Tehran University of Medical Sciences Publication
http://tums.ac.ir

Iran J Parasitol

Open access Journal at
http://ijpa.tums.ac.ir

Iranian Society of Parasitology
http://isp.tums.ac.ir

Original Article

Molecular Identification, Subtypes Distribution, and Alleles Discrimination of *Blastocystis* sp., Isolated from Immunocompromised Subjects in Iran

Hanieh Mohammad Rahimi [1], Seyed Ahmad Karamati [2], *Sara Nemati [1], *Hamed Mirjalali [1], Mohammad Reza Zali [3]

1. *Foodborne and Waterborne Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran*
2. *Department of Medical Parasitology and Mycology, Faculty of Medicine, Tehran Medical Sciences, Islamic Azad University, Tehran, Iran*
3. *Gastroenterology and Liver Diseases Research Center, Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences, Tehran, Iran*

Received 15 Oct 2021
Accepted 06 Jan 2022

Keywords:
Blastocystis sp.;
Immunodeficiency;
Opportunistic infection;
Subtypes;
Allele discrimination;
Iran

***Correspondence Email:**
hamedmirjalali@sbmu.ac.ir
sara.nemati64@yahoo.com

Abstract

Background: *Blastocystis* sp., is a prevalent protist isolated from humans and animals, which its opportunistic role in immunocompromised patients is still controversial. The current study aimed to evaluate the subtype and alleles distribution of *Blastocystis* sp., among immunocompromised patients.

***Methods*:** Totally, 33 microscopically *Blastocystis*-positive stool samples, isolated from Guilan province during April 2018 to May 2019 were investigated. Total DNA extraction was performed and the barcoding region of the small subunit ribosomal RNA (SSU rRNA) gene was amplified. Targeted fragments were sequenced to characterize subtypes and relevant alleles. Phylogenetic tree was constructed using Maximum-likelihood and Tamura 3-parameter to illustrate the correlation between subtypes and certain immunodeficiency.

***Results*:** Subtype analysis revealed the presence of ST1, ST2, ST3, and ST7 among 13/33 (39.4%), 5 (15.2%), 14/33 (42.4%), and 1/33 (3%), of samples, respectively. ST1 was the major subtype among cancer patients 5/7 (71.42%), while ST3 was the predominant subtype among rheumatoid arthritis (RA) patients 3/6 (50%), internal ward patients 5/10 (50%), and asthma and allergy patients 2/3 (66.66%). ST7 was isolated from a patient hospitalized in internal ward. No significant correlation was seen between the type of immunodeficiency and subtypes (*P*-value = 0.771). The phylogenetic tree showed no separation regarding the type of immunodeficiency.

***Conclusion*:** Among studied immunocompromised patients, ST3 was the most prevalent subtype followed by ST1. There was no specific correlation between subtypes and alleles with type of immunodeficiency. Putative zoonotic alleles were highlighted the probability of zoonotic transmission for *Blastocystis* sp.

Introduction

Blastocystis sp., is one of the most common parasite, which colonizes the intestine of its hosts. *It* is a cosmopolitan protist with a potential for pandemic distribution (1, 2). This parasite is reported from wide range of animals including amphibians, reptiles, invertebrates, birds, non-human primates, and artiodactyls, as well as humans (3, 4). Four major forms of this parasite including vacuolar, granular, amoeboid, and cyst have been reported from stool samples and cultured mediums (2). *Blastocystis* sp., was classified initially as a cyst of a flagellate, vegetable, fungus, and harmless yeast for many years, but it is now considered as an agent, which may lead to intestinal or extra-intestinal manifestations (5, 6). The fecal-oral route is considered as the main transmission mode of infection (7).

A couple of clinical features have been linked to *Blastocystis* sp., ranging from mild and chronic diarrhea to acute gastroenteritis, anemia, and urticarial (6, 8, 9). During recent years, the reports of *Blastocystis* sp., in symptomatic patients without any known causative agents signified the pathogenic role of *Blastocystis* sp., (10, 11). However, the pathogenicity of this parasite is still matter of debate and it mostly leads to a generally self-limiting gastrointestinal disorders (12, 13).

By study on the small subunit ribosomal RNA (SSU-rRNA) gene, 17 specific subtypes (ST) of *Blastocystis* sp., have been identified in humans and a wide range of animals (14, 15). *Blastocystis* sp., subtypes (ST1-ST9 and ST12) are isolated from human and animals, while the reports of ST9 is limited to humans (1, 16, 17). Subtypes ST10 to ST17 have been identified exclusively in non-human hosts including non-human primates (NHPs), mammals, birds and insects (15). The recent updates suggested the presence of at least 22 subtypes including ST21 and ST23-ST26 in mammals (18).

The pathogenicity of *Blastocystis* sp. seems to be affected by both host and parasite factors; however, evaluation of the pathogenic potential of certain subtypes is difficult (19). A probable correlation between specific subtypes and clinical manifestations has been proposed (20), and few studies suggested the presence/majority of certain subtypes in patients with background diseases or specific symptoms (21, 22).

In the current study, we aimed to evaluate the subtype distribution and frequency of relevant alleles among *Blastocystis* sp., isolated from different hospitalized immunocompromised patients receiving corticosteroids.

Materials and Methods

Ethics approval

This study received ethical approval from the Ethics Committee of the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences (SBMU), Tehran, Iran (no. IR.SBMU.RIGLD.REC.1398.048).

Sample collection and DNA extraction

Totally, 33 microscopically *Blastocystis*-positive stool samples, isolated from Guilan province during April 2018 to May 2019, were investigated (23). Briefly, 250 µL of stool suspensions was centrifuged at 2500 × g for 5 min, supernatant was discarded and total DNA was extracted using stool DNA extraction kit (Yekta Tajhiz Azma, Tehran, Iran). Extracted DNA was stored at -20°C until further molecular analysis.

PCR amplification

The ~ 620-bp fragment of the barcoding region of the SSU rRNA gene of *Blastocystis* sp., was amplified using primers RD5 (5'-ATCTGGTTGATCCTGCCAGT-3') and

BhRDr (5'-GAGCTTTTTAACTGCAACAACG-3') (24). PCR amplifications were done at standard conditions: an initial denaturing step of 95 °C for 5 min and 35 cycles consisting of 94 °C for 30 s, 58 °C for 30 s, and 30 s at 72 °C. A final extension at 72 °C was performed for 5 min. Finally, 5 µL of amplification products was fractionated on 1.5% agarose electrophoresis gel stained with ethidium bromide and then visualized by the UV transilluminator (Cleaver scientific Ltd., Warwickshire, United Kingdom).

Subtype analysis and allele discrimination

PCR products were purified and then sequenced with ABI 3130 sequencer. The obtained sequences were edited and trimmed by Chromas and BioEdit software. Homology analysis of the sequences was done using the basic local alignment search tool (BLAST; http://blast.ncbi.nlm.nih.gov/) for the most similar reference sequences to determine the relevant subtypes. Finally, the new DNA sequences have been submitted into the genetic sequence database at the national center for biotechnical information (NCBI) by the Sequin program (version 10.3). All sequences are available in the GenBank database with accession numbers MT645783 to MT645814. To identify the relevant alleles, the edited sequences were submitted to (https://pubmlst.org/organisms/blastocystis-spp/).

Phylogenetic analysis

After alignment and trimming of the sequences, a ~ 530-bp fragment was utilized for analyses. The phylogenetic tree was constructed using Maximum-likelihood algorithm and tamura-3- parameter model in MEGA10 software (http://www.megasoftware.net/) (25). To evaluate the reliability of the tree, bootstrap with 1000 replications was considered.

Statistical analysis

The frequency of subtypes and gender, and the mean of age ± standard deviation (SD) were calculated. In addition, to analyze the possible correlation between immunodeficiency and subtypes, the Fisher's exact test incorporated in SPSS Statistics software for Windows, v22 (IBM Corp., Armonk, NY, USA) was used. A probability (*P*) value < 0.05 was considered statistically significant.

Results

The studied patients were 16 (48. 5%) males and 17 (51. 5%) females. The average age ± SD was 51.82 ± 13.17. *Blastocystis* sp., was isolated from hospitalized patients, who received either corticosteroids or chemotherapy agents, including one (3%) transplantation recipient, six (18.2%) rheumatoid arthritis (RA), three (9.1%) gastrointestinal disorders, three (9.1%) systemic lupus erythematosus (SLE), seven (21.2%) cancer patients, three (9.1%) asthma and allergy patients, and 10 (30.3%) patients hospitalized in internal ward with undiagnosed immunodeficiency disorder.

Molecular detection, subtyping, and allele discriminations

Target fragment was amplified among all 33 microscopically *Blastocystis*-positive samples. Subtypes analysis revealed the presence of ST1, ST2, ST3, and ST7 among 13/33 (39.4%), 5/33 (15.2%), 14/33 (42.4%), and 1/33 (3%), of samples, respectively. The subtypes distribution among patients showed the majority of ST1 among cancer patients 5/7 (71.42%), while ST3 was the predominant subtype among RA patients 3/6 (50%), internal patients 5/10 (50%), and asthma and allergy patients 2/3 (66.66%). ST2 was seen among RA, internal, gastrointestinal, SLE, and transplantation patients. ST7 was isolated from a patient hospitalized in internal ward (Table 1).

The results of allele discrimination revealed the presence of alleles 4 (10/13) and 88 (3/13) in ST1. ST2 exhibited alleles 9 and 10 in four and one isolates, respectively (Table 2). ST3 represented alleles 34 and 36 with majority of allele 34 (10/13). The only ST7 was allele 99 (Fig. 1).

Table 1: *Blastocystis* sp., subtype distribution among immunocompromised patients

Immunodeficiency disorders	***Subtypes/No.33***			
	ST1	ST2	ST3	ST7
RA	2	1	3	-
Internal	3	1	5	1
Gastrointestinal disorders	1	1	1	-
SLE	1	1	1	-
Cancer patients	5	-	2	-
Asthma and allergy	1	-	2	-
Transplantation	-	1	-	-

Table 2: *Blastocystis* sp., and its subtypes among immunocompromised patients

No.	***Gender***	***Age(yr)***	***Blastocystis sp., (sub-types)***	***Alleles***	***Acc. No.***
1	Female	48	ST2	9	MT645783
2	Female	59	ST3	36	MT645784
3	Male	59	ST7	99	MT645785
4	Male	71	ST2	9	MT645786
5	Female	58	ST3	34	MT645787
6	Male	38	ST2	10	MT645788
7	Female	48	ST3	34	MT645789
8	Female	53	ST1	4	MT645790
9	Female	43	ST3	36	MT645791
10	Female	36	ST3	34	MT645792
11	Male	38	ST2	9	MT645793
12	Female	58	ST1	88	MT645794
13	Male	29	ST3	36	MT645795
14	Male	70	ST1	4	MT645796
15	Female	47	ST2	9	MT645797
16	Male	59	ST1	4	MT645798
17	Female	52	ST3	34	MT645799
18	Female	35	ST3	34	MT645800
19	Female	45	ST1	4	MT645801
20	Male	59	ST3	34	MT645802
21	Male	61	ST3	34	MT645803
22	Male	71	ST1	4	MT645804
23	Female	58	ST1	88	MT645805
24	Female	50	ST1	4	MT645806
25	Male	52	ST3		Not provided
26	Male	57	ST3	34	MT645807
27	Female	26	ST1	4	MT645808
28	Male	34	ST1	4	MT645809
29	Male	58	ST1	4	MT645810
30	Female	52	ST1	4	MT645811
31	Female	61	ST3	34	MT645812
32	Male	86	ST3	34	MT645813
33	Male	39	ST1	88	MT645814

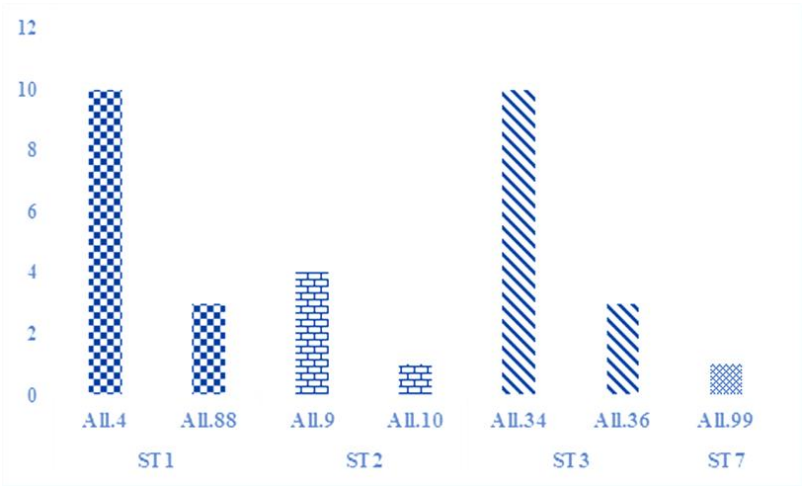

Fig. 1: Allele distribution among each studied subtype

Phylogenetic analysis

Phylogenetic analysis of the SSU rRNA gene sequences revealed that all subtypes were clearly separated into four clades regarding the currently characterized subtypes, with bootstraps ranging from 77 to 99% (Fig. 2). The phylogenetic tree also showed that there was no separation regarding the type of immune diseases or hospitalization's wards.

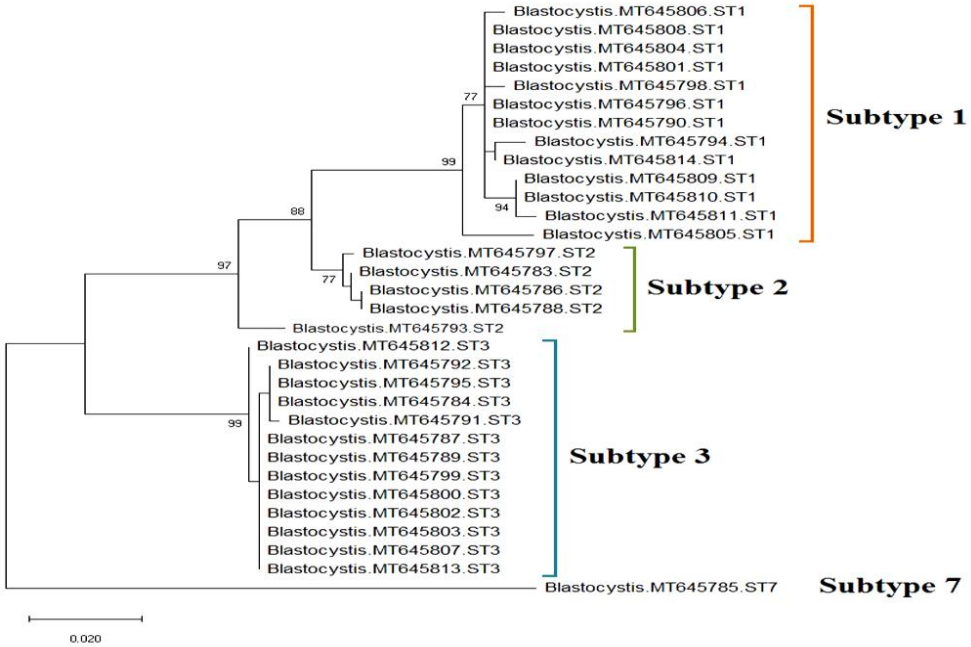

Fig. 2: The phylogenetic position of *Blastocystis* sp., ST1-3 and 7 isolated from immunocompromised patients. The phylogenetic tree was assembled based on the Maximum-likelihood and Tamura 3-parameter algorithms. Bootstrap lower than 75% were deleted

Discussion

Blastocystis sp., is a mysterious protist with a lot of unknown features in its life cycle, infectivity, and pathogenicity. The distribution of this protist is thought to be linked with the socioeconomic conditions (7), but a couple of studies indicated a high prevalence of *Blastocystis* sp., in developed countries with high levels of standard of living (26-30).

The pathogenicity of *Blastocystis* sp., is also unclear. Although some studies showed a correlation between the presence of this eukaryote and gastrointestinal, as well as extra-intestinal symptoms (6, 8, 9), most of researches have been failed to provide a strong correlation between symptoms and the presence of *Blastocystis* sp. In this line, Jalallu et al., (31) investigated the frequency of *Blastocystis* sp., and its subtypes in symptomatic and asymptomatic human subjects and claimed no significant coexistence between the protist and clinical manifestations. Dogan et al., (32) studied the prevalence of *Blastocystis* sp. among symptomatic and asymptomatic children and reported no statistical correlation between the presence of symptoms and *Blastocystis* sp. In a large-scale study conducted in France, a statistical significant correlation was not seen between the colonization of *Blastocystis* sp., and clinical manifestations (33). However, there are studies that suggested a linkage between the presence of *Blastocystis* sp., or a specific subtype with clinical symptoms. Abdulsalam et al. (34) screened outpatients who were referred to a laboratory and claimed that the prevalence of *Blastocystis* sp., in symptomatic group was higher that asymptomatic group. In addition, in a study in Lebanon the correlation between *Blastocystis* sp., subtype 1 and clinical manifestations was pointed out (20).

Although the pathogenic role of *Blastocystis* sp., in immunocompetent subjects has been remained controversial, a couple of studies suggested *Blastocystis* sp., to be an opportunistic infection in immunocompromised patients. One of the first studies highlighting the opportunistic role of *Blastocystis* sp. was performed by Ghosh et al, (35) who presented a case of myeloid leukemia underwent bone marrow transplantation, which was colonized by *Blastocystis* sp. In the current study we did not access to stool samples of immunocompromised patient hospitalized in different wards to evaluate the prevalence of the protist; nevertheless, *Blastocystis* sp., ST2 was the only subtype detected in the transplant recipient.

The correlation between colonization of *Blastocystis* sp., and its subtypes with cancer has been evaluated. Kumarasamy et al (36), showed a significant correlation between *Blastocystis* sp., and colorectal cancer (CRC) and presented a significant association between the presence of ST3 and CRC. A high frequency of *Blastocystis* sp., ST3 was determined in cancer patients in Turkey (37), as well. Indeed, Mohamed et al., (38) analyzed the correlation between colonization of *Blastocystis* sp., and its subtypes with cancer and showed that not only colonization of *Blastocystis* sp., in cancer patients was higher than controls, but also there was a significant correlation between the presence of subtype 1 and CRC. Zhang et al. (39), characterized the prevalence rate of *Blastocystis* sp., subtypes among cancer patients and although there was no statistically significant correlation between subtypes and cancer, ST3 was the most prevalent subtype in these patients followed by ST1. In the current study, a significant correlation was not found between *Blastocystis* sp., and its subtypes with type of immunodeficiency; however, in line of study performed by Mohamed et al. (38), *Blastocystis* sp., ST1 was the most prevalent subtype in cancer patients followed by ST3.

Although some studies linked the presence of *Blastocystis* sp., particularly ST3, with urticarial and cutaneous rashes (22, 40, 41), there is no study evaluating subtype distribution of *Blastocystis* sp., among patients who suffered from asthma and allergy, and SLE. In the current study, only ST1 and ST3 were detected in asthma and allergy patients, while all three

subtypes ST1-3 were characterized in SLE patients. However, due to the low number of evaluated subjects, establishing a subtype pattern in these patients, particularly among asthma and allergy patients, needs to be validated.

To the best of our knowledge, there is no study evaluating the subtype distribution of *Blastocystis* sp., among RA patients. Nonetheless, Lee et al. (42) presented a case of RA with acute diarrhea and increased inflammation harboring *Blastocystis* sp., who after successful treatment of the protist with metronidazole, the symptoms such as diarrhea, abdominal pain, and inflammation of patient's knee were ameliorated. In the current study, three subtypes ST1-3 were characterized in RA patients; however, due to the low number of samples, this study was failed to propose a probable connection between certain subtype and RA.

As a result, ST1 represented alleles 4 and 88. Allele 4 seems to be the most prevalent allele related to ST1 (43). However, there is a little data presenting allele 88 from ST1 in the world (44). For ST2, alleles 9 and 10 were identified among samples. Allele 9 was characterized as one of the most prevalent alleles reported from humans and also dogs in Iran (43, 45). This allele was also reported from other countries and seems to be the common characterized allele from ST2. In contrast, allele 10 is not common in human subjects and there is no report of this allele in Iran, while it was reported from South America (4). For ST3, alleles 34 and 36 were identified among samples. These alleles are among the most frequently reported alleles in human and animal subjects. However, in the line of previous studies, allele 34 was the most prevalent allele in our subjects. ST7 represented allele 99. There are reports of this allele in humans and animals. Melo et al. (46) successfully cultivated microscopically *Blastocystis*-positive fecal samples and isolated *Blastocystis* ST7 allele 99 from asymptomatic Brazilian subjects. In Iran, Mohammadpour et al. (45) investigated the prevalence of *Blastocystis* sp., from cats, dogs, and brown rats, and isolated ST7 allele 99 from dog samples. Most recently, ST7 allele 99 was isolated from chicken sample in Iran (47). Although reports of this allele is limited to couple of studies, the presence of that in both animal and human isolates may provide a clue of zoonotic transmission of this allele.

Conclusion

Blastocystis sp., ST3 and ST1 were characterized as the most prevalent subtypes among studied immunocompromised patients of which ST3 was the most prevalent followed by ST1. Although ST1 was more prevalent in cancer patients and ST3 was the major subtype in RA patients, asthma and allergy patients, and those who were hospitalized in internal ward, a significant correlation between certain subtypes and type of immunodeficiency was not seen. The allele discrimination showed none specific alleles among immunocompromised patients, while putative zoonotic alleles were also detected.

Acknowledgements

The authors thank all members of the Foodborne and Waterborne Diseases Research Center for their supports. This study was financially supported by the Research Institute for Gastroenterology and Liver Diseases, Shahid Beheshti University of Medical Sciences with grant number: RIGLD-1072.

Conflict of interest

The authors declare that they have no conflict of interest

References

1. Clark CG, van der Giezen M, Alfellani MA, Stensvold CR. Recent developments in *Blasto-*

cystis research. Adv Parasitol. 2013; 82:1-32. DOI: 10.1016/B978-0-12-407706-5.00001-0
2. Tan KS. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. Clin Microbiol Rev. 2008; 21:639-65. DOI: 10.1128/CMR.00022-08
3. Nemati S, Zali MR, Johnson P, Mirjalali H, Karanis P. Molecular prevalence and subtype distribution of *Blastocystis* sp. in Asia and in Australia. J Water Health. 2021;19(5):687-704. DOI:10.2166/wh.2021.011
4. Jimenez PA, Jaimes JE, Ramirez JD. A summary of *Blastocystis* subtypes in North and South America. Parasit Vectors. 2019; 12:376. DOI: 10.1186/s13071-019-3641-2
5. Scanlan PD. *Blastocystis*: past pitfalls and future perspectives. Trends Parasitol. 2012; 28:327-34. DOI: 10.1016/j.pt.2012.05.001
6. Tan KS, Mirza H, Teo JD, Wu B, Macary PA. Current views on the clinical relevance of *Blastocystis* spp. Curr Infect Dis Rep. 2010; 12:28-35. DOI: 10.1007/s11908-009-0073-8
7. Javanmard E, Niyyati M, Ghasemi E, Mirjalali H, Asadzadeh Aghdaei H, Zali MR. Impacts of human development index and climate conditions on prevalence of *Blastocystis*: A systematic review and meta-analysis. Acta Trop. 2018; 185:193-203. DOI: 10.1016/j.actatropica.2018.05.014
8. El Deeb HK, Khodeer S. *Blastocystis* spp.: frequency and subtype distribution in iron deficiency anemic versus non-anemic subjects from Egypt. J Parasitol. 2013; 99:599-602. DOI: 10.1645/12-80.1
9. Rezaei Riabi T, Haghighi A, Mirjalali H, et al. Study of prevalence, distribution and clinical significance of *Blastocystis* isolated from two medical centers in Iran. Gastroenterol Hepatol Bed Bench. 2017; 10:S102-S7.
10. Katsarou-Katsari A, Vassalos CM, Tzanetou K, Spanakos G, Papadopoulou C, Vakalis N. Acute urticaria associated with amoeboid forms of *Blastocystis* sp. subtype 3. Acta Derm Venereol. 2008; 88:80-1. DOI: 10.2340/00015555-0338
11. Vogelberg C, Stensvold CR, Monecke S, et al. *Blastocystis* sp. subtype 2 detection during recurrence of gastrointestinal and urticarial symptoms. Parasitol Int. 2010; 59:469-71. DOI: 10.1016/j.parint.2010.03.009
12. Andersen LO, Stensvold CR. *Blastocystis* in health and disease: are we moving from a clinical to a public health perspective? J Clin Microbiol. 2016; 54:524-8. DOI: 10.1128/JCM.02520-15
13. Engsbro AL, Stensvold CR. *Blastocystis*: to treat or not to treat...but how? Clin Infect Dis. 2012; 55:1431-2. DOI: 10.1093/cid/cis699
14. Stensvold CR, Suresh GK, Tan KS, et al. Terminology for *Blastocystis* subtypes--a consensus. Trends Parasitol. 2007; 23:93-6. DOI: 10.1016/j.pt.2007.01.004
15. Yoshikawa H, Koyama Y, Tsuchiya E, Takami K. *Blastocystis* phylogeny among various isolates from humans to insects. Parasitol Int. 2016; 65:750-9. DOI: 10.1016/j.parint.2016.04.004
16. Alfellani MA, Jacob AS, Perea NO, et al. Diversity and distribution of *Blastocystis* sp. subtypes in non-human primates. Parasitology. 2013; 140:966-71. DOI: 10.1017/S0031182013000255
17. Alfellani MA, Stensvold CR, Vidal-Lapiedra A, Onuoha ES, Fagbenro-Beyioku AF, Clark CG. Variable geographic distribution of *Blastocystis* subtypes and its potential implications. Acta Trop. 2013; 126:11-8. DOI: 10.1016/j.actatropica.2012.12.011
18. Stensvold CR, Clark CG. Pre-empting Pandora's Box: *Blastocystis* subtypes revisited. Trends Parasitol. 2020. DOI: 10.1016/j.pt.2019.12.009
19. Alinaghizade A, Mirjalali H, Mohebali M, Stensvold CR, Rezaeian M. Inter- and intra-subtype variation of *Blastocystis* subtypes isolated from diarrheic and non-diarrheic patients in Iran. Infect Genet Evol. 2017; 50:77-82. DOI: 10.1016/j.meegid.2017.02.016
20. El Safadi D, Meloni D, Poirier P, et al. Molecular epidemiology of *Blastocystis* in Lebanon and correlation between subtype 1 and gastrointestinal symptoms. Am J Trop Med Hyg. 2013; 88:1203-6. DOI: 10.4269/ajtmh.12-0777
21. Taghipour A, Javanmard E, Mirjalali H, et al. *Blastocystis* subtype 1 (allele 4); Predominant subtype among tuberculosis patients in Iran. Comp Immunol Microbiol Infect Dis. 2019; 65:201-6. DOI: 10.1016/j.cimid.2019.06.005
22. Casero RD, Mongi F, Sanchez A, Ramirez JD. *Blastocystis* and urticaria: Examination of subtypes and morphotypes in an unusual clinical manifestation. Acta Trop. 2015; 148:156-61. DOI: 10.1016/j.actatropica.2015.05.004

23. Karimi K, Mirjalali H, Niyyati M, et al. Molecular epidemiology of *Enterocytozoon bieneusi* and *Encephalitozoon* sp., among immunocompromised and immunocompetent subjects in Iran. Microb Pathog. 2020; 141:103988. DOI: 10.1016/j.micpath.2020.103988
24. Scicluna SM, Tawari B, Clark CG. DNA barcoding of *Blastocystis*. Protist. 2006; 157:77-85. DOI: 10.1016/j.protis.2005.12.001
25. Tamura K, Stecher G, Peterson D, Filipski A, Kumar S. MEGA6: molecular evolutionary genetics analysis version 6.0. Mol Biol Evol. 2013; 30:2725-9. DOI: 10.1093/molbev/mst197
26. Beghini F, Pasolli E, Truong TD, Putignani L, Caccio SM, Segata N. Large-scale comparative metagenomics of *Blastocystis*, a common member of the human gut microbiome. ISME J. 2017; 11:2848-63. DOI: 10.1038/ismej.2017.139
27. Mattiucci S, Crisafi B, Gabrielli S, Paoletti M, Cancrini G. Molecular epidemiology and genetic diversity of *Blastocystis* infection in humans in Italy. Epidemiol Infect. 2016; 144:635-46. DOI: 10.1017/S0950268815001697
28. Forsell J, Granlund M, Stensvold CR, Clark CG, Evengard B. Subtype analysis of *Blastocystis* isolates in Swedish patients. Eur J Clin Microbiol Infect Dis. 2012; 31:1689-96. DOI: 10.1007/s10096-011-1416-6
29. Bart A, Wentink-Bonnema EM, Gilis H, et al. Diagnosis and subtype analysis of *Blastocystis* sp. in 442 patients in a hospital setting in the Netherlands. BMC Infect Dis. 2013; 13:389. DOI: 10.1186/1471-2334-13-389
30. Stensvold CR, Christiansen DB, Olsen KE, Nielsen HV. Blastocystis sp. subtype 4 is common in Danish *Blastocystis*-positive patients presenting with acute diarrhea. Am J Trop Med Hyg? 2011; 84:883-5. DOI: 10.4269/ajtmh.2011.11-0005
31. Jalallou N, Iravani S, Rezaeian M, Alinaghizade A, Mirjalali H. Subtypes Distribution and Frequency of *Blastocystis* sp. isolated from diarrheic and non-diarrheic patients. Iran J Parasitol. 2017; 12:63-8.
32. Dogan N, Aydin M, Tuzemen NU, Dinleyici EC, Oguz I, Dogruman-Al F. Subtype distribution of *Blastocystis* spp. isolated from children in Eskisehir, Turkey. Parasitol Int. 2017; 66:948-51. DOI:10.3855/jidc.12650
33. El Safadi D, Cian A, Nourrisson C, et al. Prevalence, risk factors for infection and subtype distribution of the intestinal parasite *Blastocystis* sp. from a large-scale multi-center study in France. BMC Infect Dis. 2016; 16:451. DOI 10.1186/s12879-016-1776-8
34. Abdulsalam AM, Ithoi I, Al-Mekhlafi HM, et al. Prevalence, predictors and clinical significance of *Blastocystis* sp. in Sebha, Libya. Parasit Vectors. 2013; 6:86. DOI: 10.1186/1756-3305-6-86
35. Ghosh K, Ayyaril M, Nirmala V. Acute GVHD involving the gastrointestinal tract and infestation with *Blastocystis hominis* in a patient with chronic myeloid leukaemia following allogeneic bone marrow transplantation. Bone Marrow Transplant. 1998; 22:1115-7. DOI: 10.1038/sj.bmt.1701488
36. Kumarasamy V, Roslani AC, Rani KU, Kumar Govind S. Advantage of using colonic washouts for *Blastocystis* detection in colorectal cancer patients. Parasit Vectors. 2014; 7:162.
37. Yersal O, Malatyali E, Ertabaklar H, Oktay E, Barutca S, Ertug S. *Blastocystis* subtypes in cancer patients: Analysis of possible risk factors and clinical characteristics. Parasitol Int. 2016; 65:792-6. DOI: 10.1016/j.parint.2016.02.010
38. Mohamed AM, Ahmed MA, Ahmed SA, Al-Semany SA, Alghamdi SS, Zaglool DA. Predominance and association risk of *Blastocystis hominis* subtype I in colorectal cancer: a case control study. Infect Agent Cancer. 2017; 12:21. DOI: 10.1186/s13027-017-0131-z
39. Zhang W, Ren G, Zhao W, et al. Genotyping of *Enterocytozoon* bieneusi and subtyping of *Blastocystis* in cancer patients: relationship to diarrhea and assessment of zoonotic transmission. Front Microbiol. 2017; 8:1835. DOI: 10.3389/fmicb.2017.01835
40. Gupta R, Parsi K. Chronic urticaria due to *Blastocystis hominis*. Australas J Dermatol. 2006; 47:117-9. DOI: 10.1111/j.1440-0960.2006.00244.x
41. Lepczynska M, Chen WC, Dzika E. Mysterious chronic urticaria caused by *Blastocystis* spp.? Int J Dermatol. 2016; 55:259-66; quiz 63-4, 66. DOI: 10.1111/ijd.13064
42. Lee MG, Rawlins SC, Didier M, DeCeulaer K. Infective arthritis due to *Blastocystis hominis*. Annal Rheum Dis. 1990; 49:192-3. DOI: 10.1136/ard.49.3.192

43. Rezaei Riabi T, Mirjalali H, Haghighi A, et al. Genetic diversity analysis of *Blastocystis* subtypes from both symptomatic and asymptomatic subjects using a barcoding region from the 18S rRNA gene. Infect Genet Evol. 2018; 61:119-26. DOI: 10.1016/j.meegid.2018.03.026
44. Higuera A, Villamizar X, Herrera G, et al. Molecular detection and genotyping of intestinal protozoa from different biogeographical regions of Colombia. PeerJ. 2020; 8:e8554. DOI: 10.7717/peerj.8554
45. Mohammadpour I, Bozorg-Ghalati F, Gazzonis AL, Manfredi MT, Motazedian MH, Mohammadpour N. First molecular subtyping and phylogeny of *Blastocystis* sp. isolated from domestic and synanthropic animals (dogs, cats and brown rats) in southern Iran. Parasit Vectors. 2020; 13:365. DOI: 10.1186/s13071-020-04225-9
46. Melo GB, Roldan W, Malta FM, et al. Culture isolation and molecular identification of *Blastocystis* sp. in Brazilian human isolates: preliminary results. Rev Inst Med Trop Sao Paulo. 2020; 62:e51. DOI: 10.1590/s1678-9946202062051
47. Mohammad Rahimi H, Mirjalali H, Zali MR. Molecular epidemiology and genotype/subtype distribution of *Blastocystis* sp., *Enterocytozoon bieneusi*, and *Encephalitozoon* spp. in livestock: concern for emerging zoonotic infections. Sci Rep. 2021;11(1):17467. DOI:10.1038/s41598-021-96960-x