Original Article

Detection of *Blastocystis* Subtypes in Children with Functional Abdominal Pain and Celiac Disease in Çorum, Turkey

*Ayşe Semra Güreser [1], Atakan Comba [2], Djursun Karasartova [1], Nezahat Koşar [1], Adem Keskin [3], Christen Rune Stensvold [4], Ayşegül Taylan-Ozkan [1,5]

1. *Department of Medical Microbiology, Faculty of Medicine, Hitit University, Corum, Turkey*
2. *Department of Pediatrics, Faculty of Medicine, Hitit University, Corum, Turkey*
3. *Department of Biology, Faculty of Science and Art, Tokat Gaziosmanpaşa University, Tokat, Turkey*
4. *Department of Bacteria, Parasites and Fungi, Infectious Disease Preparedness, Statens Serum Institut, Copenhagen, Denmark*
5. *Department of Medical Microbiology, Faculty of Medicine, University of Economics and Technology University, TOBB, Ankara, Turkey*

Abstract
Background: *Blastocystis* has been associated with various symptoms of the gastrointestinal tract. We aimed to investigate the prevalence of *Blastocystis* in children with celiac disease (CeD) or functional abdominal pain (FAP) and to evaluate its subtypes (STs) with respect to demographic, socioeconomic and epidemiological factors.
Methods: Overall, 161 fecal samples were collected from healthy children and patients with FAP or CeD in Hitit University Erol Olçok Research and Training Hospital, Corum, Turkey between 2016-2018. Samples were examined using both native-Lugol (NL) and trichrome-stained (TS) smears, and further analyses by PCR and Sanger sequencing were performed. A standard questionnaire was applied to obtain demographic, socioeconomic, epidemiological data.
Results: *Blastocystis* was found in 10.6% of the total study population. Neither bacteria nor any other parasites were found, except for one *Giardia* (0.6%) in the CeD group. The presence/absence of the parasite was not found to be associated with demographic, socioeconomic and epidemiological factors. *Blastocysis* was detected in 11.5% (6/52) of the CeD, 7.7% (4/52) of the FAP, and 12.3% (7/57) of the healthy group. Diagnostic methods were similar in terms of *Blastocystis* detection ($P= 0.671$), and there was fair agreement between the NL, TS and PCR (Fleiss' Kappa=0.847, $P=0.001$). ST2 (42.8%) and ST3 (35.7%) were the predominant STs followed by ST1 (21.4%).
Conclusion: We observed no difference between study groups in terms of *Blastocystis* prevalence. ST1, ST2 and ST3 subtypes were detected. *Blastocystis* prevalence and STs were not related to any of the demographic, socioeconomic and epidemiological factors.

Introduction

*F*unctional abdominal pain (FAP) is seen in a group of diseases that do not meet the diagnostic criteria for irritable bowel syndrome, functional dyspepsia, abdominal migraine, or organic abdominal pain. Intestinal permeability is increased in patients with FAP, and that past intestinal infections may give rise to symptoms of FAP (1). On the other hand, celiac disease (CeD) is a chronic inflammatory disease of the small intestine triggered by the ingestion of gluten, which can cause significant morbidity (2).

Blastocystis is a parasite commonly found in the gastrointestinal tract of humans, and a wide variety of animals can be acquired via fecal-oral route through contaminated water and food or by exposure to animals (3). The prevalence of *Blastocystis* varies between 0.5% and 86.6% in children with respect to developmental level of the country and is associated with demographic, socioeconomic, and epidemiological factors (4, 5).

Although asymptomatic *Blastocystis* carriage is common, clinical trials and case reports have argued that *Blastocystis* can cause dermatological symptoms such as itching and rashes, as well as gastrointestinal complaints, such as abdominal pain, diarrhea, distention, bloating, nausea, constipation and anorexia in humans (3,6). These crucial characteristics warrant further investigation of the role of *Blastocystis* subtypes (STs) in patients with such symptoms. Especially considering that, prior studies have identified 22 STs of *Blastocystis*, which are morphologically indistinguishable from each other (7). Therefore, the STs may differ in terms of their clinical impact on the host (3, 6, 8).

We aimed to determine the prevalence of *Blastocystis* and molecular characterization of its subtypes in children with CeD or FAP and whether any particular STs can be linked these conditions. The secondary purpose of the study was to evaluate the presence of this parasite and its STs with respect to demographic, socioeconomic and epidemiological data.

Materials and Methods

Study outline and sample collection

The ethical approval for the study was obtained from the Clinical Research Ethics Board of Ankara Numune Training and Research Hospital, Turkey (Approval Code/Date: E.Kurul-E-15-585/01.10.2015). Written informed consent was obtained from the parents of each individual participant included in the study.

A total of 161 children, consisted of 52 patients (23% male, 77% female) with CeD, 52 (54% male, 46% female) with FAP, and 57 healthy children (58% male, 42% female) living in Çorum, Turkey aged between 1 to 18 yr were included in the study between the 2016-Nov 2018.

Children with CeD and FAP were diagnosed based on the European Society for Pediatric Gastroenterology Hepatology and Nutrition (ESPGHAN) 2012 criteria, modified Marsh (Oberhuber) classification and Rome 4 diagnostic criteria (1,9).

A standard questionnaire was applied to each participant for the collection of demographic (age, gender), socioeconomic (parent's education, economic income), and epidemiological (presence of domestic animals) data.

Microbiological examination

A total of 161 fresh fecal samples were collected (one sample from each participant) included in the study. Samples were examined macroscopically and microscopically using both native-Lugol (NL) and trichrome staining (TS) of direct smears (10, 11). Bacteriological cultures (*Salmonella* and *Shigella*) and viral antigen tests (adenovirus and rotavirus) were applied to watery stool samples. Parts of the

freshly voided samples were stored at room temperature in polyvinyl alcohol for TS, and another aliquot was stored at -80 °C for molecular studies. All stool specimens were evaluated microscopically and polymerase chain reaction (PCR) methods.

DNA extraction and PCR analysis

For molecular analysis of *Blastocystis*, DNA extraction was performed on frozen samples using the GeneMATRIX Stool DNA Purification Kit (EURx, Gdansk, Poland) according to the instructions of the manufacturer. Briefly, a total of 70 mg of fecal sample was used in each DNA extraction with a final elution volume of 100 µl. For detection of *Blastocystis*, small subunit rDNA (SSU-rDNA), and the primers BhRDr (5'-GAGCTTTTTAACTGCAACAACG-3') and RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') were used in a touchdown PCR (12). Two microliters of DNA template were added to the standard PCR mixture yielding a total reaction volume of 25µl.

DNA sequencing and phylogenetic analysis

Sequences (approximately 600 bp) obtained by Sanger sequencing of the PCR products were analyzed using the *Blastocystis* STs (18S) and sequence typing database (MLST) (http://pubmlst.org/ Blastocystis/) online software. Representative nucleotide sequences from this study were submitted to GenBank under the accession numbers MK416169–MK416182.

The evolutionary history of all raw nucleotide sequences and sequences of the various known *Blastocystis* subtypes were inferred by using the Neighbor-Joining method (13,14).

Statistical analysis

If one of the methods (NL, TS, or PCR) gave a positive result, the sample was scored as *Blastocystis*-positive.

Statistical analyses were performed where relevant using SPSS 21 (IBM Corp., Armonk, NY, USA). All tests were two-sided, with a type I error (α) set at = 0.05. Associations between *Blastocystis* colonization and categorical parameters, including demographic, socioeconomic, epidemiological and clinical factors were evaluated using Pearson χ2 tests or Fisher's exact tests. The Fleiss' Kappa method was used for comparison of the three methods with regard to diagnostic performance (15). Probability (*P*) values of < 0.05 were considered statistically significant.

Results

Prevalence of Blastocystis

By macroscopic examination, five (3.1%) stool samples were noted to have a watery appearance; whereas the remaining samples had normal consistency and color. No pathogenic bacteria were detected, and viral antigen tests were negative. Moreover, no pus, blood, or helminths were observed during macroscopic evaluation of the specimens.

Distribution of *Blastocystis* detection rates according to the diagnostic method is presented in Table 1. There was fair agreement between NL, TS microscopy and PCR results. Fleiss' Kappa value was 0.847.This was confirmed by the *P*-value (*P* < 0.0001), indicating that our calculated Kappa was significantly different from zero.

Demographic, socioeconomic, and epidemiological findings

A total of 161 children (median age, 8; interquartile range [IQR], 5–12) were enrolled in the study and 17 (10.6%) of these were *Blastocystis*-positive.

Distribution of *Blastocystis* prevalence according to gender and disease/control group is shown in Table 2.

Table 1: Distribution of *Blastocystis* detection rates according to the diagnostic method

Diagnostic method	Blastocystis-positive N (%)	Blastocystis-negative N (%)	P value
PCR	14 (8.7)	147 (91.3)	
Native-Lugol Microscopy	12 (7.4)	149 (92.6)	0.671*
Trichrome-stained Microscopy	17 (10.6)	144 (89.4)	0.001**
* Pearson's χ²			
** Fleiss' Kappa = 0.847			

Table 2: Distribution of *Blastocystis* prevalence according to gender and disease/control group

Gender	N (%)	Blastocystis positive	P value
CeD group			
Male	12 (23.1)	3 / 12	0.127*
Female	40 (76.9)	3 / 40	
FAP group			
Male	28 (53.9)	3 / 28	0.615*
Female	24 (46.2)	1 / 24	
Healthy group			
Male	33 (57.9)	4 / 33	1.000*
Female	24 (42.1)	3 / 24	
Overall			
Male	73 (45.3)	10 / 73	0.238**
Female	88 (54.7)	7 / 88	

* Fisher's Exact Test, ** Pearson's χ²
CeD: Celiac disease   FAP: Functional abdominal pain

Blastocystis prevalence in the 7–11 and 12–18 ages was 0.6% and 3.1% in the CeD group and 1.8% and 0.6% in FAP groups, respectively. In healthy group, prevalence was 1.2% in 0-11 ages and 1.8% in 12-18 ages. *Blastocystis* was more common in those aged 12–18 than in those aged 0–6, and overall *Blastocystis* prevalence was observed to increase by age: 1.9%, 3.1% and 5.6%, in the 0–6, 7–11 and 12–18 ages, respectively. However, there were no statistically significant differences between age groups in terms of *Blastocystis* detection rate. The overall median age was 12 yr for all positive cases, and median ages were similar in the CeD, FAP and healthy groups (12 yr, 9 yr, 10

yr, respectively). In the CeD group, *Blastocystis* was found most frequently detected in the 12–18 ages (9.6%), while no children with CeD or FAP in the 0–6 ages were positive for *Blastocystis*.

A minority of the parents (14.5%) were university graduates. *Blastocystis* detected in 9.3% of the children whose fathers had graduate lower than university and in 10.6% of those whose mothers had graduate lower than university. There were no significant relationships between *Blastocystis* positivity and parental education level.

Most of the parents (67.3% in the CeD group, 71.2% in the FAP group, 57.9% in the

healthy group) had low socioeconomic status (monthly income ≤400 $). *Blastocystis* prevalence in children who had low socioeconomic status was 2.5% in CeD and FAP groups, 3.2% in healthy group. While the prevalence of *Blastocystis* was 1.2% in the CeD and healthy groups in children with middle-high socioeconomic status, it was not detected in the FAP group. Although *Blastocystis* frequency was highest among children with low socioeconomic status, the distribution of the parasite was not significantly associated with demographic or socioeconomic factors.

Distribution of *Blastocystis* prevalence according to the domestic animal owners shown in Table 3.There was no significant relationship between *Blastocystis* and domestic animal ownership.

Table 3: Distribution of *Blastocystis* prevalence according to the domestic animal owners

Variable	N (%)	Blastocystis positive	P value*
CeD group			
Yes	8 (15.4)	1 / 8	1.000
No	44 (84.6)	5 / 44	
FAP group			
Yes	7 (13.5)	1 / 7	0.450
No	45 (86.5)	3 / 45	
Healthy group			
Yes	8 (14)	2 / 8	0.252
No	49 (86)	5 / 49	
Overall			
Yes	23 (14.3)	4 / 23	0.270
No	138 (85.7)	13 / 138	

*Fisher's Exact Test
CeD: Celiac disease   FAP: Functional abdominal pain

The most majority of the children were using bottled water (57.1%) for drinking. While *Blastocystis* was not detected in CeD and healthy groups in those who use tap water for drinking, the prevalence of *Blastocystis* was 0.6% in FAP group. In those who use bottled water for drinking, *Blastocystis* prevalence was 3.7%, 1.9% and 4.3% in CeD, FAP and healty groups respectively. *Blastocystis* was remarkably more common in those who used bottled water for drinking in the CeD and healthy groups (*P*=0.001).

Blastocystis subtype (ST) distribution and association with disease

Blastocystis was detected in 11.5% (6/52), 7.7% (4/52) and 12.3% (7/57) of the individuals in the CeD, FAP and healthy groups, re-

spectively, as determined by trichrome. *Blastocystis* detection rate by PCR presented in Table 4. Except one case of (1.92%) *G. intestinalis* infection in the CeD group, no other viral, bacterial and parasitic agents were found in any of the patients. Statistically significant differences were not found between the CeD, FAP and healthy groups in terms of *Blastocystis* frequency.

To determine *Blastocystis* STs, PCR products of 14 PCR-positive stool samples were sequenced bidirectionaly. There was no significant difference between the CeD and FAP groups in terms of *Blastocystis* ST frequency (Table 4). In addition, there was no relationship between *Blastocystis* ST frequencies and age (Table 5).

Table 4: Distribution of *Blastocystis* subtypes found positive by PCR according to the disease

Variable	Blastocystis positive by PCR #	Subtypes (STs)[a]		
		ST1 N (%)	ST2 N (%)	ST3 N (%)
CeD Group	5 (35.7)	–	4 (28.6)	1 (7.1)
FAP Group	4 (28.6)	1 (7.1)	1 (7.1)	2 (14.3)
Healthy group	5 (35.7)	2 (14.3)	1 (7.1)	2 (14.3)
Total	14 (100)	3 (21.4)	6 (42.8)	5 (35.7)

[a] Only 14 PCR positive samples included in the analysis
CeD: Celiac disease   FAP: Functional abdominal pain

Table 5: Distribution of *Blastocystis* subtypes found positive by PCR according to the age

Age(yr)	Blastocystis positive N (%)	Subtypes (STs)[a]		
		ST1 N (%)	ST2 N (%)	ST3 N (%)
0–6	1 (7.1)	–	–	1 (7.1)
7–11	5 (35.7)	2 (14.3)	2 (14.3)	1 (7.1)
12–18	8 (57.1)	1 (7.1)	4 (28.6)	3 (21.4)
Total	14 (100)	3 (21.4)	6 (42.8)	5 (35.7)

[a] Only 14 PCR positive samples included in the analysis

Phylogenetic analysis

The evolutionary history of *Blastocystis* was inferred using the Neighbor-Joining method (Fig. 1). The optimal tree with the sum of branch length = 0.29238781 is shown. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. The tree is drawn to scale, with branch lengths directly representing the evolutionary distances used to infer the phylogenetic tree.

Discussion

CeD is an autoimmune disease and the role of impaired intestinal epithelial permeability in the formation of CeD is well known. Colon microbiota plays a role in the regulation of the intestinal mucosa barrier and epithelial permeability (15).

Although there are several studies investigating the relationship between microbiota/probiotics and CeD in recent years, the relationship between *Blastocystis,* and CeD has not been investigated before (16-17). Our study is the first to determine the frequency of *Blastocysis* in pediatric CeD and FAP patients in the literature. *Blastocystis* was less frequent in FAP patients and most frequent in healthy children, while there was no statistically difference between patients with CeD (11.5%) or FAP (7.7%) and healthy children (12.3%) in terms of *Blastocystis* detection frequency (P = 0.7103). *Blastocystis* was more prevalent in healthy controls and in patients with inactive ulcerative colitis compared to those with active disease (18). Similarly, Rossen et al found it to be significantly less common in patients with ulcerative colitis than in healthy controls (19). Interestingly, several other recent studies have reported that *Blastocystis* is found more frequently in healthy individuals than patients with gastrointestinal disease (20).
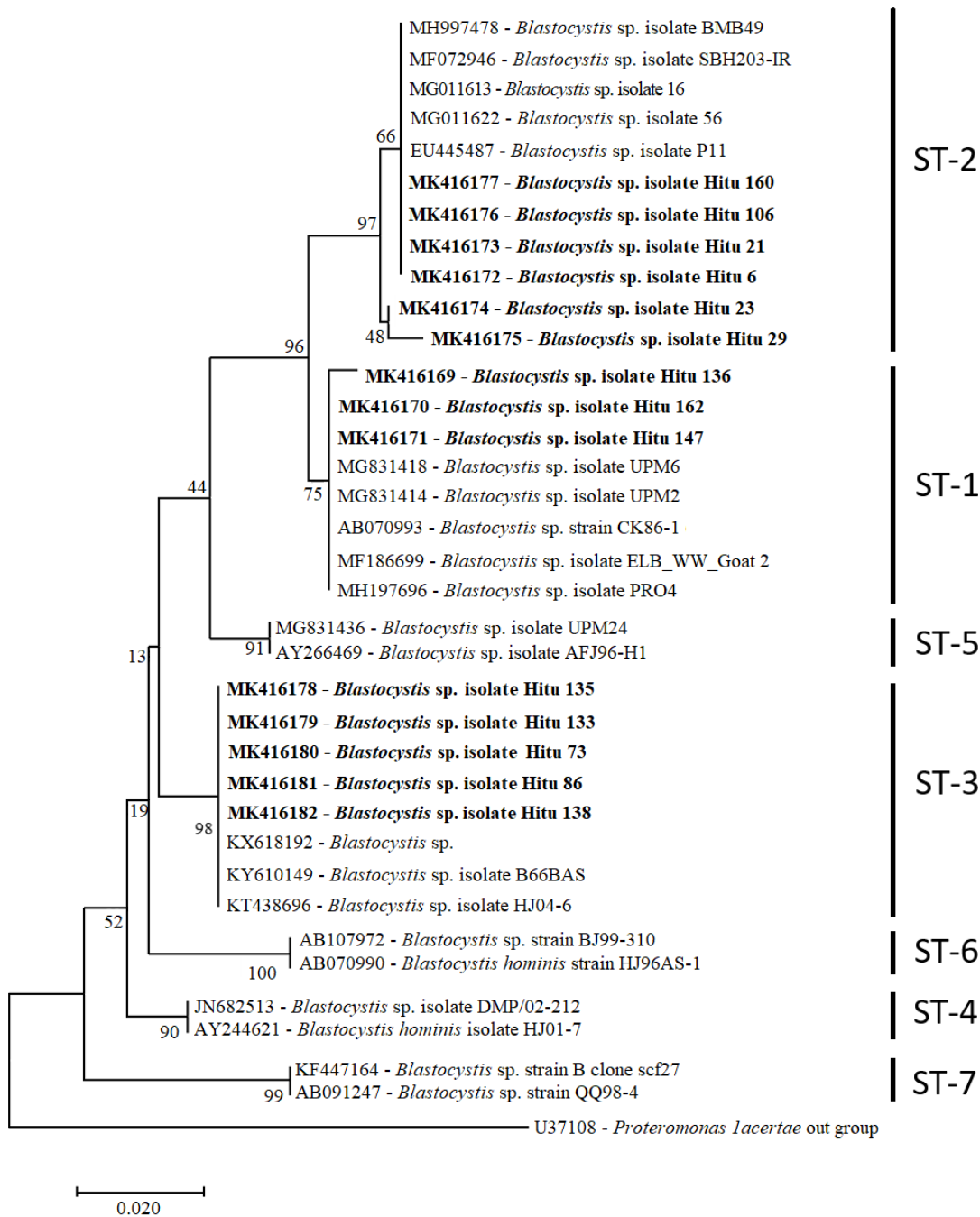
Fig. 1: Evolutionary relationships of taxa of *Blastocystis* among children with FAP and CeD in Çorum, Turkey

Children are thought to be more susceptible to intestinal infections, especially because of poor hygiene practices, frequent contact with soil, and the immaturity of their immune system (21). *Blastocystis* prevalence demonstrates considerable variation with respect to the developmental level of the country and the urban/rural nature of the research population. Sagnuankiat et al detected *Blastocystis* in 0.5% of the children in Thailand (4), while Rebolla

et al found in 86.63% of Brazilian school's children (5). There are only a few reports of *Blastocystis* prevalence in children in our country, which have reported significant variations in prevalence, from 1.1% to 37.9% (22,23). In the current study, positivity was detected in 10.6% of the children.

We found that *Blastocystis* positivity was not related to the sex of the children. The distribution of *Blastocystis* did not vary according to sex (10) however, in contrast, the prevalence of *Blastocystis* infection was higher in males compared to females (24).In our study, *Blastocystis* was most frequent in the 12–18 ages and it appeared that prevalence was increasing by age, albeit statistical significance was not observed. Forsell et al and Beyhan et al reported similar results, with their data indicating highest prevalence in those aged 7–13 yr in addition to increased frequency with age (25,26). This may mean that colonization is relatively rare among infants and toddlers, while older children are more commonly colonized.

Blastocystis positivity was not significantly related to socioeconomic status and education level of the parents in our study. *Blastocystosis* is more common in children whose parents had a basic level of education (27). On the other hand, the frequency of *Blastocystis* was not associated with socioeconomic status (28), similar to our findings.

Blastocystis transmission occurs through the fecal-oral route among humans. There may also be zoonotic transmission from animals such as, chickens, pigs and horses (29,30). In addition, increased risk for *Blastocystis* presence among individuals who have close contact with animals, including zookeepers and abattoir workers was reported (31,32). Our findings did not demonstrate any relationship between *Blastocystis* prevalence and domestic animal ownership.

Source of the drinking water is the most important risk factor for protozoon infections (33) Seyer et al did not find a link between *Blastocystis* carriage and source of drinking water (10). In our study, there was no family us-

ing water collected from the wells and *Blastocystis* was remarkably more common in bottled water users in CeD and healthy groups than the tap water users. It is controversial, since it is expected that bottled water is safer. Further study is needed on this subject because of the low positive cases in our study.

Today, based on small subunit rRNA gene analysis (SSU rDNA), more than 22 STs of *Blastocystis* have been identified (34).

In this study, ST2 (42.8%) and ST3 (35.7%) were the predominant STs, followed by ST1 (21.4%) in the study group. Although ST3 has been reported as the most common ST in our country, ST1 and ST2 have also been reported in some studies as the most common STs depending on the region of the study population and the symptomatic or asymptomatic states of the individuals analyzed (23,35-37). In our study, ST2 was the most common ST in the CeD group (28.6%). In addition, we detected predominantly ST1 (14.3%) and ST3 (14.3%) in the healthy children. We found the expected STs which were previously found in Turkey (35-37) and none of the STs were more predominant than others were overall. It seems that children were not affiliated with a particular STs detected. ST1, ST2 and ST3 were the most common STs in humans across the globe, but we could not identify any patients with ST4, which is frequently observed in studies outside Europe (10).

In our study to determine *Blastocystis* STs we used limited number of PCR products of PCR-positive stool samples, which may be a limitation of our study.

Conclusion

In the study where *Blastocystis* frequencies were assessed in children with CeD, FAP and healthy controls, we observed no statistically difference between study groups in terms of *Blastocystis* prevalence. ST1, ST2 and ST3 were the most common STs and presence of the parasite and its STs were not significantly re-

lated to any of the demographic, socioeconomic and epidemiological factors. To determine the association of STs with CeD or FAP, further studies which include a greater number of subjects are required.

Acknowledgements

Conflict of interest

The authors declare that there is no conflict of interest.

References

1. Rasquin A, Di Lorenzo C, Forbes D, et al. Childhood functional gastrointestinal disorders: child/adolescent. Gastroenterology. 2006;130(5):1527-37.

2. Rosinach M, Esteve M, Gonzalez C, et al. Lymphocytic duodenosis: aetiology and long-term response to specific treatment. Dig Liver Dis. 2012;44(8):643-8.

3. Tan KS. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. Clin Microbiol Rev. 2008;21(4):639-65.

4. Sagnuankiat S, Wanichsuwan M, Bhunnachet E, et al. Health status of immigrant children and environmental survey of child daycare centers in Samut Sakhon Province, Thailand. J Immigr Minor Health. 2016;18(1):21-7.

5. Rebolla MF, Silva EM, Gomes JF, et al. High prevalence of *Blastocystis* spp. infection in children and staff members attending public urban schools in Sao Paulo State, Brazil. Rev Inst Med Trop Sao Paulo. 2016;58:31.

6. Ozcakir O, Gureser S, Erguven S, et al. Characteristics of *Blastocystis hominis* infection in a Turkish university hospital. Turkiye Parazitol Derg. 2007;31(4):277-82.

7. Scanlan PD. *Blastocystis*: past pitfalls and future perspectives. Trends Parasitol. 2012;28(8):327-34.

8. Dogruman-Al F, Yoshikawa H, Kustimur S, et al. PCR-based subtyping of *Blastocystis* isolates from symptomatic and asymptomatic individuals in a major hospital in Ankara, Turkey. Parasitol Res. 2009;106(1):263-8.

9. Comba A, Güreser AS, Karasartova D, et al. Thiol-disulfide homeostasis in children with celiac disease. Pediatr Int. 2020 Aug;62(8):950-956. doi: 10.1111/ped.14243. PMID: 32239752.

10. Seyer A, Karasartova D, Ruh E, et al. Epidemiology and prevalence of *Blastocystis* spp. in North Cyprus. Am J Trop Med Hyg. 2017;96(5):1164-70.

11. Seyer A, Karasartova D, Ruh E, et al. Is "dried stool spots on filter paper method (DSSFP)" more sensitive and effective for detecting Blastocystis spp. and their subtypes by PCR and sequencing? Parasitol Res. 2016;115(12):4449-55.

12. Scicluna SM, Tawari B, Clark CG. DNA barcoding of blastocystis. Protist. 2006;157(1):77-85.

13. Saitou N, Nei M. The neighbor-joining method: a new method for reconstructing phylogenetic trees. Mol Biol Evol. 1987;4(4):406-25.

14. Anonymus. Avaialble from: http://entamoeba.lshtm.ac.uk/ref.blasto.txt

15. Guandalini S, Setty M. Celiac Disease.Curr Opin Gastroenterol. 2008;24(6):707-12.

16. Pecora F, Persico F, Gismondi P, et al. Gut Microbiota in Celiac Disease: Is There Any Role for Probiotics?. Front Immunol. 2020;11:957. Published 2020 May 15. doi:10.3389/fimmu.2020.00957.

17. Chibbar R, Dieleman LA. The Gut Microbiota in Celiac Disease and probiotics. Nutrients.2019;11(10):2375. Published 2019 Oct 5. doi:10.3390/nu11102375 .

18. Petersen AM, Stensvold CR, Mirsepasi H, et al. Active ulcerative colitis associated with low prevalence of *Blastocystis* and *Dientamoeba fragilis* infection. Scand J Gastroenterol. 2013;48(5):638-9.

19. Rossen NG, Bart A, Verhaar N, et al. Low prevalence of *Blastocystis* sp. in active ulcerative colitis patients. Eur J Clin Microbiol Infect Dis. 2015;34(5):1039-44.

20. Beghini F, Pasolli E, Truong TD, et al. Large-scale comparative metagenomics of *Blastocystis*,

a common member of the human gut microbiome. ISME J. 2017;11(12):2848-63.

21. LaBeaud AD, Nayakwadi Singer M, McKibben M, et al. Parasitism in children aged three years and under: Relationship between infection and growth in rural coastal Kenya. PLoS Negl Trop Dis. 2015;9(5):e0003721.

22. Degerli S, Celiksoz A, Aslan A, et al. Comparison of the results of examination of fecal samples from students at six months intervals in the Alahaci village primary school in Sivas. Turkiye Parazitol Derg. 2006;30(4):305-7.

23. Dogan N, Aydin M, Tuzemen NU, et al. Subtype distribution of *Blastocystis* spp. isolated from children in Eskisehir, Turkey. Parasitol Int. 2017;66(1):948-51.

24. Cabrine-Santos M, Cintra Edo N, do Carmo RA,et al. Occurrence of *Blastocystis* spp. in uberaba, minas gerais, Brazil. Rev Inst Med Trop Sao Paulo. 2015;57(3):211-4.

25. Forsell J, Granlund M, Samuelsson L, et al. High occurrence of *Blastocystis* sp. subtypes 1-3 and *Giardia intestinalis* assemblage B among patients in Zanzibar, Tanzania. Parasit Vectors. 2016;9(1):370.

26. Beyhan YE, Yilmaz H, Cengiz ZT, et al. Clinical significance and prevalence of *Blastocystis hominis* in Van, Turkey. Saudi Med J. 2015;36(9):1118-21.

27. Cociancic P, Torrusio SE, Zonta ML, et al. Risk factors for intestinal parasitoses among children and youth of Buenos Aires, Argentina. One Health. 2019;9:100116.

28. Oliveira-Arbex AP, David EB, Guimaraes S. Blastocystis genetic diversity among children of low-income daycare center in Southeastern Brazil. Infect Genet Evol. 2018;57:59-63.

29. Yoshikawa H, Tokoro M, Nagamoto T, et al. Molecular survey of *Blastocystis* sp. from humans and associated animals in an Indonesian community with poor hygiene. Parasitol Int. 2016;65(6 Pt B):780-4.

30. Basak S, Rajurkar MN, Mallick SK. Detection of *Blastocystis hominis*: a controversial human pathogen. Parasitol Res. 2014;113(1):261-5.

31. Parkar U, Traub RJ, Vitali S, et al. Molecular characterization of *Blastocystis* isolates from zoo animals and their animal-keepers. Vet Parasitol. 2010;169(1-2):8-17.

32. Rajah Salim H, Suresh Kumar G, Vellayan S, et al. *Blastocystis* in animal handlers. Parasitol Res. 1999;85(12):1032-3.

33. Assavapongpaiboon B, Bunkasem U, Sanprasert V, et al. A Cross-Sectional Study on Intestinal Parasitic Infections in Children in Suburban Public Primary Schools, Saraburi, the Central Region of Thailand. Am J Trop Med Hyg. 2018;98(3):763-7.

34. Stensvold CR, Clark CG. Molecular identification and subtype analysis of *Blastocystis*. Curr Protoc Microbiol. 2016;43:20A. 2. 1-20A. 2 .10.

35. Dogruman-Al F, Dagci H, Yoshikawa H, et al. A possible link between subtype 2 and asymptomatic infections of *Blastocystis hominis*. Parasitol Res. 2008;103(3):685-9.

36. Ertug S, Malatyali E, Ertabaklar H, et al. Subtype distribution of *Blastocystis* isolates and evaluation of clinical symptoms detected in Aydin province, Turkey. Mikrobiyol Bul. 2015;49(1):98-104.

37. Yersal O, Malatyali E, Ertabaklar H, et al. *Blastocystis* subtypes in cancer patients: Analysis of possible risk factors and clinical characteristics. Parasitol Int. 2016;65(6 Pt B):792-6.