Zagazig University Medical Journal
www.zumj.journals.ekb.eg

Volume 29, Issue 1, January 2023, Page (144-150) Supplement Issue

Manuscript ID ZUMJ-2006-1888 (R1)

DOI **10.21608/zumj.2020.33988.1888**

ORIGINAL ARTICLE

Evaluation of Staining Methods Versus in-Vitro Cultivation in Diagnosis of Blastocystis Hominis

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Submit Date 2020-06-30
Revise Date 2020-08-04
Accept Date 2020-08-15

ABSTRACT

Background: Blastocystis is a widely distributed, unicellular, anaerobic intestinal protozoan parasite. Blastocystis hominis accused to be possible cause of irritable bowel syndrome, inflammatory bowel diseases, and chronic diarrhea. It has many morphologic forms and is transmitted via fecal-oral route through ingestion of cysts. Laboratory diagnosis of Blastocystis infection is usually via microscopic examination of freshly stained or chemically preserved stool samples but it lacking the sensitivity. The current study is concerned with evaluation of sensitivity and specificity of culture test in comparison to other ordinary staining methods (trichrome, MZN and iodine) for diagnosis of Blastocystis hominis.

Methods: Stool samples were collected from seventy two cases, complaining of different gastrointestinal troubles and attending to different Outpatient Clinics of Zagazig Hospitals. All samples were examined microscopically by wet mount, saline, iodine then preserved for staining by Modified Ziehl-Neelsen (MZN) and trichrome stain and were cultured on Boeck and Drbohlav Locke egg serum medium (LE), then examined by light microscope with objectives x10, x40 and x100.

Results: Blastocystis hominis infection was common in younger aged males (14-35years). Conventional methods for diagnosis of Blastocystis e.g simple saline, iodine stained smears, MZN and trichrome stain are simple, specific, non-invasive methods but with low sensitivity. Stool culture was more accurate and sensitive for detection of Blastocystis than direct microscopy.

Conclusions: LE medium culture offers better sensitivity than other ordinary methods for detecting *B. hominis* infection in stool samples. It is suitable for use in large hospitals or public health laboratories.

Keywords: Blastocystis hominis, Modified Ziehl-Neelsen, Trichrome stain, Boeck and Drbohlav Locke egg (LE) serum medium.

INTRODUCTION

Blastocystis species, anaerobic enteric parasites, are one of the commonly detected parasites in a variety of vertebrates throughout the world [1]. Its prevalence ranged from 10-70% in different countries with adults being more affected than children [2, 3]. High infection rates are associated with increased population density, low socio-economic standard, inadequate clean water sources, unhygienic disposal of faeces and immune-compromised patients predominantly those infected with human immunodeficiency virus [4].The pathogenic role of Blastocystis remains debatable. Some scholars argue about the harmfulness of Blastocystis in humans due to its link with asymptomatic carriers [5], though others are with the faith of the possible pathogenic role it may play in symptomatic cases [6]. Blastocystis has been associated with irritable bowel syndrome, inflammatory bowel disease, chronic diarrhea, and ulcerative colitis [7, 8].Blastocystis hominis has multiple forms: vacuolar, granular, amoeboid, cyst, multivacuolar, and avacuolar forms [9] and a proposed precyst form [10]. So, it provides a significant challenge for laboratory diagnosis because of the possibility of confusion with yeast, Cyclospora spp. or fat globules [11]. A better detection method to distinguish therapies which eradicate the parasite from those providing short-term symptomatic improvement is an urgent requirement [12].

Diagnosis of Blastocystis infection is usually via microscopic examination of stained, or chemically preserved faecal specimens, however, they are not

frequently detected because of their variable shedding and polymorphic nature that may possibly lower the sensitivity of direct examination of stool samples with ordinary staining methods [13]. Trichrome staining is one of several permanent stains used for detection of trophic forms of protozoa in faecal specimens [14]. Since Blastocystis characteristically stained with trichrome stain, it was frequently used in several laboratories owing to its sensitivity for detection of Blastocystis spp. than iodine-stained wet mount [15]. Many scholarships suggested that stool culture may be the most sensitive method for detection of Blastocystis [16, 17], together with immunofluorescence (IFA) assay and enzyme-linked immunosorbent assay (ELISA) [18]. Short-term (24 to 72 hrs.) in-vitro cultivation increase Blastocystis detection sensitivity in comparison with direct microscopy and Lugol's iodine or trichrome stained smears [14]. In vitro techniques would be an excellent choice being economically better in screening large sample size [4], as well as obtaining a higher concentration of the requested genetic material for molecular testing [19]. Owing to the light infection with Blastocystis spp. is common and organisms can be simply missed by direct microscopy, combination of simple smear and culture method may provide the standard approach for detecting *B. hominis* in patient specimens [20]. In this work we aimed to evaluate the sensitivity and specificity of culture test (LE medium) in comparison with microscopy (iodine, MZN, trichrome) for diagnosis of Blastocystis hominis.

METHODS

The study was approved by the research ethical committee of Faculty of Medicine, Zagazig University and Elahrar hospital. The study was done according to The Code of Ethics of the World Medical Association (Declaration of Helsinki) for studies involving humans. The study was carried out in the Parasitology Department, Faculty of Medicine, Zagazig University, during the period from February to June 2019

Samples collection: Seventy-two fresh faecal specimens were collected from patients with/without gastrointestinal symptoms attending Zagazig university hospital, Elahrar hospital clinics. From all patients, data were collected including full history taking (age, sex, residence, sanitary habits, source of food/water, GIT troubles, and history of previous illness or receiving drugs), patients with diarrhea were asked about duration of illness, number of motions/day, character of stool and previous similar attacks. Patients receiving antiparasitic or immunosuppressive drugs were omitted. Samples were collected in clean dry labeled cups, with tight fitting cover.

Processing of samples: Each collected sample was divided into 2 parts, one part kept fresh to be examined microscopically by wet mount, and iodine stain using objective lenses X10 and X40 respectively [21] and for culture. The second part kept in formalin 10% then prepared for staining by Modified Ziehl-Neelsen stain [22] and trichrome stain [21] to exclude the possibility of a mixed infection with other parasites.

Isolation & Cultivation of Blastocystis:

Stool samples were cultivated immediately in 5ml screw caped tube containing a previously prepared LE media supplemented with 10% fetal bovine serum (Biowest), antibiotic mixture ready-made antibiotic mixture of penicillin (10000 U/ml) and streptomycin (10000 µg/ml) (Biowest) and antifungal (diflucan 150 mg dissolved in 30 ml distilled water) (pifzer) was added by insulin syringe 10 units at 37°C for 2-3 days [20]. Culture tubes were screened for Blastocystis multiplication rate by light microscopy every 48hr-72hr. The sediment was examined with or without iodine by (×10, ×40 and ×100) objectives, when the characteristic vacuolar/granular forms of Blastocystis were observed, a sub-cultured in a new medium was performed. Sub-cultivation was carried out every 3days.

Ethical considerations: The study was permitted by Institutional Review Board, Faculty of Medicine, Zagazig University. The purpose and procedures of the study were clarified to all participants, and a written well-versed consent was obtained from all cases and from parents on behalf of their children.

STATISTICAL ANALYSIS

Data were recorded, calculated, tabulated and statistically analyzed using statistical computer program SPSS version 18.0. Qualitative data were represented as frequencies and percentages. Chi square test was used to calculate difference between qualitative variables.

RESULTS

Out of 72 studied cases (44 males and 28 females) with an age range of (2 – 60) years, *B. hominis* was detected in 30 cases (41.7%), males were more affected than females with an average age range of 14-35 (table1). Within Blastocystis positive cases, only 19 cases (26.38 %) were only infected with *B.hominis*, and 11 cases (15.3%) were mixed infection of *B.hominis* and other protozoa. The most common associated infection was Cryptosporidia followed by Entamoeba hitolytica, Entamoeba coli and Giardia lambilia with percentage of infection was (6.94%, 4.2%, 2.8% and 1.4%) respectively (table 2)(fig1,2,5). When comparing the frequency of detection of Blastocystis by different diagnostic methods, we found that positive cases were (15.3%, 20.8%, 25% and 30.6%, 41.7%) by direct saline, iodine, MZN,

trichrome stain and LE medium respectively (table 3)(fig 3-7). Concerning the sensitivity, specificity and accuracy of different tests for detection of *B.hominis* in comparison with LE medium as gold slandered, trichrome staining was the most sensitive method followed by MZN, iodine staining and direct saline with a sensitivity of 73.3%, 60%, 50%, 36.7% and specificity 100% for all methods and accuracy of 88.9%, 83.3%, 79.2%, 73.6% respectively (tables 4,5,6,7) with statistical significance difference between them.

(Table 1) B. hominis positive (+ve) and negative (–ve) cases concerning age and sex:

Variable	**+ve cases (n=30)**		**-ve cases (n=42)**		χ^2	**P**
	No	**%**	**No**	**%**		
Age group: • **2 – 13 y** • **14 – 35 y** • **36 – 50 y** • **> 50 y**	3 14 8 5	10 46.7 26.7 16.6	6 10 16 10	14.3 23.8 38.1 23.8	4.11	**0.25 NS**
Sex: • **Male** • **Female**	18 12	60 40	26 16	61.9 38.1	0.03	**0.87 NS**
χ2: Chi square test **NS: Non significant (P>0.05)**						

Table (2): Associated infections among groups:

Variable	**(n=72)**		**χ2**	**P**
	No	**%**		
No infection (-ve cases)	42	58.33		
(+ve cases) ***Blastocystis* alone** **Mixed infection** ***B. hominis* with *Cryptosporidia*** ***B. hominis* with *Entamoeba histolytica*** ***B. hominis* with *Entamoeba coli*** ***B. hominis* with *Giardia Lambilia***	30 19 11 5 3 2 1	41.7 26.38 15.3 6.94 4.2 2.8 1.4	**18.18**	**<0.001 ****
****: Highly significant (P<0.01)** **χ2: Chi square test**				

Table (3): Assessment of different diagnostic methods for detection of *Blastocystis*

Variable	**(n=72)**			
	No +ve	**%**	**No** -ve	**%**
Direct saline wet mount	11	15.3	61	**84.7**
Direct Iodine stain	15	20.8	57	**79.2**
MZN	18	25	54	**75**
Trichrome	22	30.6	50	**69.4**
Culture on LE medium	**30**	**41.7**	**42**	**58.3**

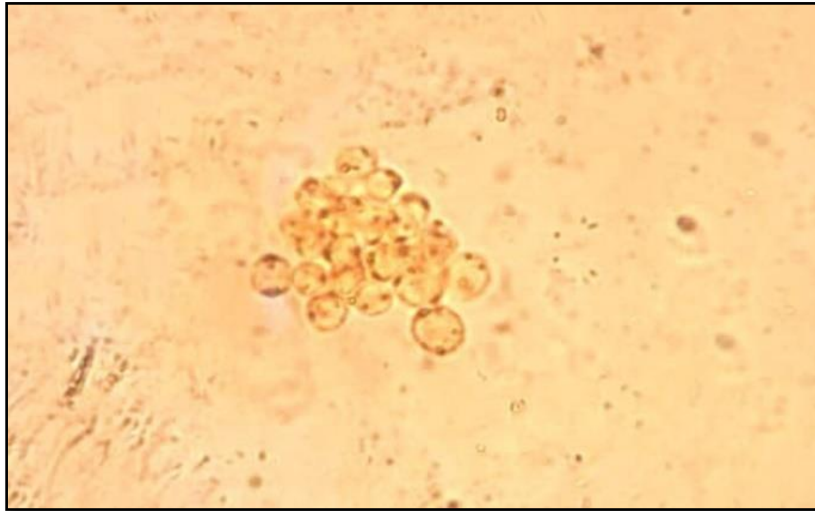

Figure (1): Culture smear stained with iodine stain presenting *B. hominis* growth with extensive variation in size (x1000).

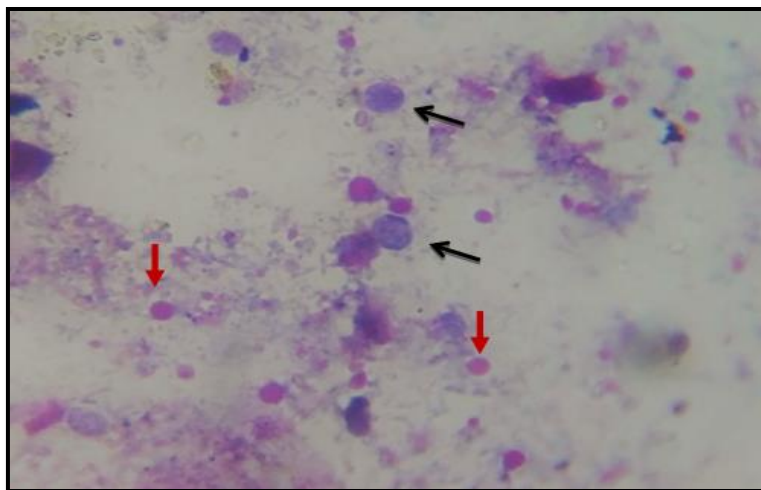

Figure (2): Stool smear stained with MZN stain showing *B. hominis* cystic form (black arrow), *C. parvum* (red arrow) (x1000).

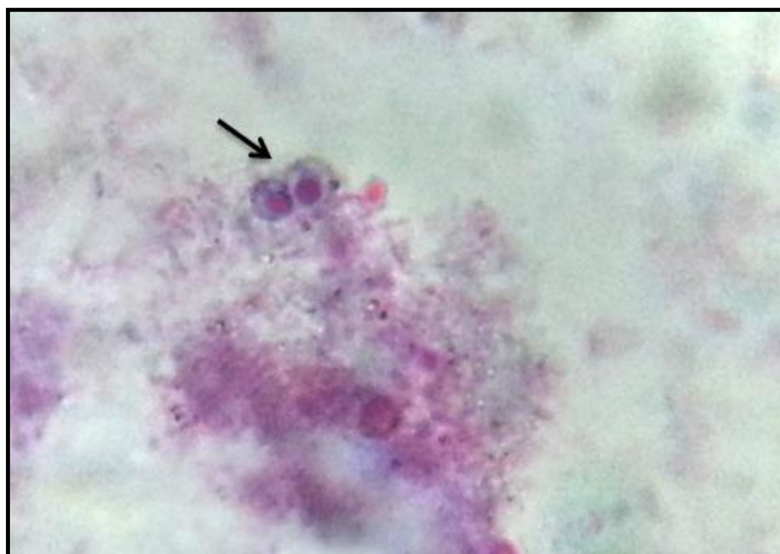

Figure (3): Stool smear stained with trichrome stain showing vacuolar form of *B. hominis* (x1000).

DISCUSSION

Blastocystis is the most common enteric protozoa detected in the stools of humans and many animals worldwide [11]. The pathogenic role of Blastocystis in humans not yet verified [23]. In our study, we found that the prevalence of *B. hominis* was 41.7% (30/72). Other studies in Egypt reported similar finding. Abd El Wahab and Selim [24], informed a prevalence rate of 46.6% in Shibin El Kom, Monofiya Governorate, and Farghaly et al. [25], who reported that *B. hominis* infection rate was 42.3%. Concerning age groups, we found that the highest prevalence of infection was among the age group 14-35 years old (46.7 %) followed by 36-50 years (26.7%), >50 year's (16.6%) and finally 2-13 years old (10%) with no statistically significant difference between different age groups table (1). our finding was in accordance with Gabr et al. [26], who reported that the highest prevalence rate of *B. hominis* infection was observed in two age groups (15-30 years and >30 years) and the age group (< 15 years) showed the lowest positive percent. On the other hand, other studies found that the Blastocystis spp. infection was more prominent in the old age group [27]. Also, El Safadi et al. [28] who reported that patients aged 0-14 years were mostly affected (26.3%) followed by patients aged 15-49 years (22.2%) and patients aged over 50 years were the least affected (13.6%). Our finding can be attributed to the more frequent exposure to sources of infections as young adults commonly favor the junk food. Among *B. hominis* infected cases, males (60%) were more exposed to infection than females (40%) with no statistically significant difference between them (table1) .This was in agreement with Farghaly et al. [25] who reported that *B. hominis* infection rate among males (69.4%) was higher than females (30.6%) and Nithyamathi et al. [4] who reported that Blastocystis infection rate among males (12%) was higher than in females (9%). This could be due to the Egyptian customs and traditions outdoor activities that make them more liable to infection than females. On the other hand, higher prevalence rates were recorded in females [29]. Diagnosis of Blastocystis relies mainly on microscopy using ordinary staining methods (trichrome, MZN, Sufarine methylene blue and iodine), but it is of low sensitivity due to its variable shedding and polymorphic nature [13]. On microscopic examination of stool samples, we observed that, the vacuolar form of Blastocystis spp. was the most common detectable form (fig 3,6,7). This was in agreement with Mehta et al. [30], Darabian et al. [31] and Gabr et al. [26].The vacuolar form of Blastocystis spp. is the diagnostic stage and could be easily distinguished from other protozoa. Direct Iodine stain was able to identify 15/72 (20.8%) positive samples (table 5) (fig 3). It was the most rapid, simplest and the cheapest method but with low sensitivity (50%). Moreover, it could identify different forms of the parasite, but the possible confusion of the parasite with fat cells, white blood cells or yeasts, in addition of being not permanent stain make it of limited diagnostic value. Lower positive values for iodine-stained smear were previously verified by El ghareeb et al. [32], who found that iodine-stained smear identified only 72/1200 (6%) positive cases, although it was not difficult to detect the parasite in wet mounts due to its variation in size and shape. Conversely, Nascimento and Mda [33], identified 36/48 (75%) by direct iodine wet mount after concentration by formalin-ether sedimentation.

MZN stain detected 18/72 (25%) samples with a significantly higher sensitivity (60%) than iodine stain (50%) (Table 6)(fig 6) although it was difficult to identify *B. hominis* because of the color contrast between the parasite and the background that had the same color. This was in accordance with El-Marhoumy et al. [34], who reported that sensitivity of MZN stain and iodine stain were (50%, 43.3% respectively). Concerning, trichrome stain, it identified 22/72 (30.6%) positive samples (table7) (fig 7). It was better and more sensitive (73.3%) than other stains iodine (50%) and MZN (60%) in identifying *B. hominis*, and it could clarify the definitive morphological details of the parasite but it was time consuming. These was more or less in agreement with Farghaly et al. [25], who compared the sensitivity of trichrome stain, MZN, iodine & Safranine-methylene blue (SMB) stains, and reported a sensitivity of 91.7%, 72.9%, 60% & 55.3% for them respectively. Elghareeb et al. [32] conveyed that trichrome stain detected (12.3%) of their studied cases, while direct smears detected only (3.5%) and iodine-stained smear detected (6%) of cases. Also, Tan et al. [11], reported that trichrome stain was more sensitive for the detection of intestinal protozoa than iodine-stained wet mounts. In our study, we considered LE culture media as the gold standard for diagnosis of *B. hominis* infection. It was proved to be the most sensitive method detecting 30 positive cases (41.7%) out of 72 examined samples. All positive samples by other methods were also positive by LE media with no false positive detection in all methods. Moreover, this method detected positive cases that were negative by other tests. Trichrome stain was time consuming, this make it unsuitable for use in the survey study.

CONCLUSION

In conclusion, Locke egg serum medium is simple, rapid, easy to perform with high sensitivity, so it could be an alternative to direct microscopy, for that reason, we recommend its use in large

hospitals and public health laboratories in developing countries.

Conflict of interest: the authors affirmed that there is no conflicts of interest.

Funding Non-Declared

Ethical approval: Institutional review boards' approval was obtained.

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To Cite:

Hegazy, L., Salama, M., Saleh, A., Maghawry, A. Evaluation of staining methods versus in-vitro cultivation in diagnosis of Blastocystis hominis. *Zagazig University Medical Journal*, 2023; (144-150): -.doi: 10.21608/zumj.2020.33988.1888.