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COMPARISON BETWEEN GROWTH MAGNITUDE OF *BLASTOCYSTIS* GENOTYPES ISOLATED FROM GASTROINTESTINAL SYMPTOMATIC EGYPTIAN PATIENTS

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Abstract

Blastocystis presents in healthy and diseased persons so its role in disease development is a subject of debate. This study compared between growth patterns of different symptomatic *Blastocystis* subtypes. Morphometric measures of the vacuolar form of STs isolated from acute GIT symptomatic patients showed that major diversity in size ranged from 4.928±1.77 among ST4 to 70.54±29.66 among wild ST3. Size in chronic group was 4±0.816 in ST-4 and 40.83±7.359 in heterozygous ST3, and in intermittent group ranged from 5.5±0.707 in ST4 to 47.5±6.45 in heterozygous ST3 with significant differences. Phenotypic variations were commonly among inter-subtypes of ST3. Generation patterns in re-culturing were 24.1% of isolates at 5th re-culturing and the 29.6% at 6th re-culturing loss of 90% viable vacuolar form numbers compared to that on first culture. Low percentage (9.3%) reached to 9th re-culture. Majority of ST-1 (40%) was lost at the 5th re-culture while 58.8% of wild ST-3 was lost in the 7th & 8th, respectively, with 29.4% per each. Meanwhile, 66.6% of ST-4 was lost in the 5th & 6th, respectively, with 33.3% per each, but without significant difference. As to metronidazole® susceptibility, the majority of ST1, wild ST3, heterozygous ST3 and ST4 isolates gave 60%, 58.7%, 57.2% & 79.9% sensitivity.

Key words: *Blastocystis*, Genotypes, Growth magnitude, Metronidazole

Introduction

Blastocystis spp. is the most prevalent anaerobic, unicellular, and obligatory intestinal protozoan in both vertebrates and invertebrates (Yan *et al,* 2007) with a prevalence rate reached to 60% among humans (Roberts *et al,* 2014). Debatable is the role that *Blastocystis* plays in the emergence of diseases because it manifests in both healthy and diseased people (Skotarczak, 2018). Although up to 38 genetic subtypes (STs) of *Blastocystis* have been found in birds and mammals based on the small-subunit ribosomal RNA gene providing its wide genetic diversity (Alfellani *et al,* 2013; Stensvold and Clark, 2020; Lepczyńska *et al,* 2021; Maloney *et al,* 2023), phylogenic, sequencing and high resolution melting curve studies added intra-subtypes and inter-subtypes genetics variants (Hussein *et al,* 2023). Only 14 subtypes were demonstrated in human and animals (Jinatham *et al,* 2021). The most common inhabitants of gastrointestinal humans are ST1 to ST4 (Hussein *et al,* 2008; Ramírez *et al,* 2016; Jiménez *et al,* 2019). These subtypes, intra-subtypes and inter-subtypes variations of *Blastocystis* had a strong effect on enhancing the pathogenicity of this parasite (Wu *et al*, 2014).

Blastocystis is an organism with various morphological forms (Stenzel *et al,* 1991). Vacuolar form has a central vacuole with a size varies from 2-200µm containing 1-4 nuclei (Popruk *et al,* 2013). Granular form contains granules within the central vacuole, its size varied from 6.5-8µm, with 1-4 nuclei and avacuolar form has no vacuole; its size is approximately 5 µm and having 1-2 nuclei it is a degenerative form (Zaman, 1998). Multivacuolar form was rarely seen in stool with the size varies from 5-8 µm and having 1-4 nuclei (Tan, 2008). Amoeboid form is associated with pathogenicity; its size varies from 3-8 µm with central vacuole and 1-2 nuclei (Tan and Suresh, 2006). Cyst is rarely found in culture ranged from 3-10 µm in size without central vacuole containing 1-4 nuclei and it is the infective stage (Yoshikawa *et al,* 2004). Thus, four are the main forms; vacuolar, granular, amoeboid and cysts (Alfellani *et al,* 2013), but *in vitro* the amoeboid form was present in higher per-

centages of symptomatic isolates (Rajamanikam and Govind, 2013).

Infection is by feco-oral route with contaminated food, or environment, and zoonosis (Stensvold and Clark, 2020). In symptomatic patients, blastocystosis might be accompanied by many symptoms like nausea, vomiting, dyspepsia, abdominal cramps, fatigue, and diarrhea (Sari *et al,* 2018).

Metronidazole® (MTZ) is the drug of choice for blastocystosis treatment (Adao and Rivera, 2018), but up to 40% resistance was recorded (Yakoob *et al,* 2004). However, subtype-dependent different degree of susceptibilities in MTZ was in vitro among the ST1 to ST4 & ST7 (Coyle *et al,* 2012; Al-Mohammed *et al,* 2013; Roberts *et al,* 2015) and by ST-3 (Rajamanikam *et al,* 2019). MTZ eliminated *Blastocystis* by inhibiting nucleic acid synthesis (Raman *et al,* 2016) led to a high proportion of clinical remission with six-month of fecal clearance (Sekar and Shanthi, 2013).

The initial stage in identifying a parasite, in particular the vacuolar form utilizing direct wet mount or after culture, is microscopic diagnosis (Stensvold and Clark, 2020). Molecular identification studies of *Blastocystis* STs provide a discriminating tool for investigating the epidemiology of the parasite such as transmission, host specificity, and chemotherapeutic resistance (Ozyurt *et al,* 2008; Seyer *et al,* 2017). Phenotypic differences of the parasite reported from symptomatic and asymptomatic cases may be related to inter- and intra-subtype variation of the *Blastocystis* (Ragavan *et al,* 2014). Others related the high morphometric measures of vacuolar forms in vitro obtained from stool samples to symptomatology (Hegazy *et al,* 2008). But, only one study assessed inter- and intra-subtype variations in size, phenotype and generation time of STs 1-3 and 6 of *Blastocystis* of symptomatic and asymptomatic subjects (Karamati *et al,* 2019).

The present study aimed to clarify the relationship between genetic diversity of *Balstocystis* Egyptian symptomatic ST1, ST3 inter-subtypes, ST4 and magnitudes *in vitro* as to morphometric, phenotypes, generation pattern in re-culturing and MTZ susceptibility.

Material and Methods

Blastocystis isolates: Stool specimens of 54 gastrointestinal symptomatic *Blastocystis* patients attended Gastroenterology and Tropical Medicine Outpatient Clinics, Suez Canal University Hospitals were selected. Their ages ranged from 18-60 years, 35 of them were females, and 30 patients were living in urban area and 14 in rural one. Patients had acute GIT symptoms were 26 (<2 weeks), 14 patients had chronic symptoms (>4 weeks) and 14 had diarrhea alternating with constipation (intermittent) symptom.

Blastocystis stool samples were propagated by stool culture and subjected to STs/ inter-subtypes identified by conventional PCR and high resolution melting curve (Hussein *et al,* 2023).

The conventional PCR divided the 54 symptomatic *Blastocystis* isolates into 10/54 (18.5%) were ST1, 29/54 (54.7%) were ST3 & 15/54 (27.8%) were ST4. PCR/ HRM discriminated 29 isolates referred to ST3 into wild 17/54 (31.5%), mutant 5/54(9.3%), and heterozygous 7/54 (12.9%) intra-subtypes. Patients with acute symptoms were seven from ST1, 17 ST-3 and two from ST4 while chronic patients included two from ST1, seven from ST3 and five from ST4, intermittent were one from ST1, five from ST3 and eight from ST4.

In vitro study: About 50mg of *Blastocystis* positive stool were inoculated in 10ml sterilized modified Jones medium with10% heat-inactivated (56°C for 30min) horse serum, 100µg/ml streptomycin, and100 IU/ml penicillin. For *Blastocystis* propagation, inoculated medium was incubated for four days at 37°C with 5% CO_2 (Jones, 1946).

Growth pattern assessment included size, phenotypes and number of vacuolar (active), common diagnostic form, growth rate in re-culture after 72hr inoculation and metronidazole susceptibility were evaluated (Karamati *et al,* 2019). *Balastocystis* was re-culti-

vated after 72hr (log phase) in 3ml of Jones' medium without rice starch, but supplied with 10% horse serum **(**Leelayoova *et al,* 2003). Culture was examined daily for 96hr till being negative.

Morphometry and Phenotyping: To measure the vacuolar forms size, 10 different portion of each slide were randomly selected by ocular micrometer and reticle scale in 400x culture (Tan and Suresh, 2006). Average size was 10 high power fields counted the iodine stained common abundant forms.

Generation time and maintains regarding re-culturing: To assess utility in relation to re-culturing per each subtype and inter-subtype, about 10.000 vacuolar form after 72hr culture were counted by haemocytometer chamber. The subculture was continued until the growth of viable vacuolar form by using trypan blue dye (0.4%) became 10% of first inoculum.

In vitro anti-parasite susceptibility: MTZ tablets (Flagyl®) (Sanofi Aventis Co.) were dissolved PBS, pH 7.2 and adjusted to 1, 10, 100, & 250µg/ml (El Deeb *et al,* 2012). MTZ resistance isolates were considered when 10µg/ml dose gave no effect, but low, intermediate and high resistances were detected in 10, 100, & 250µg/ml, respectively (Mossallam *et al,* 2021). For each isolate, 1×10^4 inoculum of *Blastocystis*/ml was re-cultured at 37°C for 72hr (Yang *et al,* 1996). Subculture tubes were divided into four groups and were performed in triplicates. GI: Non-treated infected control. GII: Infected and treated, and subdivided into subgroups III (a, b, c & d); treated with MTZ different concentrations. *Blastocystis* growth daily vacuolar form was counted by a hemocytometer after 72hr the log phase (Yakoob *et al,* 2011). Viability was evaluated by using trypan blue dye (0.4%), as completely viable or dead partially excluded dye (El-Sayed *et al,* 2017). The inhibitory concentrations (IC_{90}) were determined for isolate reduction in viable *Blastocystis* vacuolar form compared to control. The cytocidal effect was the least lethal MTZ concentrations to all vacuoles confirmed by neither stained nor growth after 72hr by 100µl samples transferred into fresh medium (Vdovenko and Williams, 2000). The *Blastocystis* average vacuolar number of three culture tubes each group at each time was considered. Inhibition% of *Blastocystis* multiplication/viability in GII & GI was calculated as the following formula: Multiplication/viability inhibition (%) = (A-B)/A×100. Where A = control number (GI), B= treated number (GII)

Statistical analysis: Data were computerized and analyzed by One-Way ANOVA and Tukey HSD for Mean ± standard deviation while Chi-square was used for dependent results. P value was < 0.05

Ethical considerations*:* Verbal consent was obtained from all patients. All procedures were carried out according to the standards approved by the Ethics Research Committee, Faculty of Medicine, Suez Canal University, with the reference number 5098#.

Results

Morphometric measures of the vacuolar form of STs isolated from acute GIT symptomatic patients revealed the major diversity in size. Size ranged from 4.928±1.77 among ST-4 to 70.54±29.66 among wild ST-3. In chronic group, size ranged from 4±0.816 in ST-4 to 40.83±7.359 in heterozygous ST-3. In intermittent one, size ranged from 5.5± 0.707 in ST-4 to 47.5±6.45 in heterozygous ST-3. Subtype and symptomatology showed significant differences in wild and heterozygous ST3.

Examination the first 5 re-culturing showed amoeboid form in 13/26(50%) of parasite isolated (ST3) from acute symptomatic patients particularly during the first 3 re-culturing. In the first 2 re-culturing, ST4 and ST3 isolates showed clumping forms in 9/15 (60%) and 12/29(41.3%), respectively, while the lowest detected ST1 was in 2/10(20%). Meanwhile, cysts were obviously shown in culture from chronic patients infected with ST3 during 4^{th} re-culturing in 4/7(57%) and at the end of 5^{th} re-culturing of ST1 and ST4 (30% each) induced chronic and intermittent

infections. Among other symptomatic patients cyst progressed until the experimental end. Granular forms were in 2/10(20%) with ST1, 6/29(20.6%) with ST3 & 2/15 (13.3%) with ST4 at the 5th re-culture.

Regarding generation organism re-culturing pattern, 24.1% of isolates at 5th re-culturing and 29.6% at 6th re-culturing lost 90% of viable vacuolar form numbers in symptomtic STs compared to first culture on 72hr growth. Low (9.3%) reached 9th re-culture. ST1 (40%) was lost at 5th re-culture while 58.8% of wild ST3 was lost in 7th & 8th, respectively, 29.4% for each. Also, 66.6% of ST4 was lost in 5th & 6th respectively, 33.3% for each, without significant difference.

MTZ susceptibility degrees were among symptomatic *Blastocystis* subtype/inter-subtypes, ST1 isolates showed 60% sensitivity; 2 (20%), 1(10%) and 1 (10%) showed low, intermediate, high resistances, respectively. ST3 wild isolates 10/17 (58.7%) were sensitive, 4/17 (23.4%), 2/17(12.6%) & 1 (6.3%) showed mild, intermediate, & high resistances, respectively. Mutant inter-subtypes isolates 3/5(60%) resisted with mild, intermediate and high degrees one/each, but 3/7of heterozygous resisted mild and intermediate, severe degrees 14.2% for each. The ST4 isolates only 3/15 (20%) showed mild, intermediate and high resistance while 12/15 (80%) were sensitive, but without significant difference in sensitivity and/or resistance degree of ST1 & ST4 compared to ST3. None *Blastocystis* was in re-culturing of ST1, ST3, & ST4 sensitive isolates exposed to this dose. Total sensitive were 62.9% while low resistance was 16.7%.

Details were given in tables (1, 2, 3 & 4) and figures (1 & 2).

Table 1: Distribution of STs among patients in relation GIT symptoms

STs		No.	Acute		Chronic		Intermittent	
			No.	%	No.	%	No.	%
ST1		10	7	22.9	2	14.3	1	7.2
ST3	Wild	17	13	50	2	14.3	2	14.3
	Mutant	5	2	7.7	2	14.3	1	7.2
	Heterozygous	7	2	7.7	3	21.4	2	14.3
ST4		15	2	7.7	5	35.7	8	57
Total		54	26	100	14	100	14	100

Table 2: Vacuolar form morphometry of symptomatic *Blastocystis* STs as to symptomatology course in 72h culture

STs		No	Acute		Chronic		Intermittent		F
			No.	Mean ±S.D	No.	Mean ±S.D	No.	Mean ± S.D	P value
ST1		10	7	17.28±6.268	2	17.5±6.454	1	12.5±3.535	F=0.5532 0.5851**
ST3	Wild	17	13	70.54±29.66	2	25±4.08	2	15 ±4.08	F=11.1146 0.0002*
	Mutant	5	2	27.5 ±6.4549	2	25±9.12	1	12.5±3.53	F= 2.8452 0.1246**
	Heterozygous	7	2	58.75±8.539	3	40.83±7.35	2	47.5±6.45	F= 6.901 0.01143*
ST4		15	2	4.928±1.77	5	4±0. 816	8	5.5 ±0. 707	0.7382 0.4927**
Total		54	26	100	14	100	14	100	

*Significant, ** Non-significant

Table 3: Duration of (90% loss) re-culturing of symptomatic *Blastocystis* STs viable vacuoles compared to first culture (10^4) on 72hr growth.

STs		No.	5th		6th		7th		8th		≥ 9th-10th	
			No.	%	No.	%	No.	%	No.	%	No.	%
ST1		10	4	40	3	30	1	10	1	10	1	10
ST3	Wild	17	2	11.8	4	23.6	5	29.4	5	29.4	1	5.8
	Mutant	5	1	20	1	20	1	20	1	20	1	20
	Heterozygous	7	1	14.3	3	42.8	1	14.3	1	14.3	1	14.3
ST4		15	5	33.3	5	33.3	3	20	1	6.7	1	6.7
Total		54	13	24.1	16	29.6	11	20.3	9	16.7	5	9.3

Not significant

Table 4: Generation rate of symptomatic *Blastocystis* STs vacuolar form as to MTZ course in culture.

STs		No.	Sensitive		Low		Intermediate		High	
			No.	%	No.	%	No.	%	No.	%
ST1		10	6	60	2	20	1	10	1	10
ST3	Wild	17	10	58.7	4	23.4	2	12.6	1	6.3
	Mutant	5	2	40	1	20	1	20	1	20
	Heterozygous	7	4	57.4	1	14.2	1	14.2	1	14.2
ST4		15	12	79.9	1	6.7	1	6.7	1	6.7
Total		54	34	62.9	9	16.7	6	11.1	5	9.3

Not significant

Discussion

Blastocystis hominis is an enteric parasite of man and many of animals, with global geographic range known since the early of the 20th century, as causative agent of traveler's diarrhea, rectal bleeding, fever, and irritable bowel syndrome (Bogitsh *et al,* 2019). In Egypt, El-Shazly *et al.* (2005) reported *B. hominis* ranged from 10.1% up to 4.73% among children. In Saudi Arabia, Alqarni *et al*. (2022) reported *B. hominis* in 78.4%. Baek *et al.* (2022) in USA reported that in horses zoonotic *Blastocystis* subtypes were identified in 88.9%.

In the present study, morphometric vacuolar form measures of STs isolated from acute GIT symptomatic patients showed that major size diversity was 4.928±1.77 among ST4 to the largest one 70.54±29.66 among wild ST3. In chronic group, size was 4±0.816 in ST4 & 40.83±7.359 in heterozygous ST3, but in intermittent one, size ranged from 5.5 ±0.707 in ST4 to 47.5±6.4 5 in heterozygous ST3, with significant differences. This agreed with Stenzel *et al.* (1991) and Hegazy *et al.* (2008), they reported that *Blastocystis* vacuolar largest size went side by side with acute diarrhea symptomatic cases than in others. Also, Ragavan *et al.* (2014) reported that *Blastocystis* ST3 isolated from IBS showed the largest diameter with a mean of 18.43 ± 2.22μm compared to other GIT symptomatic and asymptomatic isolates, which were due to the clinical course variability. But, Karamati *et al.* (2019) showed that the smallest vacuolar form size was attributed to ST1, followed by ST6 & ST2, while ST3 show-ed that most variable size compared with the other three subtypes. El-Sayed *et al.* (2017) suggested that this variability may be related to the intra-subtype differences.

In the present study, examination of the first 5 re-culturing showed amoeboid form among 50% of parasite isolated from 13/ 26 acute symptomatic patients having ST3 in all of intra-subtypes particularly during the first 3 re-culturing. During the first two re-culturing (ST4 & ST3 isolates) had clumped forms in 9/15(60%) and in 12/29 (41.3%) respectively, but the lowest was detected among ST1 2/10(20%). Besides, cysts were detected in culture from chronic infected 4/7(57%) patients with ST3 during 4th re-culturing with and at the end of 5th re-culturing of other STs. But, among other isolates cyst numbers progressed to the experimental end. Relation between phenoty-pic differences to symptomatology was reported (Tan *et al,* 2008; Chan *et al,* 2012; Ragavan *et al,* 2014). In contrast, amoeb-oid forms and parasite clumping forms were only seen in ST3- symptomatic pati-ents (Karamati *et al*, 2019). This variability in the intra-subtype differences may be attributed to different alleles in each subty-pe reflected on the phenotypes (Yakoob *et al,* 2011; Ragavan *et al,* 2014) or may be source of zoonotic or anthroponotic (Raja-manikam *et al,* 2022).

In the present study, the lowest number in re-culturing (5th & 6th) led to 90% vacuolar form decrease among ST1& ST4, but highest number was among ST3 intra-sub-types. This agreed with Nasirudeen *et al.* (2001) reported that on generation times of different STs related to re-culturing numb-er, *Blastocystis* central vacuole caused apo-ptosis, and apoptotic bodies were stored before being released into the extracellular space. Klionsky and Emr (2000) reported that during autophagy phagophore forms expanded to sequester a portion of cytopl-asm in the form of an autophagosome that fused with a lytic compartment. Besides, Yin *et al,* (2010) reported that apoptotic fe-atures, autophagy is another type of cell death demonstrated among *Blastocystis*. But, Ragavan *et al.* (2014) reported that ST3 growth profile have three separate growth profiles when compared to sympt-omatic isolates, since asymptomatic ones displayed the highest peak growth Generation time analysis showed that the number of ST1 isolated from symptomatic and asymptomatic subjects peaked higher than the other subtypes

(Karamati *et al,* 2019). The autophagic machinery may be a response to *Blastocystis* aging due nutrient deprivation and/or certain proteases (Tan *et al,* 2001; Tan and Nasirudeen, 2005). Activation of Cysteine proteases identified in the culture supernatants of *Blastocystis* particularly legumain induced autocatalytic activity, while proteolytic activity induced by recombinant cathepsin B with legume-ain (Wawrzyniak *et al,* 2012). No doubt, genetic variability of cathepsin B didn't relate to *Blastocystis* symptomatology and STs (Gonzalez-Arenas *et al,* 2018).

In the present study, metronidazole acti-on on majority of ST1, wild ST3, heterozygous ST3 &ST4 isolates gave 60%, 58.7%, 57.2% & 79.9% sensitivities, respectively. This agreed with Mossallam *et al.* (2021), who reported that *Blastocystis* multiplication was inhibited by 89.78% & 95.43% by metronidazole intermediate and high doses. Girish *et al.* (2015) found a uniform inhibi-tion among different *Blastocystis* subtypes. Mokhtar *et al.* (2019) reported that this variation explained the various *Blastocystis* different subtypes' response to same drug. El Deeb *et al.* (2012) reported that *Blastocys-tis* treatment was reached by lower concentrations. But, Roberts *et al.* (2015) reported total *Blastocystis* clearance was not reached by even at 1000µg/ml MTZ.

In the present study, Ismailia Governorate *Blastocystis* isolates (rural and urban areas) led to varying growth profiles and degree of resistance against metronidazole. This agreed withHaresh *et al.* (1999), who reported that *Blastocystis* sensitivity to various MTZ doses differed with the isolates geographical locations. They added that the least resistance of 0.01mg/ml was in isolates obtained from Singapore and Bangladesh patients, while the isolates of the Malaysian were viable at same drug dose. Indonesian isolate exhibited the highest resistance with 40% of the parasites initial inoculum viable at one day cultures. Rajamanikam *et al.* (2019) found that *Blastocystis spp.* ST3 isolates gave more parasite numbers especially the amoebic forms (urban isolates) treated with metronidazole at 0.001mg/ml at the same time high number of vacuolar form in post-treated isolates coincided with the increased apoptosis.

Conclusion

The insignificant results reflected that other factors rather than the STs variability and clinical course controlled the growth magnitude of *Blastocystis*. Besides, other factors such as the parasitic source, host-parasite relationship, and the geographical areas must be considered. However, *Blastocystis* STs genetic diversity role on pathogenicity, different morphometric, phenotypes, and drug resistance are ongoing and will be published in due time elsewhere.

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References

Adao, DV, Rivera, WL, 2018: Recent advances in *Blastocystis spp*. research. Philipp. Sci. Lett. 11: 39-60.

Alfellani, MA, Taner-Mulla, D, Jacob, AS, Imeede, CA, Yoshikawa, H, *et al.,* 2013: Genetic diversity of *Blastocystis* in livestock and zoo animals. Protist.164, 4:497-509.

Al-Mohammed, HI, Hussein, EM, Aboulmagd, E, 2013: Effect of green tea extract and cysteine proteases inhibitor (E-64) on symptom- tic genotypes of *Blastocystis hominis* in vitro and in infected animal model. Int. J. Curr. Mic-robiol. App. Sci. 2, 12:228-39.

Alqarni, AS, Wakid, MH, Gattan, HS, 2022: Prevalence, type of infections and comparative analysis of detection techniques of intestinal parasites in the province of Belgarn, Saudi Arabia. Peer J. Aug 11; 10:e13889

Baek, S, Maloney, JG, Molokin, A, George, NS, Cortés Vecino, JA, *et al,* 2022: Diversity of *Blastocystis* subtypes in horses in Colombia and identification of two new subtypes. Microorganisms Aug 24; 10(9):1693. doi: 10.3390/ microorganisms10091693.

Bogitsh, BJ, Carter, CE, Oettman, TN, 2019: Blood and tissue protistans III: Other protists. In: Human Parasitology. 5th ed., USA.

Chan, KH, Chandramathi, S, Suresh, K, Chua, KH, Kuppusamy, UR, 2012: Effects of symptomatic and asymptomatic isolates of *Blastocystis hominis* on colorectal cancer cell line, HCT116. Parasitol. Res.110:2475-80.

Coyle, MC, Varughese, J, Weiss, ML, Tanowitz, BH, 2012: *Blastocystis*: To treat or not to treat. Clin. Infect. Dis. 54, 1:105-10.

El Deeb, HK, Al Khadrawy, FM, Abd El-Hameid, AK, 2012: Inhibitory effect of *Ferula asafoetida* L. (Umbelliferae) on *Blastocystis* sp. subtype 3 growth in vitro. Parasitol. Res. 111, 3: 1213-21.

El-Sayed, SH, Amer, N, Ismail, S, Ali, I, Rizk, E, *et al,* 2017: *In vitro* and *in vivo* anti-*Blastocystis* efficacy of olive leaf extract and bee pollen compound. Res. J. Parasitol. 12, 2:33-44

Girish, S, Kumar, S, Aminudin, N, 2015: Tongkat Ali (*Eurycoma longifolia*): A possible therapeutic candidate against *Blastocystis* spp. Parasit. Vectors 8:332. doi:10.1186/s13071-015-092.

Gonzalez-Arenas, N, Villalobos, G, Vargas-Sanchez, GB, Avalos-Galarza, CA, Marquez-Valdelamar LM, *et al,* 2018: Is the genetic variability of Cathepsin B important in the pathogenesis of *Blastocystis spp.*? Parasitol. Res. 117, 12:3935-43.

Guerrant, RL, Van Gilder, T, Steiner, TS, Thielman, NM, Slutsker, L*, et al,* 2001: Practice guide-lines for the management of infectious diarrhea. Clin. Infect. Dis**.** 32, 3:331-51.

Haresh, K, Suresh, K, Khairul, A, Saminathan, S, 1999: Isolate resistance of *Blastocystis hominis* to metronidazole. Trop. Med. Int. Hlth. 4, 4:274-7.

Hegazy, MM, Maklouf, LM, El Hamshary, E M, Dawoud, HA, Eida, AM, 2008: Protein profile and morphometry of cultured human *Blastocystis hominis* from children with gastroenteritis and healthy ones. J. Egypt. Soc. Parasitol. 38, 2:453-64.

Hussein, EM, Hussein, AM, Eida, MM, Atwa, MM, 2008: Pathophysiological variability of different genotypes of human *Blastocystis hominis* Egyptian isolates in experimentally infected rats. Parasitol. Res. 102, 5:853-60.

Hussein, EM, Muhammad, MA, Hussein, A M, Elzagawy, SM, Zaki, WM, *et al,* 2023: Levels of genetic variants among symptomatic *Blastocystis* subtypes and their relationship to mucosal immune surveillance in the precancerous colons of experimentally infected rats. Acta Parasitol. 68, 1:70-83.

Jiménez, PA, Jaimes, JE, Ramírez, J, 2019: A summary of *Blastocystis* subtypes in North and South America. Parasit. Vectors 12, 1:1-9.

Jinatham, V, Maxamhud, S, Popluechai, S, Tsaousis, AD, Gentekaki, E, 2021: *Blastocystis* one health approach in a rural community of Northern Thailand: Prevalence, subtypes and novel transmission routes. Front. Microbiol. 12 :746340.

Jones, DR, 1946: A medium for investigating the breakdown of pectin by bacteria. Nature 158, 4018:625.

Karamati, SA, Mirjalali, H, Niyyati, M, Rezaei Riabi, T, Yadegar, A, *et al,* 2019: Comprehensive study of phenotypic and growth rate features of *Blastocystis* subtypes 1-3 & 6 in symptomatic and asymptomatic subjects. Iran J. Parasitol. 14, 2:204-13.

Klionsky, DJ, Emr, SD, 2000: Autophagy as a regulated pathway of cellular degradation. Science 290, 5497:1717-21.

Leelayoova, S, Rangsin, R, Naaglor, T, 2003: In-vitro cultivation: A sensitive method for detecting *Blastocystis hominis*. Patho. Glob. Hlth. 96, 8:803. doi:10.1179/000349802125002275.

Lepczyńska, M, Dzika, E, Chen, W, 2021: Prevalence of *Blastocystis* subtypes in healthy volunteers in Northeastern Poland. J. Parasitol. 107, 5:684-8.

Maloney, JG, Molokin, A, Seguí, R, Maravilla, P, Villalobos, G, *et al,* 2023: Identification and molecular characterization of four new *Blastocystis* subtypes designated ST35-ST38. Microorganism 11,1:46.doi:10.3390/microorgnisms.

Mokhtar, AB, El-Gayar, EK, Habib, ES, 2016: *In vitro* anti-protozoal activity of propolis extract and cysteine proteases inhibitor (phenyl vinyl sulfone) on *Blastocystis* species. J. Egypt. Soc. Parasitol. 46, 2:261-72.

Mokhtar, AB, Ahmed, SA, Eltamany, E, Karanis, P, 2019: Anti-*Blastocystis* activity in vitro of Egyptian herbal extracts (family: Asteraceae) with emphasis on *Artemisia judaica*. Int. J. Environ. Res. Pub. Hlth. 16, 9:1555. https:// doi. org/10.3390/ijerph16091555.

Mossallam, SF, El-Mansoury, SA, Tolba, M M, Kohla, AA, Khedr, SI, 2021: In vitro susceptibility of human *Blastocystis* subtypes to simeprevir. Saudi J. Biol. Sci. 28, 4:2491-501.

Nasirudeen, AM, Tan, KS, Singh, M, Yap, E H 2001: Programmed cell death in a human intestinal parasite, *Blastocystis hominis*. Parasitology 123, 3:235-46.

Ozyurt, M, Kurt, O, Mølbak, K, Nielsen, H V, Haznedaroglu, T, *et al,* 2008: Molecular epidemiology of *Blastocystis* infections in Turkey. Parasitol. Int. 57, 3:300-6.

Popruk, S, Pintong, A, Radomyos, P, 2013: Diversity of *Blastocystis* subtypes in humans. J. Trop. Med. Parasitol. 36:D88-97.

Ragavan, ND, Govind, SK, Chye, TT, Mahadeva, S, 2014: Phenotypic variation in *Blastocystis spp.* ST3. Parasit. Vectors 7:404.doi: 10. 1186/1756-3305-7-404.

Rajamanikam, A, Govind, SK, 2013: Amoebic forms of *Blastocystis spp.* evidence for a pathogenic role. Parasit. Vectors 6, 1:295. doi: 10. 1186/1756-3305-6-295.

Rajamanikam, A, Hooi, HS, Kudva, M, Samudi, C, Kumar, S, 2019: Resistance towards metronidazole in *Blastocystis spp*.: A pathogenic consequence. PLoS One 14, 2:0212542. doi: 10.1371/journal. pone.0212542.

Rajamanikam, A, Hooi, HS, Kudva, M, Samudi, C, Govind, S, 2022: Distinct phenotypic variation of *Blastocystis* sp. ST3 from Urban and Orang Asli Population: An influential consideration during sample collection in surveys. Biology 11, 8:1211. doi: 10.3390/biology 110 81211.

Raman, K, Kumar, S, Chye, TT, 2016: Increase number of mitochondrion-like organelle in symptomatic *Blastocystis* subtype 3 due to metronidazole treatment. Parasitol. Res. 115, 1:391-6.

Ramírez, JD, Sánchez, A, Hernándezm C, Flórez, C, Bernal, M, *et al,* 2016: Geographic distribution of human *Blastocystis* subtypes in South America. Infect. Genet. Evolut. 41:32-5.

Roberts, T, Bush, S, Ellis, J, Harkness, J, Stark, D, 2015: In vitro antimicrobial susceptibility patterns of *Blastocystis*. Antimicrob. Agents hemother. 59, 8:4417-23.

Sari, IP, Benung, MR, Wahdini, S, Kurniawan, A, 2018: Diagnosis and identification of *Blastocystis* subtypes in primary school children in Jakarta. J. Trop. Pediatr. 64, 3:208-14.

Sekar, U, Shanthi, M, 2013: *Blastocystis*: Consensus of treatment and controversies. Trop. Parasitol. 3:35-9.

Seyer, A, Karasartova, D, Ruh, E, Gu¨reser, AS, Turgal, E, *et al*, 2017: Epidemiology and prevalence of *Blastocystis spp.* in North Cypr-us. Am. J. Trop. Med. Hyg. 96, 5:1164. https:// doi.org/10.4269/ ajtmh.16-0706

Skotarczak, BL, 2018: Genetic diversity and pathogenicity of *Blastocystis*. Ann. Agric. Enivron. Med. 25, 3:411-6.

Stensvold, CR, Clark, CG, 2020: Pre-empting Pandora's box: *Blastocystis* subtypes revisited. Trends Parasitol. 36, 3:229-32.

Stenzel, DJ, Boreham, PF, McDougall, R, 1991: Ultrastructure of *Blastocystis hominis* in human stool samples. Int. J. Parasitol. 21:807-17. doi: 10.1128/CMR.00022-08

Tan, KSW, 2008: New Insights on classification, identification, and clinical relevance of *Blastocystis* sp. Clin. Microbiol. Rev. 21, 4:63965.

Tan, KS, Nasirudeen, AM, 2005: Protozoan programmed cell death- insights from *Blastocystis* death styles. Trends Parasitol. 21:5470-50.

Tan, TC, Suresh, KG, 2006: Predominance of amoeboid forms of *Blastocystis hominis* in isolates from symptomatic patients. Parasitol. Res. 98:189-93.

Tan, KS, Howe, J, Yap, EH, Singh, M, 2001: Do *Blastocystis hominis* colony forms undergo programmed cell death? Parasitol. Res. 87:362-7.

Tan, TC, Suresh, K, Smith, H, 2008: Phenotypic and genotypic characterization of *Blastocystis hominis* isolates implicates subtype 3 as a subtype with pathogenic potential. Parasitol. Res. 104, 1:85-93.

Vdovenko, AA, Williams, JE, 2000: *Blastocystis hominis*: Neutral red supra-vital staining and its application to *in vitro* drug sensitivity testing. Parasitol Res. 86, 7:573-81.

Wawrzyniak, I, Texier, C, Poirier, P, Viscogliosi, E, Tan, KS, *et al,* 2012: Characterization of two cysteine proteases secreted by *Blastocystis* ST7, a human intestinal parasite. Parasitol. Int. 61, 3:437-42.

Wu, Z, Mirza, H, Tan, KS, 2014: Intra-subtype variation in entero-adhesion accounts for differences in epithelial barrier disruption and is associated with metronidazole resistance in *Blastocystis* subtype-7. PLoS Negl. Trop. Dis. 8, 5: e2885.https://doi.org/10.1371/journal.pntd. 0002885

Yakoob, J, Jafri, W, Jafri, N, Islam, M, Asim, MB, 2004: In vitro susceptibility of *Blastocystis hominis* isolated from patients with irritable bowel syndrome**.** Br. J. Biomed. Sci. 61, 2: 75-7.

Yakoob, J, Abbas, Z, Beg, MA, Naz, S, Awan, S, *et al,* 2011: In vitro sensitivity of *Blastocystis hominis* to garlic, ginger, white cumin, and black pepper used in diet. Parasitol. Res. 109, 2:379-85.

Yan, Y, Su, S, Ye, J, Lai, X, Lai, R, *et al,*

2007: *Blastocystis* spp. subtype 5: A possibly zoonotic genotype. Parasitol. Res. 101:1527-32.
Yang, LQ, Singh, M, Yap, EH, Ng, GC, Xu, HX, *et al,* 1996: *In vitro* response of *Blastocystis hominis* against traditional Chinese medicine. J. Ethnopharmacol. 55, 1:35-42.
Yin, J, Ye, AJ, Tan, KS, 2010: Autophagy is involved in starvation response and cell death in *Blastocystis*. Microbiology 156:665-677.
Yoshikawa, H, Yoshida, K, Nakajima, A, Yamanari, K, Iwatani, S, *et al,* 2004: Fecal-oral transmission of the cyst form of *Blastocystis hominis* in rats. Parasitol. Res. 94:391-6.
Zaman, V, 1998: The differential identification of *Blastocystis hominis* cysts. Ann. Trop. Med. Parasitol. 92:233-5.

Explanation of figures

Fig. 1: Percentages of *Blastocystis spp.* phenotypes among different subtypes during first 5 re-culturing. Amoeboid form presents among 50 % of parasite isolated from patients (13/26) with acute symptoms and having ST3 in all of intrasubtypes particularly during first 3 re-culturing. First two re-culturing, ST4 & ST3 isolates clumped forms with 9/15(60%) and 12/29(41.3%), respectively, lowest one ST1 2/10(20%). Cysts were showed in culture of ST3chronic patients infected during 4th re-culturing with 4/7 (57%) and at 5th re-culturing of other STs induced chronic infection. Cysts among other isolates progressed to experimental end.

Fig. 2: Microscopic eamination of recultured *Blastocystis spp*. isolated from patients showed different forms (arrows). A- clumping vacular forms x100, b- cyst form x400, c- ameoboid form x400, d- vacuolar form x400 and e) granular form x400. Scale bar =100µ.