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ASSESSMENT OF MICROSCOPIC EXAMINATION AND *IN-VITRO* CULTURE COMPARED TO PCR FOR DIAGNOSING BLASTOCYSTOSIS

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Abstract

Blastocystosis is incriminated in chronic gastrointestinal diseases, as irritable bowel syndrome, and colorectal cancer with subtype (ST7) that reduced beneficial intestinal microbiota.

This study compared the diagnostic performances of microscopic examination and *In-vitro* culture compared with RCR as a golden standard. A total of 70 fecal samples were obtained from diarrheic patients attending Pediatric, Gastrointestinal and Oncology Unit Suez Canal University Hospitals. *Blastocystis* was microscopically examined (direct smear, formalin ethyl-acetate concentration & trichrome stain) and cultured on Jones' culture medium compared to golden standard PCR using newly-developed primers as a reference technique.

The result showed that 8/70 (11.4%) were positive by wet mount microscopy with 19.5% & 100% sensitivity and specificity, 15 samples (21.4%) were positive with trichrome stain with 36.5% & 100% sensitivity and specificity and 29 samples (70.7%) were positive by Jones' culture medium with 70.7%, & 100% sensitivity and specificity respectively.

Keywords: *Blastocyst* sp., *in vitro* culture, polymerase chain reaction.

Introduction

Blastocystis sp. is the commonest single celled eukaryote in intestinal tract of humans and many animals exceeding 50% in many developing countries **(**Jimenez *et al,* 2023). In Egypt, rates as low as 19.1% was reported in humans (Eassa *et al,* 2016), but prevalence in animals and birds was 72.2% (Mokhtar and Youssef 2018). Blastocystosis has a wide range of clinical pictures: asymptomatic, or present with nonspecific gastrointestinal (GIT) symptoms, including diarrhea, nausea, vomiting, flatulence, cramps, discomfort, abdominal pain, fever, anorexia urticaria (Ahmad *et al,* 2022). It is transmitted feco-orally when contaminating food and water in both humans and animals (El Safadi *et al,* 2016). Infections highly associated with irritable bowel syndrome (Graczyk *et al,* 2005), and significant association with colorectal cancer (Mohamed *et al*, 2017) especially high positivity in stages 3 & 4 (Hawash *et al,* 2020).

There are many methods to detect *Blastocystis* including direct microscopic examination and concentration techniques of fecal samples with or without staining, rapidly multiplies in culture medium (El Shazly *et al,* 2006) and molecular techniques (Nascimento *et al,* 2005). However, both microscopic examinations and culture cannot differentiate morphologically the species and subspecies of *Blastocystis* exclusively without the use of molecular techniques, as PCR **(**Yoshikawa *et al,* 2007). But, most of the developing countries didn't use PCR as a routine diagnosis (Mohammad *et al,* 2018).

The present study aimed to compare diagnostic accuracy of microscopic examinations as stained (Lugol's iodine, methylene blue & trichome) smears and concentration technique, and *in-vitro* Jones' culture medium as compared to PCR assay (as a golden standard) using newly developed primers as a reference technique.

Material and Methods

Study population: This is cross sectional study on 70 patients (32 male & 38 female aged from 6 to70 years). They attended the Internal Medicine Unite at Suez Canal University Educational Hospitals from January 2022 to January 2023 with manifestations suggesting blastocystosis.

Sample processing: Morning stool samples were collected and divided into four parts, the first part was sent to microbiology dep-

artment to be cultured using MacConey agar, Helton agar, TCBS and *Salmonella- Shigella* agar cultures to confirm no micro biological pathogens responsible for clinical manifestations (Cheesbrough, 2006). Second part was fixed in polyvinyl alcohol (PVA) for microscopic examination (wet mount, iodine, methylene blue, trichrome stain and formalin ethyl acetate concentration), third one for culture and last one was kept at -20°C for molecular analysis.

Culture in Jones' medium: About 1g of fresh stool, or 750µl for formed stools was processed into 5ml screw-capped tubes containing 3ml Jones's medium using sterile applicator stick. The culture medium was enriched with 10% heat-inactivated horse serum (Gibco, USA) before usage and incubated at 37°C growth was examined microscopically at 24, 48, & 72hr incubation positive samples were considered when vacuolar, granular, amoeboid or cyst forms were seen (Padukone *et al*, 2018).

DNA extraction: DNA was extracted from frozen stool samples by QIAamp™ DNA (Minikit, Qiagen, Hilden, Germany), each concentration was measured by Nano-Drop according to manufacturer instructions.

PCR amplification: Amplification was done by using automated thermo-cycler forward primer: 5′GGA GGT AGT GAC AAT AAA TC-3′, and reverse primer: 5′TAA GAC TAC GAG GGT ATC TA-3′, specific for SSU rDNA of *Blastocystis*. PCR was involved denaturation at 95°C for 7 minutes, 35cycles at 94°C for one minute, annealing at 56°C for 45 seconds followed by extension at 72°C for 7 minutes. Amplicons of 580 base-pairs were observed after electrophoresis on Agarose Gel Electrophoresis (Roberts *et al,* 2011).

Statistical analysis: Data were collected, computerized and analyzed using statistical software program IBM SPSS 19.0, evaluating the diagnostic accuracies of laboratory assays for *Blastocystis* detection was done, as well as estimation of sensitivity, specificity, PPV & NPV for each test compared to PCR. Kappa test identified agreement between different diagnostic tests. A significant difference was considered when $P < 0.05$.

Ethical consideration: All procedures were approved by the Institutional Human Ethics Committee, Faculty of Medicine, Suez Canal University; number (Research 4370#).

After explaining the study purpose, an informed written consent was signed from each patient; an appropriate treatment was given for infected patients.

Results

B. hominis was detected in 41 patients (58.6%), females (22) were more affected than males (19) with an average age range of 16-33 years. The most affected age group was 10-20 without significance to residences. Co-infection with *Giardia lamblia*, *Entamoeba histolytica,* and *Cryptosporidum parvum* was in 4, 2 & 1 patients respectively.

Blastocystis infection was most prevalent in cancer colon patients 17/23 and irritable bowel disease 15/21, but lowest one was in nephrotic syndrome patients (1/5) only. Diarrhea and abdominal pain were the most frequent presented symptoms while fatigue and weight loss were the least frequent.

The least detected *Blastocystis* sp. was by microscopic examination of FECT 8/70 samples followed by trichrome stain 15 cases (21.4%), and Jones' culture medium showed 29 positive cases (41.4%). PCR detected 41 positive cases (58.6%), at a band of 580bp in gel. Granular and vacuolar forms were abundant in smears stained with Lugol's iodine, methylene blue and trichrome samples, vacuolar forms were most dominant in culture, cyst stages were rare in culture media.

Jones' culture medium showed high sensitivity and specificity (70.73% & 100%, respectively), which had good agreement with PCR (0.667), followed by trichrome stained ones with sensitivity and specificity (36.59% &100% respectively), fair agreement when compared to PCR (0.323). FECT (stained or unstained) microscopy gave least sensitivity (19.51%), but specificity (100%), with poor agreement (0.167).

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

Table1: Sociodemographic data of *Blastocystis* positive and negative patients (n = 70).

Demographic data	Positive (No. %)	Negative (No. %)	Total (No. %)	P value
Sex: Male	19(27%)	13(19%)	32(46%)	
: Female	22(31%)	16(23%)	38(54%)	0.66
Total	41(59.5%)	29(41.5%)	70(100%)	
Residence: Rural	18(26%)	14(20%)	32(46%)	
: Urban	23(33%)	15(21%)	38(54%)	0.46
Total	41(59.5%)	29(41.5%)	70(100%)	
Age (years) :<10	1(1.5%)	1(1.5%)	2 (3%)	
:10-20	11(16%)	5(7%)	16 (23%)	
:21- 30	10(14%)	4(6%)	14 (20%)	
:31- 40	7(10%)	5(7%)	12 (17%)	0.32
:41- 50	6(8%)	2(3%)	8 (11%)	
:51- 60	4(6%)	8(11%)	12 (17%)	
:61- 70	2(3%)	4(6%)	6 (9%)	
Total	41(58.5%)	29(41.5%)	70(100%)	

Table 2: Prevalence of *Blastocystis* in patients' groups and in different clinical presentations

Variable	Positive	Negative	Total	P value
Hemodialysis patients	2	8	10	0.081
Cancer colon	17	6	23	0.007
Gastroenteritis	6	5	11	0.646
Irritable bowel disease	15	6	21	0.005
Nephrotic syndrome	1	4	5	0.071
Total	41	29	70	
Clinical presentation				
Abdominal pain	11	4	15	0.0132
Diarrhea	17	8	25	0.0117
Vomiting	3	7	10	0.0812
Flatulence	7	4	11	0.1994
Fatigue	2	3	5	0.8905
Weight loss	1	3	4	0.1859
Total	41	29	70	

Table 3: Results of microscopy, trichrome and culture in detection of *Blastocystis* sp. compared to PCR

PCR		Direct smear & stained*		Trichrome stain		Culture	
		Positive	Negative	Positive	Negative	Positive	Negative
Positive	41	8	33	15	26	29	12
Negative	29	0	29	0	29	0	29
Total	70	8	62	15	55	29	41
Sensitivity		19.51 (8.8-34.9)		36.59 (22.1-53.1)		70.73 (54.5-83.9)	
Specificity		100 (88.1-100)		100 (88.1-100)		100 (88.1-100)	
Positive predictive value		100 (63.1-100)		100 (78.2-100)		100 (88.1-100)	
Negative predictive value		46.77 (34-59.9)		52.73 (38.8-66.3)		70.73 (54.5-83.9)	
Diagnostic accuracy		52.86 (41.1-64.5)		62.86 (51.5-74.2)		82.86 (74-91.7)	

*direct smear and methylene blue or iodine stained after FECT.

Discussion

The present study showed overall prevalence of 58.6%, which was lower than that of Hamdy *et al,* (2020) in Egypt who, reported 72%, but more or less agreed with Maghawry *et al,* (2023), who reported a rate of 41.7%. Abroad, the rate was 10.5% in Saudi Arabia (Mohamed, *et al,* 2017), 13% in Tunisia (Sebaa *et al,* 2018) and 17% in France (Menu *et al,* 2019). However, high infection rates were 71.1% in Qatar (Abu-Madi *et al,* 2015), 85% in Lebanon (Osman *et al,* 2016) and up to 100% in Syria (Darwish *et al,* 2023).

In the present study, female patients (31%) were more infected than males (27%). Also, most positive cases were among age group 10-20 years, this was inconsistent with Salehi *et al,* (2021) in Iran who found a prevalence of 7.7% in women and 37.8% in men without significant differences, their high positive cases were in age group 30- 39 years. This may explained by different ethnicities as the preva-

lence in Egypt is lower than in Iran which reflected on gender and age.

In the present study, *Blastocystis* co-infected with other parasites were in four patients with *G. lamblia*, two patients with *E. histolytica* and one patient with *C. parvum*. This agreed with Boutahar *et al.* (2023) in Morocco and Maghawray *et al.* (2023) in Egypt, they reported that blastocystosis patients were commonly co-infected with other pathogens especially in immunocompromised patients and for parasites sharing the same route of infection.

In the present study, *Blastocystis* infection was more prevalent in cancer colon patients (24.3%) followed by irritable bowel disease (21.4%) and least one was in nephrotic patients (1.4%). This agreed with Mahmoudvand *et al,* (2021) in Iran they found that *B. hominis* among colorectal cancer patients (23.9%), and Kumarasamy *et al.* (2022), who found blastocystosis was among (2%-28%) in colorectal cancer patients.

Previously Galizia *et al.* (2002) in Italy found that subtype 3 *Blastocystis* propagated positive expression of Cathepsin B (CTSB) in cancer cells. Chung and Chang (2003) in China found that IL-6 expression was associated with proliferation of colon carcinoma. Herszényi *et al.* (2008) in Hungary showed that CTSB expression was significant in blastocystosis patients with colorectal cancer. Kumarasamy *et al.* (2013) found that solubilized antigen extracted from blastocystosis subtype 2 & 3 isolates added to colon cancer cells showed significant IL-8 & IL-6 expression. Rajamanikam *et al.* (2019) reported that release of inflammatory cytokines such as IL-8 together with reactive oxygen trigger the pathogenesis of cancer.

The irritable bowel diseases was associated with *Blastocystis* by growth predilection for intestinal environmental changes were in IBD, *Blastocystis* predispose to chronic low grade inflammation due to sustained antigenic release and proteases release that devastate IgA antibody allowed *B. hominis* colonization (Fouad, *et al,* 2011). Romero-Valdovinos *et al.* (2012) in Mexico reported association between IL-8 &IL-10 gene polymorphisms in IBS-*Blastocystis* carriers. Also, Rojas-Velázquez *et al.* (2022) in Mexico correlated between pathophysiology of IBS and polymorphisms in inflammatory cytokines encoding genes predisposing to IBS.

In the present study, abdominal pain and diarrhea were the most common symptoms. This agreed with Abaza, *et al.* (2014) and Khorshidvand *et al.* (2021), while Matovelle *et al.* (2022) reported association of vomiting, flatulence, fatigue, and weight loss. Kumarasamy *et al.* (2018) and Tito *et al.* (2019) reported that the blastocystis subtypes detection with variable pathogenic potential clarified the symptoms diversity.

The present study showed granular form in iodine stained smear. This agreed with Katsarou-Katsari *et al.* (2008), who found that older culture showed granular form that may arose from vacuolar one, Thergarajan *et al.* (2018) assumed similarity between the two forms except for presence of granules. Also, the present study showed vacuolar form in microscopic FECT and in culture. Zhang *et al,* (2007) found that vacuolar form was the classical diagnostic form. El-Ghareeb *et al.* (2015) reported that vacuolar form was more prevalent in all the stained stool smears. Undoubtedly, blastocystosis diagnosis depends on such techniques. The present study showed that *B. hominis* was (11.4%, 21.4% & 41.4% by the microscopic examination of FECT, trichrome stained and Jones' culture medium respectively. However, microscopic examination of FECT totally gave low sensitivity of 19.51%. This agreed with Stensvold *et al.* (2006), who found that FECT had very poor sensitivity and should be avoided in *Blastocystis* diagnosis. But, Elghareeb *et al.* (2015) explained that lower microscopy sensitivity by low parasite load or ambiguity of recognition due to parasitic polymorphic nature in wet mounts. Süli *et al.* (2018) attributed low sensitivity to irregular parasite shedd ing or delayed examination of stool samples. But, Nascimento and Mda (2005) reported that direct iodine wet mount after concen-

tration by formalin-ether sedimentation gave high sensitivity (75%). Bart *et al,* (2013) reported microscopic sensitivity of 96.3% (103/107), and that the use of triple feces test for two fixed samples increased microscopic sensitivity, also Suresh *et al. (*2009), who reported *Blastocystis* shedding fluctuation.

In the present study, the trichrome stain detected 15/70 (21.4%) cases with (36.59%) sensitivity and (100%) specificity. This agreed with Termmathurapoj *et al.* (2004), who found that simple smear and trichrome stained showed sensitivities (16.7% & 40.2%) and specificities (94% & 80.4%), respectively. Also, Tan (2008) found that trichrome stain gave high sensitivity than the iodine stained wet, and that trichrome was more sensitive than microscopic FECT. Elghareeb *et al.* (2015) detected (12.3%) by trichrome stain, they added that although being inexpensive and clarified the parasite, but not used in epidemiological studies due to time consuming.

In the present work, Jones' culture medium showed high sensitivity (70.73%) as the parasite had enough time to grow and replicate. This agreed with Dogruman *et al.* (2010), and Robert *et al.* (2011), who reported the effectiveness of Jones' culture media for *Blastocystis* sp. growth than other media. Santos and Rivera (2013) found that Jones' culture medium gave sensitivity (67.6%) and specificity (100%). Elghareeb *et al.* (2015) reported that *in vitro* cultivation using Jones' medium detected 274/1200 (22.8%) positive cases with a sensitivity (100%) and specificity (88%). However, Stark *et al.* (2011) reported lower sensitivity rates that explained by preferential growth of *Blastocystis* strains than others in different cultures.

In the present study, the used primers were valuable in diagnosing *Blastocystis* species detected infection in 41 samples (58.6%). Roberts *et al.* (2011) reported that some primers have the ability of specific subtypes' amplification and other subtypes could be missed. They used two sets of PCR with two different primers, which gave two contradictive results as PCR1 had a sensitivity of 66% and PCR2 had a sensitivity of 94%. Also, Poirier *et al.* (2011), Padukone *et al.* (2018) and Khademvatan *et al.* (2018) reported that PCR gave higher sensitivity than other methods. However, Sari *et al.* (2018), who isolated DNA from fresh stool and compared between culture and PCR methods reported that PCR sensitivity was not preferable than culture method. The lower sensitivity may be due to lower quality and quantity of DNA isolated from stools than isolated from culture, also presence of DNA amplification biological inhibitors in stool as bile salts and polysaccharide complex.

Conclusion

PCR has a high sensitivity and specificity, but it is costly and complex, however it was valuable in immuno-compromised patients

Culture gave relative high sensitivity and specificity low cost and easy performance. It may afford satisfying sensitivity especially in some developing countries.

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Explanation of figures

Fig. 1: *B. hominis* from a stool samples. a- vacuolar form (methylene blue Stain X 400), b- granular form (Iodine stained X 1000), c- vacuolar form (trichrome stain X1000), d- vacuolar form black arrow cyst form blue arrow (trichrome stain X1000)
Fig. 2: *B. hominis* in Jones' medium culture X1000: showed numerous vacuolar forms with variable sizes; each with central vacuole, a thin peripheral rim of cytoplasm & multiple nuclei (black arrow) and cyst with smaller size and thick outer layer (blue arrow)
Fig. 3: Gel electrophoresis stained with ethidium bromide showed PCR amplification of *Blastocystis* SSU rDNA from fecal samples. Lane (1)1000-bp DNA ladder, Lanes (2-7) positive *Blastocystis* isolates at 580 base-pairs.
Fig, 4: Gel electrophoresis stained with ethidium bromide showed PCR amplification of *Blastocystis* SSU rDNA from fecal samples.