wide-mouth plastic containers with tight lids. Date of collection, patient`s name, and serial number were labeled on each container, and the sample was subdivided into three parts. The first part of the specimen (50 mg) was microscopically examined using saline and iodine wet mounts using 40 × and 100 × objectives. The second part about (50 mg) of each specimen was preserved in 5% buffered formalin solution followed by staining with modified trichrome stain. Procedure of staining was performed according to Garcia [16]. Microscopic examination of

stained smears was done using oil immersion and high-power objectives.

Culture and Subculture of Blastocystis sp.

The third part of the specimen was in vitro cultured on modified Jones' medium for *Blastocystis* sp. identification [17]. Fifty milligrams from each stool specimen were inoculated into sterile 7 ml screw caped tubes containing 5 ml Jones' medium and were incubated at 37 °C for 2–3 days [18]. Examination of the culture was done after 24, 48, 72, and 96 h by taking one drop from the tube under sterile conditions, with a sterile Pasteur pipette, and examined with low-power (×100), then with high-power (×400) magnification. The culture was considered negative and discarded if *Blastocystis* sp. was not detected after 96 h. Meanwhile, 1 ml of the positive culture samples were transferred to fresh medium using sterile Pasteur pipette under complete sterile conditions (under the UVR-Laminar flow hood) for 48–72 h. [19]. Suspensions of *Blastocystis* were centrifuged at 500×g for 5 min. The pellet was resuspended 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 [20].

Molecular Identification of Blastocystis sp.

Blastocystis positive subculture stool samples were used for genomic DNA extraction of the parasite utilizing Gene JET Genomic DNA Purification Kit (Thermo Scientific) following the manufacturer's instructions. DNA of *Blastocystis* sp. was amplified by PCR assay. Extracted DNA concentrations were assessed, adapted to 5 ng/ul, and were kept at − 20 °C until processed. A forward primer, RD5 (ATCTGGTTGATC CTGCCAGT) [21], and reverse primer, BhRDr (GAGCTT TT TAACTGCAACAACG) [22] were used.

The primers amplified a 550–585 bp fragment of SSU rDNA sequence of *Blastocystis* STs. Thermocycler following the PCR cycles and conditions described formerly [21] was performed for *Blastocystis*-DNA amplification with minor modifications. DNA amplified products were revealed using 1.5% agarose gel electrophoresis with ultraviolet transillumination after staining by ethidium bromide.

Sequencing and Phylogenetic Analysis of Isolated Blastocystis

Genome DNA purification kit was used for purification of only 20 PCR products and the primer pair (RD5 and BhRDr) with Big-Dye® Terminator v3.1 was used for sequencing. Ready Reaction Cycle Sequencing Kit (Applied Biosystems, Foster City, CA, USA) also was used following the manufacturer's instructions of the ABI Prism 310 genetic analyzer.

Blastocystis isolates sequences were coincided with reference sequences in the GenBank database. The online BLAST program available at the National Center for Biotechnology Information (NCBI) (http://www.ncbi.nlm.nih.gov/BLAST) was employed. The ClustalW program of the BioEdit software was used to align all sequences [23]. The method of neighbor joining [24] utilizing the Molecular and Evolution Genetic Analysis v7 (MEGA7) software [25] was utilized to create the phylogenetic tree for the sequences.

Evaluation of the phylogenetic tree reliability was done using bootstrapping (1000 replicates). Computing the evolutionary distances was made by the Maximum–Likelihood algorithm with Tamura-3 parameter substitution model using MEGA7.

Statistical Analysis

Data were collected, tabulated, and coded for statistical analysis using statistical package for social sciences (SPSS) IBM software (version 25), USA. Descriptive analysis of the results in the form of: frequency and percentage for qualitative data and mean ± standard deviation for quantitative data was calculated. Cross tabulation and Chi-square test ($\chi 2$): for comparison between categorical variables and percentage values were done. Calculation of sensitivity, specificity, and positive and negative predictive values was done. *P* values equal to or less than 0.05 were considered statistically significant.

Results

This study was conducted on a total of two hundred (200) individuals, ranging between 23 and 79 years, with a mean age of (48.14 ± 12.4) years. Males were 56%, while females represented 44% of participants. *Blastocystis* sp. was identified by culture in 52% and 42% of CRC and non-cancer individuals, respectively. Regarding patients categories, the present study revealed non-significant prevalence of *Blastocystis* infection in CRC group as compared to non-cancer control group ($P = 0.101$).

As shown in Table 1 and Fig. 1**,** sensitivity in detecting *Blastocystis* sp. for direct wet preparation and modified trichrome staining was 61.7% and 100% and specificity was 72.3% and 100%, compared to culture on Jones' medium as a golden standard test with diagnostic accuracy rates (82% and 87% respectively).

Blastocystis sp. infection were higher in males (53.8%) than females (46.2%), in the ≥ 50– < 65 years age group (40.4%) than other age groups in the study and more in rural areas (76.9%) than urban areas (23.1%). Nevertheless, no statistically associated relation was detected between any of the studied socio-demographic data and

Table 1 Detection of *Blastocystis* sp. positive cases among CRC patients and healthy controls (N = 200) using different diagnostic techniques

		Culture		Total	Accuracy measures				
		Positive N = 94	Negative N = (106)		Variable	%	OR	95% CI	*P* value
Direct microscopy	Positive	58 (61.7)	0 (0.00)	58 (29.0)	-Sens -Spec	61.7 100	1.61	47.3 – 66.7	**< 0.001***
	Negative	36 (38.3)	106 (100.0)	142 (71.0)	-PPV -NPV -Acc - LR + - LR-	100 74.65 82 61.7 38.29			
Modified trichrome stain	Positive	68 (72.3)	0 (0.00)	68 (34.0)	-Sens -Spec	72.34 100	2.62	32.3 – 75.7	**< 0.001***
	Negative	26 (27.7)	106 (100.0)	132 (66.0)	-PPV -NPV -Acc - LR + - LR-	100 80.30 87 72.3427.66			
Total		94 (47%)	106 (53%)	200 (100%)					

(Sens.): Sensitivity, (Spec.): Specificity, (PPV): Positive predictive value, (NPV): Negative predictive value; (Acc.): Accuracy, (LR +): Positive likelihood ratio, (LR-): Negative likelihood ratio, (OR): Diagnostic odds ratio; (CI): Confidence Interval. **P values* ≤ 0.05 were considered statistically significant

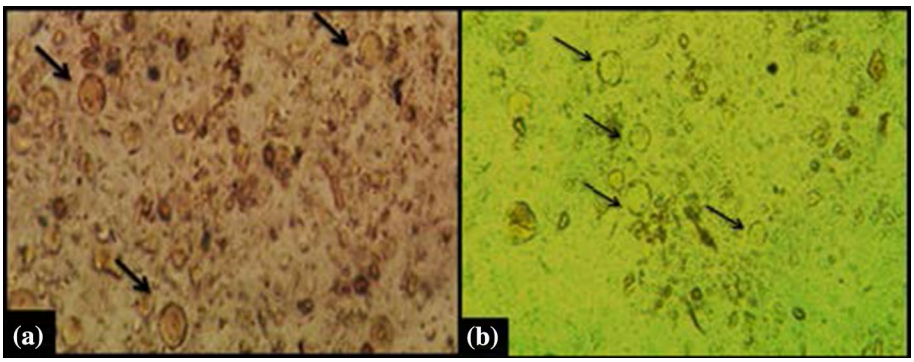

Fig. 1 **a** Vacuolar forms of *Blastocystis* sp. (black arrows) in stool sample stained with iodine by (×400); **b** in vitro cultivation of *Blastocystis* isolates on Jones′ medium after 3 day culture showing the predominance of vacuolar forms by (×400)

Blastocystis sp. infection among studied CRC patients. Urticaria was the only clinical symptom that showed statistical difference among studied CRC patients with positive *Blastocystis* sp. infection ($P = 0.014$), as shown in (Table 2).

The studied CRC patients had a significant history of previous surgeries compared to control group, with a high statistically significant value ($P < 0.001$). Other than two patients who had orthopedic surgery, the majority of patients with previous surgical history had surgeries related to colon and gastrointestinal tract diseases (colectomy, piles, hernia, appendectomy, or varicocele ligation). Chronic diseases which include diabetes mellitus (DM) and/or hypertension (HTN) were nearly similar among cases and controls without statistical significance. There was no statistically significant difference detected between *Blastocystis* sp. infection and CRC patients received radio- and chemotherapy.

Figure 2 demonstrates a significant association between colonoscopy grading of CRC patients and *Blastocystis* sp. infection. Grade 3 had more positive *Blastocystis* infection as compared with Grade 2 (65.4 vs. 34.6%) with a statistically significant difference ($P = 0.034$). Significant association between staging of CRC patients and *Blastocystis* sp. infection also was identified. With increasing stage, more infection rates were significantly detected in late stage (stage 4) (38.5%) than stage 3 (34.6%), and stage 2 (26.9%) ($P = 0.031$).

Out of 94 cultured *Blastocystis* (52 cases and 42 control), *Blastocystis*-DNA was amplified in 44 (84.6%) and 36 (85.7%) of cultured stool from CRC patients and controls, respectively. Four STs (ST1, 2, 3 and 7) of *Blastocystis* sp. were identified in the study individuals (Table 3). ST1, 2 and 3 were isolated from both cancer and non-cancer individuals; however, ST7 was only isolated from CRC

Table 2 Association between socio-demographic, past-history data, and different clinical symptoms with *Blastocystis* infection among studied CRC patients; ($N=100$) considering in vitro culture as a golden reference test

Socio-demographic and past-history data		Culture		Total	*P value*
		Positive N = 52	Negative N = 48		
Sex	Male	28 (53.8)	28 (58.3)	56 (56.0)	0.401
	Female	24 (46.2)	20 (41.7)	44 (44.0)	
Age	20—< 35	10 (19.2)	8 (17.6)	18 (18.0)	0.899
	≥ 35—< 50	18 (34.6)	20 (41.7)	38 (38.00	
	≥ 50—< 65	21 (40.4)	17 (35.4)	38 (38.0)	
	≥ 65	3 (5.8)	3 (6.3)	6 (6.0)	
Residence	Urban	12 (23.1)	10 (20.8)	22 (22.0)	0.489
	Rural	40 (76.9)	38 (79.2)	78 (78.0)	
Occupation	Working	28 (53.8)	28 (58.3)	56 (56.0)	0.401
	Not Working	24 (46.2)	20 (41.7)	44 (44.0)	
Special habits	Yes	7 (13.5)	11 (22.9)	18 (18.0)	0.166
	No	45 (86.5)	37 (77.1)	82 (82.0)	
Contact with animals	Yes	38 (73.1)	33 (68.8)	71 (71.0)	0.399
	No	14 (26.9)	15 (31.3)	29 (29.0)	
Previous surgery	Yes	38 (73.1)	39 (81.3)	77 (77.0)	0.232
	No	14 (26.9)	9 (18.8)	23 (23.0)	
Chronic disease	Yes	10 (19.2)	8 (16.7)	18 (18.0)	0.472
	No	42 (80.8)	40 (83.3)	82 (82.0)	
Different clinical symptoms					
Abdominal pain		48/52 (92.3)	40/48 (83.3)	88/100 (88.0)	0.142
Anorexia		1/52 (1.90)	3/48 (6.30)	4/100 (4.00)	0.279
Nausea		22/52 (42.3)	14/48 (29.2)	36/100 (36.0)	0.123
Vomiting		25/52 (48.1)	25/48 (52.1)	50/100 (50.0)	0.421
Flatulence		5/52 (9.60)	9/48 (18.8)	14/100 (14.0)	0.152
Passage of mucus in stool		5/52 (9.60)	7/48 (14.6)	12/100 (12.0)	0.324
Diarrhea		7/52 (13.5)	7/48 (85.4)	14/100 (14.0)	0.549
Constipation		38/52 (73.1)	28/48 (58.3)	66/100 (66.0)	0.089
Tenesmus		2/52 (3.8)	4/48 (8.3)	6/100 (6.00)	0.301
Bleeding per rectum		25/52 (48.1)	27/48 (56.3)	52/100 (52.2)	0.269
Arthritis		16/52 (30.8)	8/48 (16.7)	24/100 (24.0)	0.078
Urticaria		14/52 (26.9)	4/48 (8.30)	18/100 (18.0)	**0.014***

*P *values* ≤ 0.05 were considered statistically significant; analysis was carried out using Chi-square test

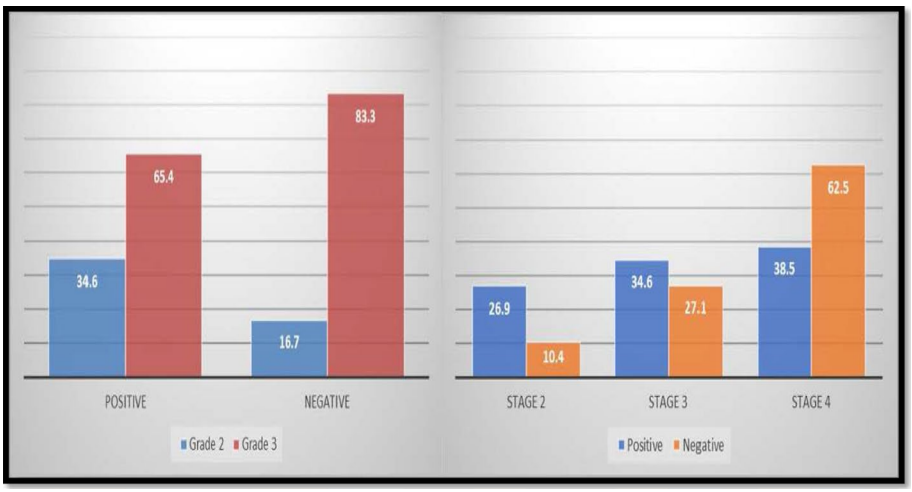

Fig. 2 Association between *Blastocystis* sp. infection and both endoscopic grading of CRC ($P=0.034$) and staging of CRC ($P=0.031$) showing statistically significant difference

Table 3 Frequency of different *Blastocystis* STs isolated from CRC patients and control group ($N=20$)

	Studied Population N (%)		Total N (%) 20	*P value*
	Cases 10	Control 10		
ST1	1(10%)	4(40%)	5(25%)	0.142
ST2	2(20%)	2(20%)	4(20%)	2.557
ST3	4(40%)	4(40%)	8(40%)	0.399
ST7	3(30%)	NO	3(15%)	**0.04***

*P *values* ≤ 0.05 were considered statistically significant. Analysis was carried out using Chi-square test

Table 4 Multivariable analysis using logistic regression demonstrates associated risk factors for *Blastocystis*-infected CRC patients

	B	Sig	OR	95% C.I. for (OR)	
				Lower	Upper
Clinical Symptoms					
Abdominal pain	− 0.737	0.474	0.478	0.063	3.607
Anorexia	1.098	0.660	2.999	0.023	398.451
Nausea	− 1.318	0.243	0.268	0.029	2.449
Vomiting	2.413	**0.044***	11.169	1.067	116.866
Flatulence	2.302	**0.033***	9.997	1.203	83.101
Passage of mucus in stool	− 0.910	0.478	0.403	0.033	4.981
Diarrhea	− 1.907	0.099	0.149	0.015	1.435
Constipation	− 2.816	**0.008***	0.060	0.008	0.473
Tenesmus	1.735	0.383	5.670	0.115	280.672
Bleeding per rectum	0.634	0.329	1.886	0.528	6.740
Arthritis	− 0.722	0.731	0.486	0.008	29.517
Urticaria	− 1.592	0.505	0.203	0.002	21.987
Past history					
Radio- or chemo-therapy	− 0.876	0.311	0.417	0.076	2.270
Previous surgery	2.730	**0.012***	15.333	1.819	129.244
Chronic disease	− 0.695	0.405	0.499	0.097	2.557
*Colonoscopy					
Grade 2	0.753	0.283	2.123	0.537	8.398
*Staging					
Staging 2	1.362	0.172	3.904	0.553	27.537
Staging 3	2.820	**0.006***	16.783	2.269	124.132
Constant	− 0.801	0.620	0.449		

Overall model P *value* = 0.002*, Cox & Snell R Square = 0.329. CRC grade 2 was compared to grade 3, CRC stage 2 and 3 were compared to stage 1, and the whole staging variable was statistically significant with P value = 0.009

stool samples, with significant association ($P=0.04$). In CRC patients, ST3 was the most prevalent (40%), followed by ST7 (30%), then ST2 (20%), and ST1 (10%). While in healthy controls, ST1, ST3, and ST 2 were detected in (40%, 40%, and 20%), respectively.

Table 4 demonstrates multivariable analysis using logistic regression containing the associated risk factors for *Blastocystis*-infected CRC patients. Relevant variables entered for prediction were clinical symptoms, past history, colonoscopy, and cancer staging. All prediction variables were categorical; in all variables, the last category was considered as the reference category. In this model, the overall statistically significant value was (0.002).

The presence of vomiting with flatulence in this regression model was considered statistically significant predictor for positive *Blastocystis*-infected cases ($P=0.044$, 0.033/ OR = 11.169, 9.997, respectively). Constipation was considered a high statistically significant predictor for positive *Blastocystis*-infected cases ($P=0.003$, OR = 0.060). Previous surgery with stage 3 CRC in the current regression model was considered statistically significant predictors for positive *Blastocystis*-infected cases ($P=0.012$, 0.006 / OR = 15.333, 16.783, respectively).

Figure 3 illustrates a dendrogram representing neighbor-joining phylogenetic tree of *Blastocystis* STs isolates from CRC patients and non-cancer individuals based on the rDNA gene sequences compared to reference strains (their accession number is presented before their name). Bootstrap analysis was based on 1000 replicates. All obtained sequences were submitted to the GenBank database under accession numbers OL375676–OL375695.

Discussion

The annual incidence of CRC worldwide is over 1 million in men and nearly 79 500 in women, with 475 000 mortalities in men and 387 000 in women [26]. The prevalence of *Blastocystis* infection in the current study is 52% in CRC patients and 42% in control group considering in vitro culture as a golden reference test. Although this difference is statistically non-significant, it agrees with findings from previous studies [1, 11, 27]. Mohamed et al. [1] reported a significant high prevalence of *Blastocystis* in CRC patients as compared to control population. In Uzbekistan, higher prevalence (80%) of *Blastocystis* sp. infection was detected in CRC patients [11]. Lower prevalence of blastocystosis was detected in Saudi Arabia CRC patients (29.7% and 26.6%) [1, 27]. These findings confirm the association between *Blastocystis* sp. and CRC patients, and assure that *Blastocystis* infection is common and should be routinely screened for immunocompromised patients.

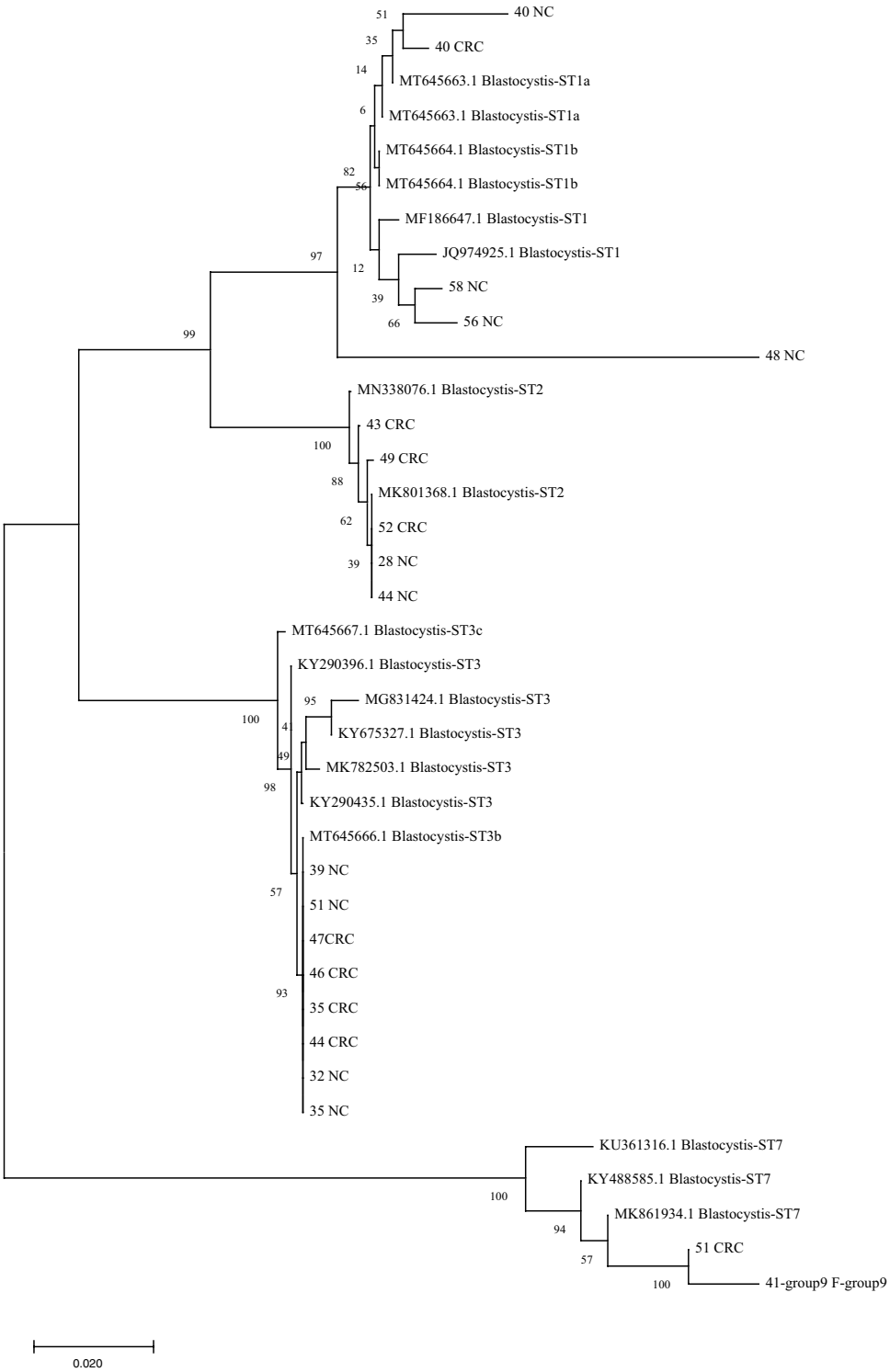

Fig. 3 Dendrogram representing neighbor-joining phylogenetic tree of *Blastocystis* STs isolates from CRC patients and non-cancer individuals based on the rDNA gene sequences compared to reference strains (their accession number is presented before their name). Bootstrap analysis was based on 1000 replicates

Blastocystis sp. was detected in the current study in 30 (30%), 36 (36%), and 52 (52%) by wet mount preparation, modified trichrome staining and culture, respectively, in stool specimens of CRC patients. Culture on Jones' medium detected the highest number of *Blastocystis* sp. in human fecal samples, in agreement with the results of previous studies [17, 28–30]. Similarly, a detection rate of in vitro culture of 53%, 53.6%, and 51.3% were reported in previous studies [28, 31, 32]. On the other hand, other studies reported that PCR assay had the highest percentage of *Blastocystis* sp. detection [14, 33, 34]. Khalifa [35] reported that modified trichrome stain was considered to be the best stain

to identify *Blastocystis*. In the present study, modified trichrome stain detected 72% of total cases detected by culturing with better quality of staining, but it has the disadvantage of being time-consuming and expensive.

Blastocystis sp. infection in CRC patients was higher in males (53.8%) than females (46.2%), and this result was non-significant but in agreement with results which reported that *Blastocystis* frequency was higher in males than females (19% vs. 6.5%) among cancer patients [36] and showed that *B. hominis* infection was more frequent in male than in female CRC patients [27]. These findings may be attributed to more outdoor activities and exposure to infection sources in male patients. Moreover, it was reported that the overall incidence of bowel cancer is higher in males than in females [37, 38]. On the other hand, some investigators reported an equal ratio of *Blastocystis* sp. infection for males and females cancer patients [39].

Blastocystis infection was higher for the age group 50–65 years (40.4%) without statistically significant difference. Similarly, a previous study has found that the *Blastocystis* sp. infection was more prominent in the older age group with mean age of 57 years [40]. Meanwhile, *Blastocystis* sp. was more common in age group of 45–65 years [36, 41]. In contrast, others concluded that *Blastocystis* sp. infection was common in 15–30 years age group [42, 43]. However, other studies recorded that *Blastocystis* sp. infection was common in individuals younger than 15 [44, 45]. This variation in the results regarding age may be explained by the variability of the number and category of patients participated in each study.

Patients living in rural areas had a higher rate of *Blastocystis* infection than urban areas (76.9% vs 23.1%) without statistical significance. This finding was in agreement with other researchers [17, 28]. This can be due to frequent contact with animals and soil, low sanitation level, large family size, and unhealthy drinking water sources [28]. This parasitic infection could be an index to the level of public health [46]. On the other side, *Blastocystis* sp. infection was more common in urban than in rural habitants [27].

Presence of vomiting and flatulence in the current regression model was considered statistically significant predictor for positive *Blastocystis* infection in CRC patients, which coincides with Hamdy et al. [28] who demonstrated that only vomiting and anorexia showed significant association with blastocystosis. Similarly, the previous studies reported that only flatulence showed statistical significance with blastocystosis [17, 47].

Constipation was also considered a statistically significant predictor for positive *Blastocystis* infection, a finding that may be attributed to the prevalence of constipation in CRC patients [48].

The most frequent symptom was abdominal pain in 92.3% of the studied CRC patients, followed by constipation in 73.1% of cases. The least frequent symptoms in studied CRC patients were anorexia in (1.9%) and tenesmus in (3.8%). All other clinical symptoms except urticaria ($P=0.014$) showed non-statistically significant differences ($P>0.05$). This result agrees with another study where there was no significant difference in gastrointestinal symptoms of *Blastocystis*-infected cancer patients [14].

Pathogenesis of urticaria can be attributed to involvement of multiple protozoan parasites [49]. *Blastocystis* is one of the most encountered enteric parasites in asymptomatic and symptomatic individuals [50]. Previous research correlates *Blastocystis* infection with skin disorders and acute or chronic urticaria [51–55]. The mechanism by which *Blastocystis* sp. induces dermal lesions is still not clear; certain immune responses or some alterations of gut microbiota may be incriminated [56–58]. Many studies associate cutaneous pathology with the amoeboid form of *Blastocystis* [52, 53]. Furthermore, it has been found that *Blastocystis* eradication results in complete resolution of dermal lesions which may indicate the role of *Blastocystis* in the skin disorders. However, despite prolonged antimicrobial therapy, recurrence of symptoms has been documented [59].

In the current study, CRC patients had history of previous surgeries (77%) compared to controls (10%) with a statistically significant difference. Previous surgery was considered a statistically significant predictor for *Blastocystis* infection. This contradicts a previous study which found that the entire number of CRC patients infected with *Blastocystis* sp. was the same after surgery and excision of tumors did not significantly reflect on the intensity or frequency of *Blastocystis* sp. infection [11].

In the present study, *Blastocystis* sp. infection was frequently detected among CRC patients who had received radio or chemotherapy without statistical significance. Two studies showed that *Blastocystis* was more frequent in patients who had received eight or more cycles of chemotherapy than those had received less or none cycles of chemotherapy [14, 36]. On the contrary, Toychiev et al., [11] reported that the number of *Blastocystis*-infected CRC patients after chemotherapy was constant (75%) and showed significant difference as compared to control group [11].

It has been documented that *B. hominis* has a pathogenic role in CRC due to induction of colonic epithelial cell mutation in addition to other factors, such as genetic predisposition, may trigger CRC [60]. The possible carcinogenic role of *Blastocystis* infection in humans, especially in CRC patients, has been previously discussed [61, 62].

The present study demonstrates a significant association between colonoscopy grading of CRC patients and *Blastocystis* infection. Grade 3 had more positive *Blastocystis* infection as compared with Grade 2 (65.4 vs. 34.6%) with a statistically significant difference ($P=0.034$). Our results agree with others which demonstrate a notable inflammatory

cellular infiltration and greater presence of mucin in grade 3 CRC patients infected with *Blastocystis* [27]. Kumarasamy et al. suggested that *Blastocystis* sp. enhanced Azoxymethane induced carcinogenesis by promoting oxidative damage to the intestinal epithelium in *Blastocystis* sp. infected rats [5].

There is a significant association between staging of CRC patients and *Blastocystis* infection. With each increasing tumor stage, more *Blastocystis* infection rates were detected, 38.5%, 34.6%, and 26.9% in stages 4, 3, and 2, respectively ($P = 0.031$). Moreover, stage 3 CRC in the multivariate regression model was considered a statistically significant predictor for positive *Blastocystis* infection. This was reported by previous researches which suggest that the infection may promote growth of CRC cells [11, 62]. *Blastocystis* infection was detected in all the cancer stages in a previous study; however, it showed higher positivity in stages 3 and 4 [27]. On the other hand, the prevalence *B. hominis* infection in CRC patients of each stage had no statistical significance among different stages [60].

Our findings support the hypothesis that the infection with *Blastocystis* sp. has a potential role in the pathogenesis of CRC and this is supported by several studies [1, 11, 14, 27]. The pathophysiological mechanism of *Blastocystis* sp. infections in CRC patients is attributed to production of multiple immunological components of inflammatory cells, as well as a result of the downregulation of the inflammatory cytokines and host immune response in the early stages of CRC, which improve its survival [27].

B. hominis infections can initiate free radical formation, such as superoxide anion and nitric oxide to CRC; however, its chronic infections lead to multiple pathophysiological disorders involving mutations with long-lasting oxidative stress causing cancer [63, 64]. Nevertheless, another study suggested that blastocystosis does not stimulate the colonic tissue to form a tumor, but it may intensify an established colorectal tumor [65]. There is a higher marked degree of antioxidant processes and intensification of the oxidative protein damage associated with *Blastocystis* infection [66].

Since PCR is known as an expensive, laborious technique and inefficient tool in screening diagnostic targets for large of sample sizes [67], in the current study, culture was more sensitive than PCR, PCR was positive for 85% of the 94 individuals that were positive for *Blastocystis* using culture, which may be attributed to the disintegration of the parasite DNA during storage process or the inability of STs primer to detect certain STs (majority of ST4, ST8, and ST9) [43, 68]. In earlier literature, *Blastocystis* ST1, 2, and 3 were isolated from both cancer and non-cancer individuals; however, ST7 was only isolated from CRC stool samples, with significant association. In the current study, for CRC patients, ST3 was most prevalent (40%), followed by ST7 (30%) which was found in CRC patients for the first time, ST2 (20%), and ST1 (10%). The obtained results of the present study were similar to others which reported that ST3 was the most prevalent subtype in Iranian cancer patients (37.5%), followed by ST2 (33.3%), ST1 (20.9%), and ST7 (8.3%) in two patients with hematological and cranial malignancies, with ST3 was the only subtype found in CRC patients in their study [14], while ST3 was the most abundant subtype in Turkey and Malaysia in cancer patients [36, 69–71].

In this study, there is no difference in the distribution of ST3 for CRC patients and control group. Research has indicated that ST3 is more anthropogenic; the most frequently isolated subtype in humans, and may be present in symptomatic and asymptomatic individuals [72–74]. ST1 was the most common subtype for Saudi Arabia CRC patients, followed by ST2 and ST5, respectively [1]. Moreover, a strong association risk was demonstrated between *Blastocystis* ST1 and CRC which confirmed the carcinogenic postulated effect of certain *Blastocystis* STs and their probable reflection on CRC [1]. For CRC patients, it was reported that ST1 was the most common subtype in Iraq followed by ST3 [40]. Similarly, in China, ST1 was more prevalent than ST3 in cancer patients with diarrhea [41].

Interestingly, our study revealed the rare subtype (ST7) in CRC patients. The three patients having ST7 were males, came from urban areas, were 54, 51, and 53 years old, had colonic adenocarcinoma grade 2, and previous history of colectomy. This subtype, as the ST5, ST6, and ST8, is rarely found in humans, and it has previously been proposed that these are rare zoonotic subtypes detected in humans [75]. Also, ST7 has been previously recognized in farm animals, supporting its zoonotic transmission through handling of animal [76]. Moreover, ST7 has potentially caused gut microbiota imbalance and decreased the level of beneficial gut bacteria like *Lactobacillus* and *Bifidobacterium* [77, 78]. ST7 has been detected in humans in many countries, with prevalence rates extending from 0.8% in Nigeria [79] to 17.9% in Thailand [80]. A recent study in Singapore has identified ST7 as the predominant subtype in diarrheal patients [81].

Conclusion

Blastocystosis plays an essential role in the CRC morbidity. It is highly prevalent in CRC patients, predominantly in the latest grades and stages of CRC. *Blastocystis* ST1, 2, and 3 were isolated from both cancer and non-cancer individuals; however, the rare pathogenic ST7 was isolated for first time in CRC stool samples, with significant association. Cancer growth and progress is highly correlated with gut inflammatory microenvironment; consequently, CRC patients require screening for *Blastocystis*

sp. infection. Although the substantial role of intestinal microbiota is accepted as a significant component in promoting and development of CRC, studies evaluating the parasitic biology of the gut microbiota are still limited, with contradictory findings. To determine whether certain STs of *Blastocystis* are associated with CRC, or are actual carcinogens would require further research.

Authors' Contribution All manuscript authors contributed to every aspect of it; idea of the research, study design, collection of materials, methodology, writing the paper, and revising/editing it. All authors read and approved the final manuscript.

Availability of Data and Materials Not applicable.

Code Availability Not applicable.

Declarations

Conflict of Interest The authors have no relevant financial or non-financial interests to disclose.

Ethics Approval This study was performed in line with the principles of the Declaration of Helsinki. Approval was granted by the Ethics Committee of Beni-Suef University, Faculty of Medicine (No. FWA00015574).

Consent to Participate Informed consent was obtained from all individual participants included in the study. The aim of the study was explained to all included participants. Participants who had positive results were personally informed or through their physicians to receive proper medications.

Consent for Publication Not applicable.

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