Journal of the Egyptian Society of Parasitology, Vol. 53, No.3, December 2023
J. Egypt. Soc. Parasitol. (JESP), 53(3), 2023: 467 – 474
Online: 2090-2549

BLASTOCYSTIS SUBTYPE 3 AMONG ADOLESCENTS WITH GASTROINTESTINAL SYMPTOMS IN FAYOUM GOVERNORATE, EGYPT

By
FATEN AHMED MAHMOUD MOHAMED[1]* AND KHOLOUD ADEL KHALIL[2]
[1*]Department of Medical Parasitology, Faculty of Medicine, Fayoum University, and
[2]Department of Public Health, Damanhour National Medical Institute, Damanhour, Egypt (Correspondence: fam02@fayoum.edu.eg),

Abstract

Blastocystis is a common polymorphic protozoan infecting human intestine with a zoonotic potential. Despite the unclear pathogenicity, its existence may be linked to a wide range of gastrointestinal disorders. The present cross sectional study was conducted to analyze *Blastocystis* subtypes among 110 adolescents suffering from disturbing GIT symptoms in stool samples. Positive samples (21 (19.1%)) by microscopy were subjected to DNA isolation and subtyped by Sequence Tagged Site (STS)-PCR analysis, which successfully subtyped 10 (62.5%) of 16 isolates. The dominant *Blastocystis* subtype was ST3 in 6 (40%) samples followed by 3 (30%) for ST2, but one sample (10%) was amplified for ST1. The results revealed significant association between nausea and vomiting and *Blastocystis* infection. However, there was no difference between the *Blastocystis* subtypes and demo-clinical characteristics.

Keywords: *Blastocystis*, Sequence-Tagged Sites, PCR, Subtyping, Microscopy

Introduction

Blastocystis is unique obligate anaerobic protist that classified within the phylum *Stramenopiles* (de Melo *et al,* 2021). Its distinguished features include a large membrane bound central vacuole, multiple nuclei, and cytochrome free mitochondria (Arisue *et al,* 2002). *Blastocystis* is an intestinal inhabitants which commonly isolated from stool samples of man and other animals (Jha *et al,* 2021). It is widely prevalent in the world (Hammood *et al,* 2016). Prevalence rate varies according to the study population, geographical area and method of detection, linked to poor hygienic practices, contaminated food or water or animals contact (Nithyamathi *et al,* 2016). Infection rate was up to 100% in poor countries (Kaczmarek *et al,* 2020). In Egypt, rate was 33.3% (Abdel Hameed *et al,* 2011) up to 46.1% among schoolchildren (Abdo *et al,* 2023), and even was reported in water (El Shazly *et al,* 2014). In Jordan Abdel-Dayem *et al.* (2014) found *Blastocystis hominis* among food handlers in the luxurious hotels. In Saudi Arabia Amin (1997) in Jeddah found (13.99%), and among the apparently healthy food-handlers was 61.0% in the symptomatic patients in Riyadh (Abdel kareem *et al,* 2022).

Although, its pathogenic potential was not clear (Valença Barbosa *et al,* 2017), others linked between blastocystosis and gastrointestinal symptoms (Alinaghizade *et al,* 2017), angioedema (Lugović-mihić *et al,* 2019), urticaria (Aykur *et al,* 2022), palmoplantar pruritus (Kick *et al,* 2002), infective arthritis (Lee, 1990), iron deficiency anemia (Fonte *et al,* 2022) and irritable bowel (Kesuma *et al,* 2019). Besides, blastocystosis was detected in subjects without any apparent symptoms (Mardani Kataki *et al,* 2019). *Blastocystis* is a polymorphic and genetically variable parasite (Mohamed *et al,* 2017). Its basic diagnosis is by microscopy and cultures to differentiate between the four morphologic forms (Dogruman-Al *et al,* 2008). Diagnosis of *Blastocystis* by PCR is more sensitive than conventional basic methods (Chen *et al,* 2023). Genetic identification of *Blastocystis* spp. and subtyping has been approached mainly by a small subunit of ribosomal RNA (*SSU rRNA*) gene or by diagnostic subtype-specific sequence-tagged-site (STS) primers (Yoshikawa *et al,* 2004). The STS primers are advantageous as they eliminate the need for sequencing of the PCR products (Stensvold, 2013). Besides, *Blastocystis* subtyping is important in clarification linked between

epidemological factors, clinical aspects and infection (Pandey *et al,* 2015)

Now, within the genus *Blastocystis*, there are 32 genetic groups (subtypes) has been identified in mammalian (including humans) and avian hosts (Munsaka *et al,* 2022). Ten subtypes were isolated from human stool; subtypes1-9 & ST12 (Lhotská *et al,* 2020). More than 90% of patients caused by ST1-ST4 (Stensvold and Clark, 2016). Others (ST5–ST9) were rare and linked to possible zoonotic transmission (Cian *et al,* 2017).

The current study aimed to analyze the *Blastocystis* frequency, subtypes infection, and the correlation with demo-clinical variables among of adolescents.

Materials and Methods

Study design and data collection: The study was a cross-sectional design with total of 110 labeled stool samples. Fresh samples were collected from adolescents (aged 10-19 years old) having different gastrointestinal complaints and attending outpatient clinics in El-Fayoum Teaching Hospitals, from May 2023 to July 2023.

Before sampling, the study aims and protocol were explained and informed consent was obtained from the participants' parents or guardians. So, demographic variables and gastrointestinal symptoms were in a questionnaire by direct interview. Patients received antiparasitic treatment was excluded.

Ethical consideration: The protocol was approved by the Scientific Research Ethics Committee, Faculty of Medicine, Fayoum University (NO: R 450/2023), which agreed with Helsinki Declaration (2008).

Direct Microscopy: Each stool sample was divided into 2 portions. The 1st portion was immediately mixed with 1-2 drops of Lugol's iodine (Merck, Germany) and smeared on clean glass slide, and microscopy examined with magnification of 40× & 100× for *Blastocystis* spp. (Calissendorf and Falhammar, 2017). The presence of vacuolar form in more than one smear field was an indication of a positive sample (Darwish *et al,* 2023). The 2nd positive portion was stored at -20°C until subsequent extraction.

DNA Extraction: Total genomic DNA was extracted from microscopy positive samples using a commercial kit, QIAamp DNA Stool Mini Kit (Qiagen GmbH, Hilden, Germany) after the manufacturer's instructions. Extracted DNA was eluted in 200µl buffer and stored at -20°C until PCR amplification.

Polymerase chain reaction (PCR): 2µl of genomic DNA was used in PCR to detect *Blastocystis* at genus level by using the forward primer (5'-GGA GGT AGTG AC AAT AAA TC-3') and the reverse primer (5'-CGT TCA TGA TGAACA ATT AC-3') primers (Böhm-Gloning *et al,* 1997). PCR conditions consisted of one initial cycle of denaturation at 94ºC for 4 min, 35 cycles of amplification of 30s at 94ºC, 30s at 54ºC, 30s at 72ºC and an elongation cycle for 5min at 72ºC. Amplified products were visualized on 1.5% agarose gel electrophoresis under UV light. Amplicons were stored at 4ºC until needed.

Subtyping by sequence-tagged site-PCR assay: The 2ul of each positive amplicons was subjected to sequence-tagged site-PCR assay using sequence-tagged site (STS) primers (Maleki *et al,* 2022). The amplicons in 20µl reaction was mixed with 0.5µM of each primers, 1UTaq DNA polymerase (Thermo-Scientific, USA). PCR conditions was done with one denaturing cycle at 94°C for 5min, 40 cycles including denaturing at 94°C for 30s, annealing at 57°C for 30s, extending at 72°C for 60s and additional elongation cycle at 72°C for 7min. followed by documentation on 1.5% agarose gel electrophoresis.

Statistical analysis: Data were collected, tabulated, computerized and analyzed by the SPSS software (version 20, IBM Inc., USA). To determine the frequency and the correlation between *Blastocystis* infections and subject variables, the Chi-square test was used. A P-value < 0.05 was statistically significant (Gouda, 2015).

Details were given in tables (1, 2 & 3).

Table 1: Sequence-tagged site (STS) primers used for *Blastocystis* subtyping

Subtype	Primer sequence	Product (pb)
1	F-GAAGGACTCTCTGACGATGA	351
	R-GTCCAAATGAAAGGCAGC	
2	F-ATCAGCCTACAATCTCCTC	704
	R-ATCGCCACTTCTCCAAT	
3	F-AGGATTTGGTGTTTGGAGA	526
	R-TTAGAAGTGAAGGAGATGGAAG	
4	F-GCATCCAGACTACTATCAACATT	338
	R-CCATTTTCAGACAACCACTTA	
5	F-TGTTCTTGTGTCTTCTCAGCTC	704
	R-TTCTTTCACACTCCCGTCAT	
6	F-GTGGGTAGAGGAAGGAAAACA	317
	R: AGAACAAGTCGATGAAGTGAGAT	
7	F-GTCTTTCCCTGTCTATTCTGCA	487
	R-AATTCGGTCTGCTTCTTCTG	

Results

Among 110 adolescents; 69 males (62.7%) and 41 females (37.3%) with ages of 14.23±3.1 years (range 10-19years), microscopy showed that 21 (19.1%) were positive. The infection was found in 5(23.8%) in females and in 16 (76.19%) males. Parasite was detected in 13 (61.9%) adolescences lived in rural area and 8 (38%) in urban one, without significant association between age, gender, residence and blastocystosis, highly significant association ($p < 0.005$) with found between vomiting, nausea and infection.

All microscopy positive *Blastocystis* samples were confirmed by diagnostic PCR primers and amplification was detected in 16/21 (76.1%) samples. Only five samples couldn't be amplified by diagnostic PCR. These PCR positive samples were subsequently screened with STS primers-PCR to identify *Blastocystis hominis* subtypes. Amplification with STS primers was observed in 10 (62.5%) of 16 isolates. There was a significant association ($p < 0.000$) between the microscopy and STS-PCR results. The dominant *Blastocystis* subtype was ST3 in six (40%) samples followed by three (30%) for ST2, and one sample (10%) was ST1 amplified. The ST4-7 and mixed subtypes was not detected. No association was found between *Blastocystis* subtypes and any demo-clinical presentations.

Details were given in tables (1, 2 & 3) and figure (1).

Table 2: Descriptive demo-clinical criteria of participants and *Blastocystis* infection:

Variations		Presenting symptoms	Positive cases	P-value
		110 (No. %)	21 (No. %)	
Age	10-14	57(51.8%)	8(38%)	0.162
	15-19	53(48.2%)	13(61.9%)	
Sex	Male	69(62.7%)	16(76.1%)	
	Female	41(37.3%)	5(23.8%)	0.156
Residence	Rural	51(46.4%)	13(61.9%)	0.112
	Urban	59(53.6%)	8(38%)	
Diarrhea	Yes	63 (57.2)	15(71.4%)	0.145
	No	47(42.7)	6(28.5%)	
Abdominal pain	Yes	50 (45.4%)	13(61.9%)	0.092
	No	60 (54.5)	8(38%)	
Vomiting	Yes	29(26.4%)	13(61.9%)	*0.000
	No	81(73.6%)	8(38%)	
Flatulence	Yes	35 (31.8)	9(42.8%)	0.227
	No	75(68.2%)	12(57.1)	
Nausea	Yes	24(21.8)	9(42.8%)	*0.009
	No	86(78.2%)	12(57.1)	

* $P < 0.05$ significant

Table 3: Association between *Blastocystis* subtypes and clinical factors.

Variations		ST3	ST2	ST1	P-value
		No.	No.	No.	
Age	10-14	2	1	0	0.475
	15-19	4	2	1	
Sex	Male	4	3	1	0.467
	Female	2	0	0	
Residence	Rural	3	2	1	0.623
	Urban	3	1	0	
Diarrhea	Yes	5	1	1	0.364
	No	1	2	0	
Abdominal pain	Yes	4	2	0	0.465
	No	2	1	1	
Vomiting	Yes	3	2	1	0.060
	No	3	1	0	
Flatulence	Yes	3	2	1	0. 300
	No	4	0	0	
Nausea	Yes	2	1	1	0.214
	No	4	2	0	

Discussion

The PCR, in human blastocystosis, was as fast as the formalin ether concentration techniques and the cultures (Dagci *et al,* 2014). PCR has a high accuracy without parasite viability (Sekar and Shanthi, 2015). Moreover, the PCR technique in epidemiological studies proved to be more realistic estimates of any parasitic prevalence (Matovelle *et al,* 2022)

In the present study, for identification of seven subtypes of *Blastocystis,* a total of seven pairs of STS primers were used and the Blastocystis subtypes were determined by PCR in the stool positive samples.

Blastocystis is a an enteric protozoan with worldwide distribution (Cian *et al,* 2017). The frequency of this polymorphic parasite was variable between studies in the same and in different localities around the world. In the present study, *Blastocystis* infection was screened with Lugol's iodine smear of freshly collected stool samples collected from adolescents. With this tool, the infection was microscopically detected in 19.1% of the participants' samples. This more or less agreed with El Safadi *et al.* (2013); El-Badry *et al.* (2018); Mardani Kataki *et al.* (2019); Ahmed *et al.* (2022). High records (61.1%), (33/5%) and (35.7%) were reported in other Egyptian studies done by Abdel Hameed *et al.* (2011); Fouad e *et al.* (2011); Mokhtar and Youssef (2018).

In the present study, only 16 positive samples were confirmed by diagnostic PCR. This finding could be explained be the polymorphic natures of the parasite with possibility of misdiagnosis with other intestinal parasite and the presence of artifacts (Delshad *et al,* 2020). Besides, the PCR results may be affected by the used extraction methods, paucity of parasites in the sample and presence of polymerase inhibitory factors in stool samples (Mülayim *et al,* 2021).
The results of present study couldn't reach to a significant relationship between *Blastocystis* infection and demo-clinical presentations except for nausea and vomiting as similar as reported by Souppart *et al.* (2009); Abdulsalam *et al.* (2013); and Hamdy *et al.* (2020).But this disagreed with El Safadi *et al.* (2013). Again, Asghari *et al.* (2021) revealed that the infection in males was significantly higher than females ($p = 0.024$), without association of age and residence. Also, El-Badry *et al.* (2018) found a significant risky association between *Blastocystis* and living in rural areas.

In the present study, 3 *Blastocystis* subtypes are identified in 10 amplified samples by STS –PCR technique among the examined adolescents without a correlation with demo-clinical presentations. Although the nonspecific nature of symptoms, studies have reported abdominal pain and diarrhea were two major symptoms in *Blastocystis*-patients

(Tan, 2008; Mohammad *et al,* 2018).

In the current study, ST3 identified as dominant (40%) subtype among the amplified positive samples followed by ST2 (30%) then ST1 (10%). These dominant subtypes suggested that they were adolescents living in urban areas (Fouad *et al,* 2011).

Also, the ST4-7 and mixed subtypes could not be detected in current study. These results could be explained by parasitic polymorphism (Melo *et al,* 2017); Mardani Kataki *et al,* 2019; Mülayim *et al,* 2021; Asghari *et al,* 2021; Ahmed *et al,* 2022). This superiority was reversed as reported by Abdulsalam *et al.* (2013) and Delshad *et al.* (2020) as the ST1 was the most prevalent followed by ST2 then ST3. Moreover, Vassalos et al. (2010); Deeb *et al.* (2012); El-Badry *et al.* (2018); Rahimi *et al.* (2022) reported a predominant ST3, followed by subtype 1.But, Badparva *et al.* (2014) gave subtypes 2 the superiority followed by ST3 and ST4 plus mixed infection by ST3,ST5 and ST2 ,ST3. Moreover, all seven *Blastocystis* subtypes were reported by Yakoob *et al.* (2010) and Seyer *et al.* (2017) in addition to mixed subtypes infection. Theses variability in subtypes in different studies in different location could be explained by the heterogenic nature of parasite in different hosts (Delshad *et al,* 2020). The ST1-ST3 was a common subtypes identified in humans, but without specificity (Munsaka *et al,* 2022).

Conclusion

Blastocystis subtype 3 was the most prevalent subtypes in the adolescents with variable enteric symptoms. More studies are ongoing to identify *B. hominis* subtypes in man and animals to explore the zoonotic transmission and clinical associations and will be published in due time elsewhere.

Authors' Declaration: The authors declared that they neither have any conflict of interest nor received any funds. They added that they both equally contributed in the study.

References

Abdel Hameed, DM, Hassanin, OM, Zuel-Fakkar, NM, 2011: Association of *Blastocystis hominis* genetic subtypes with urticaria. Parasitol Res. 108, 3:553-60.

Abdel-Dayem, M, Al Zou'bi, R, Hani, RB, Amr, ZS, 2014: Microbiological and parasitological investigation among food handlers in hotels in the Dead Sea area, Jordan. J. Microbiol. Immunol. Infect. 47, 5:377-80.

Abdelkareem, YE, Abohashem, AH, Memish, ZA, Binjomah, AZ, Takroni, FM, *et al,* 2022: Common intestinal parasitic infections among patients living in Riyadh, Saudi Arabia: Prevalence and demographic associations (A cross-sectional retrospective study). Ann. Med. Surg. (Lond). Apr 28;77:103677. doi: 10.1016/j.amsu.2022.103677

Abdo, SM, Ghallab, MMI, Zaghloul, MS, Elhadad, H, 2023: Efficacy of nitazoxanide, nitazoxanide-garlic combination and tinidazole in treatment of *Giardia duodenalis and Blastocystis hominis*: Coproscopic assessment. Iran J. Parasitol. 18, 1:48-55.

Abdulsalam, AM, Ithoi, I, Al-Mekhlafi, HM, Al-Mekhlafi, AM, Ahmed, A, *et al,* 2013: Subtype distribution of *Blastocystis* isolates in Sebha, Libya. PLoS One 8, 12: https://doi.org/10.1371/ jour- nal.pone.0084372

Ahmed, SA, El-Mahallawy, HS, Mohamed, S F, Angelici, MC, Hasapis, K, *et al,* 2022: Subtypes and phylogenetic analysis of *Blastocystis* sp. isolates from West Ismailia, Egypt. Sci. Rep. 12, 1:1-12.

Alinaghizade, A, Mirjalali, H, Mohebali, M, Stensvold, CR, Rezaeian, M, 2017: Inter- and intra-subtype variation of *Blastocystis* subtypes isolated from diarrheic and non-diarrheic patients in Iran. Infect. Genet. Evol. 50:77-82.

Amin, AM, 1997: Blastocystis hominis among apparently healthy food handlers in Jeddah, Saudi Arabia. J. Egypt. Soc. Parasitol. 27, 3:817-23.

Arisue, N, Hashimoto, T, Yoshikawa, H, Nakamura, Y, Nakamura, G, *et al,* 2002: Phylogenetic position of Blastocystis hominis and of stramenopiles inferred from multiple molecular sequence data. J. Eukaryot. Microbiol. 49:142-53.

Asghari, A, Hassanipour, S, Hatam, G, 2021: Comparative molecular prevalence and subtypes distribution of *Blastocystis* sp. a potentially zoonotic infection isolated from symptomatic and asymptomatic patients in Iran: A systematic review and meta-analysis. Acta Parasitol. 66,

3:745-59.
Aykur, M, Camyar, A, Gerceker, B, Zerrin, A, Dagci, H, 2022: Evaluation of association with subtypes and alleles of *Blastocystis* with chronic spontaneous urticaria. Acta Trop. 231: 106455.https://doi.org/10.1016/j.actatropica.2022.106455
Badparva, E, Sadraee, J, Kheirandish, F, Frouzandeh, M, 2014: Genetic diversity of human *Blastocystis* isolates in Khorramabad, central Iran. Iran. J. Parasitol. 9, 1:44-9.
Böhm-Gloning, B, Knobloch, J, Walderich, B, 1997: Five subgroups of *Blastocystis hominis* isolates from symptomatic and asymptomatic patients revealed by restriction site analysis of PCR-amplified 16S-like rDNA. Trop. Med. Int. Hlth. 2, 8:771-8.
Chen, H, Hao, Y, Liu, Y, Xu, M, Zhang, W, *et al,* 2023: The frequency and subtype distribution of *Blastocystis* sp. in humans and domestic animals in households in Heilongjiang Province, China. Acta Trop. 240, 106844. https://doi.org/10.1016/j.actatropica.
Cian, A, El Safadi, D, Osman, M, Moriniere, R, Gantois, N, ***et al,* 2017:** Molecular epidemiology of *Blastocystis* sp. in various animal groups from two French Zoos and evaluation of potential zoonotic risk. PLoS One 21, 1:1-29.
Dagci, H, Kurt, Ö, Demirel, M, *et al,* 2014: Epidemiological and diagnostic features of *Blastocystis* infection in symptomatic patients in Izmir province, Turkey. Iran J. Parasitol. 9:519-29.
Darwish, B, Aboualchamat, G, Nahhas, SAl, 2023: Molecular characterization of *Blastocystis* subtypes in symptomatic patients from the southern region of Syria. PLoS One 18, 283291:1-11.
de Melo, GB, Bosqui, LR, da Costa, IN, de Paula, FM, Gryschek, RCB, 2021: Current status of research regarding *Blastocystis* sp., an enigmatic protist, in Brazil. Clinics 76:1-8.
Deeb, HKE, Salah-Eldin, H, Khodeer, S, 2012: *Blastocystis hominis* as a contributing risk factor for development of iron deficiency anemia in pregnant women. Parasitol. Res. 110, 6:2167-74.
Delshad, A, Saraei, M, Alizadeh, SA, Niaraki, SR, Alipour, M, *et al,* 2020: Distribution and molecular analysis of *Blastocystis* subtypes from gastrointestinal symptomatic and asymptomatic patients in Iran. Afr. Hlth. Sci. 20, 3:1179-89.
Dogruman-Al, F, Dagci, H, Yoshikawa, H, Kurt, Ö, Demirel, M, 2008: A possible link between subtype 2 and asymptomatic infections of *Blastocystis hominis*. Parasitol. Res. 103, 3: 685-9.
El-Badry, AA, Abd El Wahab, WM, Hamdy, DA, Aboud, A, 2018: *Blastocystis* subtypes isolated from irritable bowel syndrome patients and co-infection with *Helicobacter pylori*. Parasitol. Res. 117, 1:127-37.
El Safadi, D, Meloni, D, Poirier, P, Osman, M, Cian, A, *et al,* 2013:. Short report: Molecular epidemiology of *Blastocystis* in Lebanon and correlation between subtype 1 and gastrointestinal symptoms. Am. J. Trop. Med. Hyg. 88, 6:1203-6.
Fonte, L, Aleaga, Y, Hernandez, Y, Ginori, M, Garcia, G, *et al,* 2022: *Blastocystosis* and iron deficiency anemia in pregnant women: A call to deep in a little-known association. J. Gynecol. Reprod. Med. 5, 2:55-9.
Fouad, SA, Basyoni, MMA, Fahmy, RA, Kobaisi, MH, 2011: The pathogenic role of different *Blastocystis hominis* genotypes isolated from patients with irritable bowel syndrome. Arab. J. Gastroenterol. 12, 4:194-200.
Calissendorff, J, Falhammar, H, 2017: Lugol's solution and other iodide preparations: perspectives and research directions in Graves' disease. Endocrine 58, 3:467-73.
Gouda, MA, 2015: Common Pitfalls in Reporting the Use of SPSS Software. Med. Princ. Pract. 24, 3:300. doi: 10.1159/000381953
Hamdy, DA, Abd El Wahab, WM, Senosy, SA, Mabrouk, AG, 2020: *Blastocystis* spp. and *Giardia intestinalis* co-infection profile in children suffering from acute diarrhea. J. Parasit. Dis. 44, 1:88–98.
Hammood, AM, Ahmed, BA, Salman, YJ, 2016: *Blastocystis hominis* detection among gastrointestinal disorders' patients in Kirkuk Province using three different laboratory methods. Int.J.Curr.Microbiol.App.Sci, 5, 7:883-900.
Jha, S, Gupta, P, Bhatia, M, 2021: *Blastocystis* spp. infection in cases of diarrhea: A pilot study from a tertiary care teaching hospital in Rishikesh, Uttarakhand, with a brief review of literature. Trop. Parasitol. 11, 2:113-21.
Kaczmarek, A, Rocka, A, Wesołowska, M, Gołąb, E, Sałamatin, R, 2020: *Blastocystis* isolates from a dog and their owners presenting with chronic diarrhoea. Dogs as reservoirs of *Blastocystis*: Research in Poland and worldwide. Ann. Parasitol. 66, 4:573-9.

Kesuma, Y, Firmansyah, A, Bardosono, S, Sari, IP, Kurniawan, A, 2019: *Blastocystis* ST-1 is ass-ociated with irritable bowel syndrome-diarrhoea (IBS-D) in Indonesian adolescences. Parasite Epidemiol. Control 6:e00112. https://doi.org/10.1016/j.parepi.2019.e00112

Kick, G, Rueff, F, Przybilla, B, 2002: Palmoplantar pruritus subsiding after *Blastocystis hominis* eradication. Acta Derm. Venereol. 82, 1: 60.https://doi.org/10.1080/00015550275360094 8

Lee, MJ, 1990: Letters to the editor pathogenicity of *Blastocystis hominis*. J. Clin. Microbiolol. 29, 9: *2089.*

Lhotská, Z, Jirků, M, Hložková, O, Brožová, K, Jirsová, D, *et al,* 2020: A study on the prevalence and subtype diversity of the intestinal protist Blastocystis sp. in a gut-healthy human population in the Czech Republic. Front. Cell. Infect. Microbiol. 10:1-14.

Lugović-mihić, L, Bukvić, I, Bulat, V, Japundžić, I, 2019: Factors contributing to chronic urticaria / angioedema and nummular eczema resolution which findings are crucial ?, Acta Clin. Croat., 58,4:595–603.

Maleki, B, Sadraei, J, Asl, AD, Pirestani, M, 2022: High occurrence of *Blastocystis* sp. subtype 3 in individuals referred to medical laboratories in Kermanshah, Iran. Gastroenterol. Hepatol. Bed Bench 15, 2:164-71.

Mardani Kataki, M, Tavalla, M, Beiromvand, M, 2019: Higher prevalence of *Blastocystis hominis* in healthy individuals than patients with gastrointestinal symptoms from Ahvaz, southwestern Iran. Comp. Immunol. Microbiol. Infect. Dis. 65:160-4.

Matovelle, C, Tejedor, MT, Monteagudo, LV, Beltrán, A, Quílez, J, 2022: Prevalence and associated factors of *Blastocystis* sp. infection in patients with gastrointestinal symptoms in Spain: A case-control study. Trop. Med. Infect. Dis. Sep 3;7(9):226. doi: 10.3390/tropicalmed7090 226.

Melo, GB, Paula, FM, Malta, FM, Maruta, CW, Criado, PR, *et al,* 2017: Identification of Blastocystis subtypes in clinical stool samples from Sao Paulo City, Brazil. Parasitol. Open, 3:1-6.

Mohamed, RT, El-Bali, MA, Mohamed, AA, Abdel-Fatah, MA, El-Malky, MA, *et al,* 2017: Subtyping of *Blastocystis* sp. isolated from symptomatic and asymptomatic individuals in Makkah, Saudi Arabia. Parasit. Vectors 10, 1:1-7.

Mohammad, NA, Al-Mekhlafi, HM, Anuar, TS, 2018: Genetic diversity of *Blastocystis* isolates from symptomatic and asymptomatic orang asli in pahang, malaysia. Southeast Asian J. Trop. Med. Publ. Hlth. 49, 2:189-97.

Mokhtar, A, Youssef, A, 2018: Subtype analysis of Blastocystis spp. isolated from domestic mammals and poultry and its relation to transmission to their in-contact humans in Ismailia Governorate, Egypt. P.U. J. 11, 2:90-8.

Mülayim, S, Aykur, M, Dağcı, H, Dalkılıç, S,

Aksoy, A, *et al,* 2021: Investigation of Isolated Blastocystis Subtypes from Cancer Patients in Turkey. Acta Parasitol. 66, 2:584-92.

Munsaka, G, Hayashida, K, Mubemba, B, Simulundu, E, Mulunda, N, *et al,* 2022: Molecular subtyping of *Blastocystis* sp. detected in patients at a large tertiary referral hospital in Lusaka, Zambia. Front. Parasitol. 1:1-7.

Nithyamathi, K, Chandramathi, S, Kumar, S, 2016: Predominance of *Blastocystis* sp. infection among school children in Peninsular Malaysia. PLoS One 11, 2:1-14.

Rahimi, HM, Karamati, SA, Nemati, S, Mirjalali, H, Zali, MR, 2022: Molecular Identification, Subtypes Distribution, and Alleles Discrimination of *Blastocystis* sp., Isolated from Immunocompromised Subjects in Iran. Iran. J. Parasitol.17, 2:184-93.

Sekar, U, Shanthi, M, 2015: Recent insights into the genetic diversity, epidemiology and clinical relevance of *Blastocystis* species. J. Med. Res. 1, 1:33-9**.**

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

Souppart, L, Sanciu, G, Cian, A, Wawrzyniak, I, Delbac, F, *et al,* 2009: Molecular epidemiology of human *Blastocystis* isolates in France. Parasitol. Res. 105, 2413-21.

Stensvold, CR, 2013: Comparison of sequencing (Barcode Region) and sequence-tagged-site PCR for *Blastocystis* subtyping. J. Clin. Microbiolol. 51, 1:190-4.

Stensvold, CR., Clark, CG, 2016: Current status of *Blastocystis*: A personal view. Parasitol. Int., 65, 6:763-71.

Tan, KSW, 2008: New insights on classification, identification, and clinical relevance of *Blastocystis* spp. Clin. Microbiol. Rev. 21, 4:639-

65.

Valença Barbosa, C, De Jesus Batista, R, Pereira Igreja, R, D'Avila Levy, CM, Werneck De Macedo, H, *et al,* 2017: Distribution of *Blastocystis* subtypes isolated from humans from an urban community in Rio de Janeiro, Brazil. Parasit. Vectors 10, 1:1-9.

Vassalos, CM, Spanakos, G, Vassalou, E, Papadopoulou, C, Vakalis, N, 2010: Differences in clinical significance and morphologic features of *Blastocystis* sp subtype 3. Am. J. Clin. Pathol. 133, 2:251-8.

Explanation of figure

Fig. 1: Amplified samples by the sequence-tagged site (STS) primers. Lanes 1, 100 bp DNA ladder; Lane 2 subtype 1 (351 bp); Lanes 3-4 subtype 2 (704 bp); Lanes 5-6 subtype 3 (526 bp); Lane 7 unamplified sample.