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

By

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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-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 & 29 samples (70.7%) were positive by Jones’ culture medium with 70.7%, & 100% sensitivity and specificity respectively.

Keywords: Blastocystis 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 & trichrome) 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 to 70 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 microbiological 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 *Cryptosporidium 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).

Table 1: Sociodemographic data of Blastocystis positive and negative patients (n = 70).

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

Table 2: Prevalence of Blastocystis in patients’ groups and in different clinical presentations.

<table>
<thead>
<tr>
<th>Variable</th>
<th>Positive</th>
<th>Negative</th>
<th>Total</th>
<th>P value</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hemodialysis patients</td>
<td>2</td>
<td>8</td>
<td>10</td>
<td>0.081</td>
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<tr>
<td>Cancer colon</td>
<td>17</td>
<td>6</td>
<td>23</td>
<td>0.007</td>
</tr>
<tr>
<td>Gastroenteritis</td>
<td>6</td>
<td>5</td>
<td>11</td>
<td>0.646</td>
</tr>
<tr>
<td>Irritable bowel disease</td>
<td>15</td>
<td>6</td>
<td>21</td>
<td>0.005</td>
</tr>
<tr>
<td>Nephrotic syndrome</td>
<td>1</td>
<td>4</td>
<td>5</td>
<td>0.071</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>29</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

Clinical presentation

<table>
<thead>
<tr>
<th>Abdominal pain</th>
<th>Positives</th>
<th>Negatives</th>
<th>Total</th>
<th>P value</th>
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</thead>
<tbody>
<tr>
<td>11</td>
<td>4</td>
<td>15</td>
<td>0.0132</td>
<td></td>
</tr>
<tr>
<td>Diarrhea</td>
<td>17</td>
<td>8</td>
<td>25</td>
<td>0.0117</td>
</tr>
<tr>
<td>Vomiting</td>
<td>7</td>
<td>4</td>
<td>11</td>
<td>0.1994</td>
</tr>
<tr>
<td>Flatulence</td>
<td>2</td>
<td>3</td>
<td>5</td>
<td>0.8905</td>
</tr>
<tr>
<td>Fatigue</td>
<td>1</td>
<td>3</td>
<td>4</td>
<td>0.1859</td>
</tr>
<tr>
<td>Total</td>
<td>41</td>
<td>29</td>
<td>70</td>
<td></td>
</tr>
</tbody>
</table>

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

<table>
<thead>
<tr>
<th>PCR</th>
<th>Direct smear &amp; stained*</th>
<th>Trichrome stain</th>
<th>Culture</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Negative</td>
<td>Positive</td>
</tr>
<tr>
<td>Positive</td>
<td>41</td>
<td>8</td>
<td>33</td>
</tr>
<tr>
<td>Negative</td>
<td>29</td>
<td>0</td>
<td>29</td>
</tr>
<tr>
<td>Total</td>
<td>70</td>
<td>8</td>
<td>62</td>
</tr>
</tbody>
</table>

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 (34.9-59.9) 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, (201) 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 be explained by different ethnicities as the preva-
ience 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 Mahmoudvandi *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* coloniziation (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 Khorshidvandi *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 Katzarou-Katsari *et al.* (2008), who found that older culture showed granular form that may arise 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 *B. hominis* was (11.4%, 21.4% & 41.4% by the microscopic examination of FECT, trichrome stained 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 shedding 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 Temmathurapoj 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 contradictory 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 sample. 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.