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Original Article

Epidemiological and Diagnostic Features of *Blastocystis* Infection in Symptomatic Patients in Izmir Province, Turkey

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Abstract

Background: The aims of this study were to identify *Blastocystis* subtypes (STs) in a cohort of Turkish patients with various gastrointestinal symptoms using a novel Real Time PCR method developed recently for *Blastocystis* detection and assess the relationship between *Blastocystis* STs and patient symptoms.

Methods: Totally, 617 stool samples of patients with gastrointestinal symptoms were examined with microscopy and inoculated in Jones medium. *Blastocystis*-positive samples were further assessed to identify coinfections with other possible pathogens, including bacteria and viruses. Diagnostic efficacies of microscopy, culture and Real-Time PCR were compared. PCR products were sequenced to identify the subtypes of *Blastocystis* isolates.

Results: Totally 94 (15.24%) samples were positive for *Blastocystis* after all methods. Among these, 83 of 94 (88.3%) samples were identified with all methods, while 11 were positive only with Real Time PCR. Diarrhea and abdominal pain were the leading symptoms in the patients. The only pathogenic agent identified in 76 of 94 (80.9%) patients was Blastocystis. Subtype 3 was the leading *Blastocystis* subtype (44.6%), while subtypes 6 and 7 were firstly isolated from symptomatic patients in our region.

Conclusion: Comparison of three diagnostic methods indicated Real Time PCR as the most sensitive and specific method. *Blastocystis* was the only pathogenic agent among symptomatic patients, with subtype 3 being predominant. Patients with subtypes 6 and 7 need further assessments concerning the zoonotic potential of *Blastocystis*.

Introduction

Blastocystis is the species name of common intestinal protists identified in the stools of humans and many animals worldwide. Although discovered more than 100 years ago, many biological properties of *Blastocystis* are still unresolved. They are probably the most common intestinal protozoa in parasitological surveys throughout the world, with prevalence rates ranging between 3% and 60% in different countries (1, 2).

There is a controversy about the pathogenicity of *Blastocystis*. Whether pathogenic or not, the parasite is remarkable in that it is capable of establishing chronic infections, for which there is no known eradication strategy (3). Patients infected with *Blastocystis* may remain asymptomatic, or suffer from gastrointestinal symptoms such as abdominal pain, diarrhea, nausea, vomiting, bloating and anorexia. To a lesser extent, patients may report dermatological complaints such as urticaria and intense itching (4-6). Many studies indicated the presence of *Blastocystis* only if five or more microorganisms were identified under x400 magnification (7). However, owing to the variation of the daily excretion of the microorganism, this measure may not be reliable to decide whether *Blastocystis* is pathogenic or not. Common use of molecular methods in Parasitology in the last decade improved the sensitivity and specificity of the laboratory diagnosis; in addition, molecular methods helped to clarify the transmission route, zoonotic potential and thus the significance of that parasitic infections in terms of public health. There is an extensive genetic diversity among the *Blastocystis* isolates and molecular analyses demonstrated 17 distinct *Blastocystis* subtypes, at least 9 of which have been found in humans with varying pathogenicity (3, 8-10). Clinical assessments on the relationship between *Blastocystis* subtypes and their pathogenicity have been on the rise in recent years (11-13).

Diagnosis of blastocystosis relies mainly on microscopy; however, variable shedding and polymorphic nature of *Blastocystis* may lower the sensitivity of direct examination of stool samples with saline-Lugol's iodine solution as well as with concentration and trichrome staining methods (14). Short-term culture of stool samples is a practical and sensitive method to identify *Blastocystis* in stool samples (2). Recently, molecular methods have been used in the diagnosis, and the Real-Time PCR was found highly sensitive and specific for *Blastocystis* infection (15-17).

The aims of the present study were to determine the prevalence of *Blastocystis* infection as well as the subtypes of *Blastocystis* in a cohort of Turkish patients with gastrointestinal complaints from Manisa and Izmir provinces; compare the efficacies of diagnostic methods for blastocystosis, assess the relationship between the symptoms and *Blastocystis* subtypes, and assess whether there are subtype differences in the culture and stool isolates of the same patients, between April 2009 and December 2010.

Materials and Methods

Study Group

The study was conducted with the patients admitted to Ege University Medical School's Hospital in Izmir and Ministry of Health's Moris Sinasi International Pediatric Hospital in Manisa. All patients (n=617; 492 from Izmir and 125 from Manisa) reported gastrointestinal complaints; they were included in the study after they gave consent and answered the questions on the "Patient Information Form", on their demographic features, risk factors and symptoms. The assessments of both prevalence and sociodemographic features of all patients (n=617) were determined initially. Efficacies of direct stool examination with saline and Lugol's iodine solutions and

culture were compared to Real Time PCR, retrospectively, for 314 randomly selected samples, 189 from Izmir and 125 from Manisa.

Parasitological Examination

All patient samples (n=617) were initially examined directly with saline and Lugol's iodine solutions, formalin ethyl acetate concentration and cultivated within 30 minutes in Jones medium (18, 19). Samples considered positive by any method were examined for the presence of other intestinal parasites, including enzyme immunoassay (EIA) with RIDASCREEN®-C 1701 and RIDASCREEN®-C 1201 for *Entamoeba sp.* and *Cryptosporidium* sp., respectively. The remaining samples were preserved in -20°C for further assessments.

Microbiological Examination

Stool samples were initially examined microscopically for the presence of red and white blood cells. They were then inoculated in Eosin Methylene Blue (EMB) and Gram-Negative (GN) Broth media for *Salmonella sp.* and *Shigella sp.*, and in Sorbitol Mac Conkey Agar medium for *E. coli* O157: H7 (20). The presence of Rotavirus and Adenovirus was assessed with EIA (RIDASCREEN® Rota Virus Enzyme Immune Assay, RIDASCREEN® Adeno Virus Enzyme Immune Assay).

Culture of Blastocystis

All stool samples were cultured using Jones medium (19). Two grams of fresh stool samples were added in culture tubes and kept in 37°C for 48 hours. Positivity of the culture samples was checked microscopically at 48 and 72 hours after cultivation. Positive culture samples were centrifuged at 1000 rpm for 5 minutes and 1.5-2.0 ml of the pellet were collected for DNA isolation and kept at -20°C. Culture method was chosen as the gold standard for diagnosis of *Blastocystis* infection (2).

Molecular Assessments

Limited financial resources of the study necessitated the application of molecular tests to only about 350 samples collected in the study. Therefore, apart from those 83 *Blastocystis* (+) samples identified initially with saline-Lugol and culture methods, we selected 273 more samples, reaching 356 samples for molecular assessment within the whole budget of the project. To prevent any bias in sample selection, the individuals were initially classified according to age, sex, profession and life-standard groups and certain number of samples were selected to represent each subgroup equally.

Real Time PCR procedure, derived from a recently-developed protocol (21), was applied to amplify a target sequence of 18S rRNA gene of *Blastocystis* using Light Cycler 480 (Roche® Applied Science, Germany) with the Taqman Assay, according to instructions of the manufacturer's. The primers and the Taqman probe used for the assay were as follows: (21)

Blas-F CGTTGTTGCAGTTAAAAAGCTCGT
Blas-R GATTAATGAAAACATCCTTGG-TAAATGC.
Blas-P CAgTTgggggTA+T+TCA+TA+T+TC

Taqman PCR conditions of amplification were 50°C for 2 minutes, 95°C for 10 minutes, 95°C for 15 seconds and 60°C for 1 minute. The last two steps were 45 cycles.

Sequencing of the Real Time PCR products of Blastocystis

Sequence analyses of positive stool and culture samples were conducted separately to assess any variation within the sequence results. The primers used for the sequencing of PCR-products were as follows:

Blasto_seq_F TTgTTgCAgTTAAAAAgCTCg-TAgTTgA
Blasto_seq_R CgCACTTgTTCATCTTCCA-TAAATC

ABI PRISM® BigDye™ Terminator v3.0. (Applied Biosystems, USA) was used for sequencing of the products and the results were analysed on an ABI 3900 Sequencer (Applied Biosystems, USA). Total volume of the sequence reaction was 10 µl. For sequencing, 4

µl MQ, 3 µl Sequencing Buffer, 1µl primer (1,6 µM), 1 µl BigDye Terminator, 1 µl PCR-product (1:10 diluted) was used. The PCR-program used for sequencing was as follows (21);

96°C 1 min
96°C, 10 sec ┐
50°C, 5 sec ├ 25 cycles
60°C, 4 min ┘
12°C ∞

Resulting sequences will be analysed and compared to previous Genbank entries using CodonCode Aligner® program (CodonCode Corporation, USA), and MEGA® (The Biodesign Institute, USA).

Statistical Analyses

The data were statistically assessed using SPSS® 13.0. Chi square test and percentages were used for data analysis, the *P* values below or equal to 0.05 were regarded as significant. Accuracy was calculated as sensitivity, specificity and positive and negative predictive values (PPV and NPV), with 95% confidence intervals (CI) calculated. Disease prevalence and positive and negative likelihood ratios (LR+ and LR-, respectively) were calculated, as well. All computations regarding accuracy were performed at Vassar Statswebsite (http://faculty.vassar.edu/lowry/clin1.html). The kappa coefficients were used to test the agreement between culture and direct stool examinations with saline and Lugol's iodine solutions/PCR results.

Results

Patients enrolled in the study (n=617) were aged between 0 and 87 years (mean, 25.56 ± 25.4 years), and the ratio of male patients was slightly higher (51.4%). Among the age groups, *Blastocystis*-positive patients were predominantly between 20-29 years old (χ^2: 13.68, *P*=0.03) (Table 1). Parasitological examination of the stool samples of these patients (n=617) with microscopy and culture revealed that 83 (13.5%) were positive for *Blastocystis*. Compared to microscopy, culture yielded significantly more *Blastocystis*-positive samples (microscopy: n=11; culture: n=80; χ^2:10.44, *P*: 0.01). Vacuolar form was the most common morphological form of the parasite in microscopic examinations of direct smears and culture, as well.

Despite microscopic and molecular examinations were done with 617 and 356 stool samples, respectively, comparison of all methods used in the study were done with 314 stool samples due to the insufficiency of some of the stools submitted. This revealed 11 new positives, which were initially negative with microscopy and culture but turned out to be positive with Real Time PCR, making the final number of positives reach 94 (Table 1.). Sequence analyses that aim to identify the STs were conducted with 70 of 94 samples; the remaining 24 were not assessed due to low-quality DNA products or even negative PCR (Fig. 1; Table 1).

It was noted that sequence analyses of two of the 70 positive samples revealed different subtypes with stool and culture samples (Subtype 1 in culture while subtype 3 in stool in one sample, and subtype 2 in culture while subtype 3 in stool in the other sample).

Statistical analyses demonstrated that the agreement between the culture and PCR was excellent (K=0.86), while the agreement between the culture and O&P examination was weak (K=0.19). Compared to culture, the sensitivity of O&P examination was 12% (95% CI: 4% - 27%) and the specificity was 100% (95% CI: 98 % - 100%). O&P examination had a positive likelihood ratio of 0.87 (0.78-0.98); however, the negative likelihood ratio calculation was not possible due to the values included one instances of zero. Real-Time PCR had a sensitivity of 100% (95% CI: 89%-100%), specificity of 95% (95% CI: 92%-97 %), positive likelihood ratio of 24.72 (13.86-44.11), and negative likelihood ratio of zero.

Seventy-six of 94 patients (80.9%) were found to be infected only with *Blastocystis*, while 14 (14.8%) were coinfected with other parasites such as *Giardia lamblia*, *Entamoeba histolytica/dispar*, *Cryptosporidium* sp., *Hymenolepis nana* and *Enterobius vermicularis*, while 4 (4.8%) were coinfected with viruses such as Rotavirus and Adenovirus.

The correlation between the *Blastocystis* subtypes and some personal and environmental factors was assessed as well. Some demographic features of *Blastocystis*-positive individuals were shown in Table 1. An interesting outcome of the study was that *Blastocystis* infection was more common among university and secondary school graduates, compared to primary school graduates and no school graduates (χ^2: 17.67, *P*=0.014). It was significantly more common as well, among the patients having cesspools instead of sewage system in their toilets (χ^2: 4.31; *P*= 0.38). Patients with daily habits such as less hand-washing especially before meals, more eating outside and using well water for drinking (instead of bottled water) at home, were found to be more susceptible to *Blastocystis* infection, without a significant difference, as well (Table 1).

Table 1: Some demographic features of all (n=617) and *Blastocystis*-positive patients (n=94)*

Demographic Feature		All Patients (n=617) (%) **	*Blastocystis*-positive patients (n=94) (%) ***	*P*
Sex	Female	48.6	13.3	*P*>0.05
	Male	51.4	13.6	
Age Groups (yr)	0-1	16.4	8.9	*P*=0.030
	2-9	26.4	11.0	
	10-19	14.4	16.9	
	20-29	7.3	28.9*	
	30-39	5.5	17.6	
	40-49	7.9	14.3	
	50+	22.0	11.0	
Education	Non-literate	30.0	9.7	*P*=0.014
	Literate	3.9	13.3	
	Primary School graduate	20.9	10.9	
	Primary School student	22.4	13.8	
	Secondary School graduate	3.4	33.3*	
	High School Graduate	11.7	15.3	
	University graduate	7.8	25.0*	
Drinking water resource	Well	7.1	15.9	*P*>0.05
	Bottled Water	64.3	12.8	
	Tap Water	25.1	13.5	
Hand washing habit	Present	86.2	13.7	*P*>0.05
	Absent	7.1	15.9	
Toilet type	Sewage system of the city	90.6	12.5	*P*= 0.38
	Cesspool	6.8	23.8*	
Frequency$^{\alpha}$ of eating outside	High	45.5	14.2	*P*>0.05
	Low	51.2	12.7	
Animal raising	Yes	9.1	9.4	*P*>0.05
	No	87.3	12.7	
Poultry animal raising	Yes	7.8	22.9*	*P*=0.04
	No	92.2	12.7	

* Frequency of statistically significant *Blastocystis* infection/** % of column
*** % of line/$^{\alpha}$ Eating ≥3 times a week outside home was considered "high"

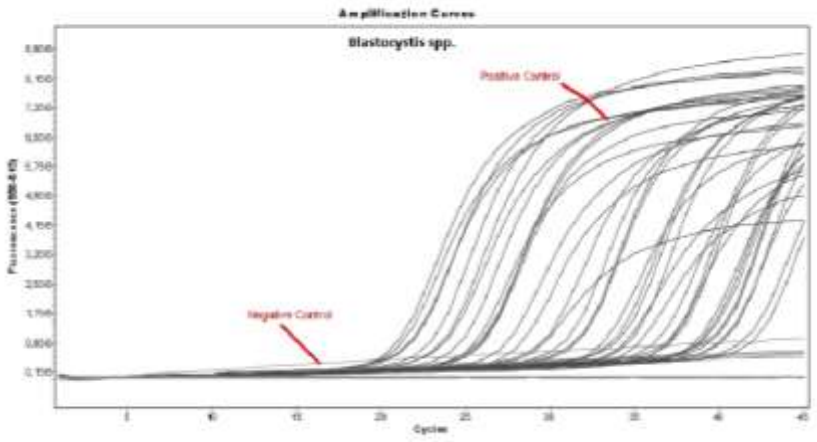

Fig. 1: Real Time PCR curves of the patient samples and the controls

One of the five patients in the study group reported close contact with various domestic animals; among them, *Blastocystis* infection was significantly more common among the owners of poultry animals (bird, chicken, quail) (χ^2: 4.005, *P*=0.04). Subtype 3 was found to be the leading *Blastocystis* subtype among these patients, as expected. Subtype 7, which is unique to avians, was identified in one patient who owned domestic birds (Table 2).

Patients reported many symptoms all of which belonged to gastrointestinal system. The leading symptoms in all as well as only *Blastocystis*-positive patients were found to be abdominal pain and diarrhea (Table 3).

There was no significant difference between the *Blastocystis*-positive group and all study groups for the frequency of gastrointestinal symptoms.

Table 2: Distribution of *Blastocystis* – positive patients according to *Blastocystis* subtypes and whether they were raising animals or not at the time of the survey (n=94)

***Blastocystis* STs**	**Patients raising animals**				**Patients NOT raising animals**	**Total**
	Avian animals (Birds, poultry, etc.)	**Cat**	**Dog**	**Farm Animals**		
ST1	1	0	1	1	10	13 (13.8)
ST2	1	0	0	0	10	11 (11.7)
ST3	5	2	1	0	34	42 (44.7)
ST6	0	0	0	0	1	1 (1.1)
ST7	1	0	0	0	0	1 (1.1)
Mixed ST2/ST3	0	0	0	0	2	2 (2.1)
Undefined	3	1	1	1	18	24 (25.5)
Total	11	3	3	2	75	94 (100.0)

Table 3: Frequency of symptoms in all-study group and *Blastocystis*-positive group

Symptoms	All-study group (n=617) (%)	*Blastocytis*-positive group (n=94) (%)
Abdominal pain	58.0	60.6
Diarrhea	64.2	56.4
Nausea / vomiting	40.6	37.2
Change in appetite	23.1	26.6
Fever	21.3	12.8
Itching	8.2	6.4

Discussion

The prevalence of *Blastocystis* infection is relatively higher in developing countries owing to poor hygiene, exposure to animals and consumption of contaminated water and food (2, 22). The prevalence rates of *Blastocystis* infection range between 1.05% and 15.0% among the symptomatic patients in different regions of Turkey (23). However, only the direct stool examination using saline and Lugol's iodine solutions were used in some of these studies. In the present study, *Blastocystis* was found to be present in 94 of 356 (26.4%) stool samples with at least one method; this figure is between the reported data in developed and developing countries (3% - 60%), and relatively higher than the figures of previous studies in Turkey (1, 2, 23). It was also the leading parasite in our study group, which confirmed our initial hypothesis that *Blastocystis* was the most common intestinal parasite in patients with gastrointestinal symptoms.

This study is unique in that coinfections with *Blastocystis* were sought not only with routine parasitological methods, but also with molecular methods. This brought up an interesting finding of the study: the only infectious agent in the stool samples of 76 of 94 patients (80.8%) was *Blastocystis*. Regarding the current conflicting data about the pathogenicity of *Blastocystis*, we think that this is a significant finding to indicate the potential pathogenicity of this protozoon.

Reports suggest that *Blastocystis* infection should be considered as a prominent causative agent of gastrointestinal disturbances in children. The prevalence rates of *Blastocystis* infection among pre-school children were reported as 18.9% in Venezuela and 25% in Jordan; among the primary school children, they were 6.7% in Libya, 13.5% in Thailand, 16% in Venezuela and 22.4% in Colombia (22). In a previous study in Turkey, the prevalence of *Blastocystis* infection among the primary school children was 14.6% (24). In the present study, the prevalence of *Blastocystis* infection was 8.9% in 0-1 years, 11% in 2-9 years, and 16.9% in 10-19 years old groups; the differences were not statistically significant. However, the highest prevalence was found in the 20-29 years group, with a statistically significant difference.

Diagnosis of *Blastocystis* infection relies mainly on microscopic examination of stool samples; however, as there are many forms of the parasite, including the cysts which are often very small, the sensitivity of microscopic examination even with the stained smears may be rather low (2, 14). Cultivation is a good option to overcome this drawback, especially in laboratories with limited financial resources. Short-term culture (24-72 hours) of stool samples is reported to be more sensitive than microscopic examination, and suggested as the "gold standard" for the diagnosis of *Blastocystis* infection (2, 25). Thus, in the present study, culture was taken as the gold standard and found to be more sensitive significantly, compared to microscopic examination of stool samples for *Blastocystis* recovery. It is found almost as reliable as PCR to identify *Blastocystis*.

Another advantage of culture method is the production of large amounts of parasites for further molecular genotyping studies, by which it is possible to get more precise data about the transmission route and origins of *Blastocystis* isolates that caused the infection (26, 27).

On the other hand, one drawback of the culture method is that in some instances it may allow the preferential growth of one subtype of a parasite over another if more than one subtype is present in the stool (28). In the present study, discordance in the subtypes of stool and culture samples was noted in 2 of 70 samples sequenced. This discordance is noteworthy, and warrants further assessments in future studies.

Application of molecular methods to Parasitology improved the sensitivity and specificity of diagnosis of *Blastocystis* infections (3, 22). Recently, several studies have described the use of conventional PCR for *Blastocystis*. Parkar and colleagues (28) demonstrated that culture followed by PCR was three times more sensitive than culture alone. In contrast, using conventional PCR alone, other studies demonstrated the ability to detect *Blastocystis* at concentrations as low as 13 and 32 parasites per 200 mg of stool (29). Today, real-time PCR is becoming more common for the diagnosis of parasitic infections. Sensitivity of Real Time PCR in the diagnosis of *Blastocystis* infection was found to be 95% in a trial in which real-time quantitative PCR was taken as the golden standard, while sensitivities of microscopy and culture were found as 29% and 52%, respectively (16). In the present study, assessments showed that the sensitivity and specificity of Real Time PCR was 100% and 95% respectively, whereas 12% and 100% for O&P examination. These results confirm that microscopy on a single sample is not reliable for the diagnosis of *Blastocystis* infections. Real Time PCR is both sensitive and specific. On the other hand, PCR methods may detect DNA rather than living parasites.

Molecular analyses demonstrated that *Blastocystis* had extensive genetic diversity; analyses of small subunit of ribosomal RNA (SSU rRNA) identified 17 distinct *Blastocystis* subtypes, nine of which have been identified in humans but also in many animal species (8, 17, 25, 28, 30). The identification of *Blastocystis* subtypes contributed to the unveiling of the transmission routes and zoonotic potentials of this mysterious parasite. Subtype 3 seems to be the most common subtype in humans, followed by subtype 1 (22). Sequencing of 70 samples in our study group which comprised only of patients with gastrointestinal symptoms revealed that subtype 3 was again the most common *Blastocystis* subtype. The overall distribution of *Blastocystis* subtypes reflects those of Middle East, more than it shows the European countries (3, 8). As all patients in our study had gastrointestinal symptoms, all subtypes identified in our study (subtype 1, 2, 3, 6 and 7) may have varying degrees of pathogenicity. Recent data suggest that subtypes 1, 4 and 7 are pathogenic whereas subtypes 2, 3 and 6 were non-pathogenic (8, 22). However, recent reports state the possibility of intra-subtype variations in patients with and without symptoms, infected with *Blastocystis* subtypes known as pathogenic (11, 31). Thus, the detection of the subtypes 2, 3 and 6, which were reported as non-pathogenic in many previous studies, in symptomatic patients in our study may be due to intra-subtype variations, which warrants further assessments.

Epidemiologic studies revealed that some *Blastocystis* subtypes were identified in various animals and humans, whereas subtype 3 is probably anthropophylic only. Some animals are reservoirs of *Blastocystis*, which may constitute some human infections (2), and close contact with animals may be the source of some human infections. In the present study, raising cattle in a farm elevated the risk for *Blastocystis* infection, but the difference was not significant. Yet, close contact with the poultry animals (birds, chicken and quail) was found

to be associated with higher risk, with a statistically significant difference.

Some recent surveys suggested that the incidence of *Blastocytis* infection was higher among the patients with gastrointestinal complaints, such as abdominal pain, diarrhea, flatulence and nausea, compared to non-symptomatic patients (22, 32, 33). In the present study, abdominal pain (59%) and diarrhea (55.4%) were the leading symptoms reported by the patients. Analyses of the symptoms revealed no statistically significant correlation between a symptom and the infection. It should be noted that 14.8% of the patients in our study group were coinfected with other parasitic agents, and 4.8% were coinfected with virus infections (Adenovirus and/or Rotavirus). Seventy-eight of 94 patients (82.9%) was infected only with *Blastocystis*, suggesting that *Blastocystis* may be the only causative agent of the symptoms in these patients. *Blastocystis* is a significant cause of diarrhea and other symptoms related to gastrointestinal tract, and thus it should be included in the evaluation of the patients (22, 32).

Conclusion

Blastocystis is a common parasitic infection, with varying levels of pathogenicity. The symptomatic profile of the patients infected only with *Blastocystis* in the present study is almost the same as the profile of the patients infected with other intestinal protozoa. More data should be reviewed to assess the pathogenicity of the subtypes. We believe that such studies will improve the clinicians' awareness about the significance of *Blastocystis* and other parasitic infections in routine practice.

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