Detection of *Blastocystis* sp. in stool samples by different diagnostic methods

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Abstract

Blastocystis sp. is an intestinal parasite that is commonly identified in asymptomatic individuals, as its pathogenic role is underestimated. In Syria, studies on this parasite are very few, and it is not documented in the laboratory reports. The present study aimed to evaluate the sensitivity of three different diagnostic methods in the detection of *Blastocystis* spp. among patients with non-specific gastrointestinal symptom. Stool samples were collected from 70 patients suffering from various gastrointestinal symptoms. All samples were examined microscopically using iodine staining smears, and after *in vitro* cultivation at 37°C for 48- 72 h using Jones' medium. Molecular detection of *Blastocystis* sp. was determined by fragment amplification of the *SSU rRNA* gene using PCR. *Blastocystis* sp. was identified in: 49 cases (70%) by direct microscopic examination, in 60 isolate (85.7%) by *in vitro* culture and in 64 (91.4%) of cases using molecular detection. Comparative analysis revealed that the sensitivity of microscopic detection for *Blastocystis* sp. was 73.4% while it was 90.6% for *in vitro* culture and approximately 96.7% using PCR detection method. *Blastocystis* sp. was found alone in 32 (65.3%) of cases, while co-infection was detected in 17 (34.7%) samples. Our findings highlighted the importance of considering *Blastocystis* sp. in laboratory diagnosis. Molecular methods are recommended for screening clinical specimens for *Blastocystis* sp. infection especially among individuals with no

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common particular symptoms. If not applicable, two different diagnostic techniques are required for accurate diagnose of this parasite.

Key words: *Blastocystis* sp; Parasite; Culture; Polymerase chain reaction; Syria

الكشف عن الأكياس الأريمية البشرية .*Blastocystis* sp في عينات برازية باستخدام طرائق تشخيصية مختلفة

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الملخص

تعد المتكيسة الأريمية (المتبرعمة الكيسية) .*Blastocystis* sp من الطفيليات المعوية الانتهازية، وتوجد بشكل شائع عند الأفراد اللاعَرَضيين مما أدى بالباحثين للخلاف حول قدرتها الإمراضية (بين إمراضيتها وعدمها). ونظراً لقلة الدراسات حول هذا الطفيلي في سوريا ولعدم توثيق وجوده في التقارير المخبرية، فقد هدفت هذه الدراسة إلى تقييم حساسية ثلاث طرق تشخيصية مختلفة في الكشف عن المتكيسة الأريمية البشرية لدى مرضى يعانون من أعراض هضمية غير نوعية. جُمِعَت عينات برازية من 70 مريضاً يعانون من أعراض هضمية متنوعة. فُحِصَت جميع العينات مجهرياً بعد تلوينها بمحلول اليود اليودي ، كما فُحِصت بعد استنباتها في وسط Jones's عند درجة حرارة 37 مئوية، خلال 48 و 72ساعة. تمّ التشخيص الجزيئي عن طريق تضخيم شدفة من المورثة *SSU rRNA* باستخدام تقانة الـ PCR. حُدِّدَ وجود المتكيسة الأريمية البشرية في 49 عينة (70%) بنتيجة الفحص المجهري المباشر، ليرتفع هذا العدد إلى 60 عينة (85.7%) في مستنبتات الوسط الصنعي، وإلى 64 عينة (91.4%) بنتيجة الكشف الجزيئي. وعند مقارنة حساسية الطرق السابقة في الكشف عن هذا الطفيلي، تبيّن أن درجة حساسية كل من الاستنبات في الوسط الصنعي والطريقة الجزيئية كانت مرتفعة (90.6%، 96.7% على الترتيب) في حين بلغت درجة حساسية الفحص المجهري 73.4%. كما أظهرت النتائج وجود طفيلي المتكيسة الأريمية البشرية منفرداً في 32 عينة

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(65.3%) ووجوده إلى جانب طفيليات أخرى في 17 عينة (34.7%). استطعنا بوساطة هذه الدراسة تسليط الضوء على ضرورة إدراج المتكيسة الأريمية البشرية في التقارير المخبرية وعلى أهمية استخدام الطريقة الجزيئية في تشخيص الإصابة بالمتكيسة الأريمية البشرية أو استخدام طريقتين تشخيصيتين مختلفتين عند تعذر القيام بالتشخيص الجزيئي.

الكلمات المفتاحية: المتكيسة الأريمية البشرية؛ طفيلي؛ استنبات؛ التفاعل السلسلي للبوليميراز؛ سوريا

Introduction:

Blastocystis sp. is a unicellular parasite that infects the lower gastrointestinal tract of humans and a wide range of animals (Clark *et al*., 2013; Greige *et al*., 2018). Poor hygiene practices, exposure to animals and consumption of contaminated food or water (Li *et al*., 2007; Leelayoova *et al*., 2008; Osman *et al*., 2016; Al Nahhas and Aboualchamat, 2020) could explain its high prevalence in developing countries (30– 50%) compared with developed countries (1.5–10%) (Bart *et al*., 2013; El Safadi *et al*., 2016).

Blastocystis sp. pathogenicity is controversial; for many years, this parasite was considered with no clinical relevance due to its high prevalence in asymptomatic individuals (Ben Abda *et al*., 2017). However, several studies reported its presence in both asymptomatic and symptomatic patients (Tan, 2004; Eida and Eida, 2008; Moosavi *et al*., 2012). No specific gastrointestinal symptoms are associated with the presence of this parasite; some individuals show abdominal pain, acute or chronic diarrhea, while others show flatulence, bloating, anorexia, and weight loss as well as urticarial lesions (Souppart *et al*., 2010; Lepczyn′ska *et al*., 2016). Recently, an association has been reported with irritable bowel syndrome (IBS) and inflammatory bowel disease (IBD) (Stensvold *et al*., 2009; Poirier *et al*., 2012).

Blastocystis sp. has a highly polymorphic appearance, direct microscopic examination of fecal samples, is considered the traditional diagnostic method of human infection with this parasite (Ben Abda *et al*., 2017; Padukone *et al*., 2018). In Syria, *Blastocystis* sp. epidemiology, diagnose, infection sources and the way of its transmission are not well studied. Therefore, we aimed in this study to employ a comparative analysis between three different diagnostic methods, especially among patients with no specific or clear symptoms in order to shed light on this parasite and to show the importance of its both pathogenicity and diagnosing.

Materials and Methods

Clinical samples

Seventy patients, who presented to the clinics of internal medicine at three major hospitals in the city of Damascus (Al Assad University Hospital, Al-Mouwasat University Hospital and Kids Hospital), participated in this study. All patients signed informed consent and completed simple questionnaire including gender, age, presence of symptoms (i.e. abdominal pain, diarrhea, vomiting, fever, nausea, headache and discomfort) and environmental

conditions, such as type of water supply and contact with household animals. Stool samples were collected into sterile containers, then each obtained sample was divided into three parts for use in microscopy, culture, and PCR assays. Samples were collected in the period between February and December 2020. This study has been approved by the ethical committee of Damascus University (number: 4031).

Macroscopic and Microscopic examination

Firstly, stool specimens were examined by naked eye to determine color and presence of blood or mucous. Then, Lugol's iodine stained smears were prepared from each stool sample to detect *Blastocystis* and other parasite forms using light microscopy at 40× and 100×. To avoid obscurity in microscopic diagnosis of the polymorphic *Blastocystis* sp., only the presence of vacuolar form in more than one field of stool smear was considered as positive.

Culture

Approximately 50-100 mg of stool samples were subjected to culture in Jones' medium (Liofilchem, Italy) supplemented with 10% horse serum and antibiotics (penicillin 100u/ml, streptomycin 100 µg/ml, GeneDirex Inc, Taiwan). Samples were incubated at 37°C in OSK incubator (OSK 9639b, Japan). The growth of *Blastocystis* and the distinct morphological and reproductive stages were confirmed by microscopic observation of culture at 48, and 72 h of incubation using Lugol's iodine staining and light microscopy at 400×.

Genomic DNA extraction and PCR amplification

Total genomic DNA was extracted from 200 to 250 mg of each stool sample, using QIAamp DNA stool mini kit (Qiagen, Valencia, CA) as described previously (Skhal *et al.*, 2016). The DNA concentration was determined using NanoDrop-2000 spectrophotometer (Thermo-Scientific Inc; USA). A pair of diagnostic primers (Stensvold *et al*., 2006) was used to amplify a 310 bp fragment of the small subunit ribosomal RNA gene (*SSU-rRNA*) in case the presence of *Blastocystis.*

The PCR reaction contained 12.5 µl One PCRTM master mix 2X (GeneDirex Inc, Taiwan ROC), 1 µl of each primer pairs, 10.5 µl nuclease-free water, and 4 µl (~70 ng/µl) of the extracted gDNA.

Each PCR experiment contained a negative control (4 µl of nuclease-free water) for contamination detection. PCR cycling conditions were as follows: initial denaturation at 94◦C for 3 min, then 30 cycles of 94◦C for 60 s, 58 ◦C

for 60 s and 72◦C for 60 s. The final extension was at 72◦C for 5 minutes. The PCR products were electrophoresed in 2% agarose gel stained with ethidium bromide (Sigma-Aldrich, USA) along with a 100 bp DNA ladder (GeneDirex Inc, Taiwan ROC) as a size standard. The final results were visualized under a UV transilluminator and photographed for documentation

Statistical study

Diagnostic accuracy was calculated as: sensitivity, specificity, positive and negative predictive values of microscopy, culture and PCR techniques were calculated using MEDCALC® online statistical software (https://www.medcalc.org/calc/diagnostic_test.php).

Results

Our sample study consisted of 70 feces specimens. 28 were collected from males (age ranged 3-75 years, median age 30 years) and 42 were collected from females (aged ranged 5-76 years, median age 37 years). The major clinical symptom of patients was abdominal pain (40/70; 57%) while the minor one was skin rash (6/70; 8.6%) (Table 1).

Table 1. Summary of the studied samples features

Study group	No. examined	%
Gender		
Male	28	(40%)
female	42	(60%)
Total	70	(100%)
Age		
>50 years	34	(69.4%)
<50 years	15	(30.6%)
Total	49	(100%)
Symptoms		
Abdominal pain	40	57%
Diarrhea	24	34.3%
Abdominal cramps	28	40%
Bloating	32	45.7%
Nausea	29	41.4%
Weight loss	22	31.4%
Rash skin	6	8.6%

The macroscopic examination results showed that 24 of the stool samples were noted to have watery appearance, whereas the remaining had normal

consistency and color. Also, no pus, blood, and helminth were observed in the morphological evaluation of the specimens.

Out of the 70 samples, 49 samples (70%) were determined positive for the presence of *Blastocystis* sp., using direct microscopic examination with Lugol's staining. The vacuolated form of *Blastocystis* was the most common, in which cellular structures as the central vacuole, band of cytoplasm, nuclei and surface coat were clearly distinguishable (Fig 1: A). Co-infection with one or more other parasites was detected in 34.7% (17/ 49) of isolates, (Fig 1: B; Table 2), while 65.3 % (32/49) showed the existence of only *Blastocystis* sp. parasite.

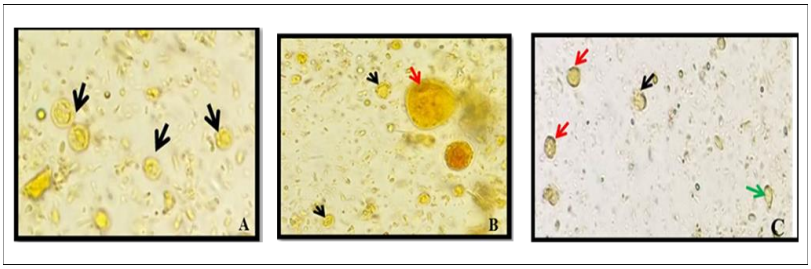

Figure 1. Iodine stained smears: A. *Blastocystis* (vacuole forms "arrows") 100×; B: co-infection *Entamoeba* cysts "red arrow" with *Blastocystis* "black arrows" 100×; C: *Blastocystis in vitro* culture in Jones' medium (granular "red arrows"; vacuolar "black arrow"; amoeboid form "green arrow" 40×.

Table 2. Parasites detected by microscopic examination in conjunction with *Blastocystis*

Parasites	No. of positive samples
Blastocystis sp.	32 (65.3%)
Blastocystis sp.+ *Entamoeba coli*	7 (14.3%)
Blastocystis sp.+ *Entamoeba histolytica* complex*	5 (10.2%)
Blastocystis sp.+ *E. histolytica* complex* + *E. coli*	3 (6.2%)
Blastocystis sp.+ *E. histolytica* complex* +*Giardia*	1 (2%)
Blastocystis sp.+ *E. histolytica* complex* +	1 (2%)
Total	49 (100%)

* *Entamoeba histolytica* complex = *E. histolytica/E. dispar/E. moshkovskii*

Our *in vitro* cultured samples results showed the growth of *Blastocystis* sp. in 60 (85.7%) stool isolates. The most detected forms of *Blastocystis* in culture media were the vacuolar and granular forms (Fig 1: C), while the cystic and amoeboid forms were present in a smaller proportion.

PCR amplification of the 310 bp fragment of the *SSU rRNA* gene was successfully obtained from 64/70 studied cases (91.4%) (Fig 2). Two samples only showed no amplification.

Comparative result analysis revealed that sensitivity and specificity identified by microscopy in comparison with PCR assay were 73.4% and 66.7%, respectively. However, the *in vitro* culture showed 90.6% sensitivity and 66.7% specificity when compared with PCR (Tables 3-4).

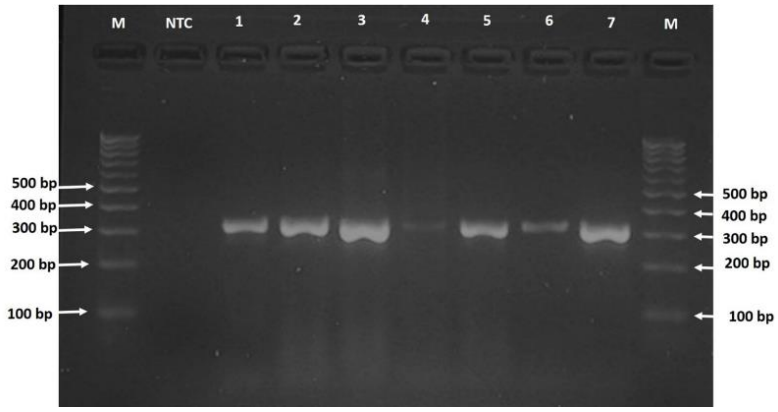

Figure 2. Ethidium Bromide-Stained 2% agarose gel electrophoresis of PCR products. Lanes M; molecular weight marker (100bp). Lanes 1-7 PCR Product; a single specific PCR fragment of 310 bp. NTC: negative control for contamination detection.

Table 3. Comparison between microscope and PCR assay in detection of Blastocystis sp.

Microscope	PCR		Total (%)
	Positive (%)	Negative (%)	
Positive	47 (67.1)	2 (2.9)	49 (70)
Negative	17 (24.3)	4 (5.7)	21 (30)
Total	64 (91.4)	6 (8.6)	70 (100)

Positive predictive value (PPV) = 95.9%

Negative predictive value (NPV) = 19.1%

Table 4. Comparison between *in vitro* cultures and PCR tool in detection of *Blastocystis* sp.

Culture	PCR		Total (%)
	Positive (%)	Negative (%)	
Positive	58 (82.9)	2 (2.9)	60 (85.7)
Negative	6 (8.6)	4 (5.7)	10 (14.3)
Total	64 (91.4)	6 (8.6)	70 (100)

Positive predictive value (PPV) = 96.7%
Negative predictive value (NPV) = 40%

Discussion

Blastocystis sp. is an enteric parasite found in animals and humans with a worldwide distribution (Menounos *et al*., 2008; Alfellani *et al*., 2013). Many studies have emphasized the importance of its pathogenic role (Stensvold and Clark 2016; Skotarczak 2018; Popruk *et al*., 2020). In Syria, *Blastocystis* sp. is considered part of the intestinal flora; it is not yet recognized as a pathogen and it is not specified usually, in the results of any laboratory analysis of the fecal sample. Hence, we aimed to focus on the different diagnostic methods and its sensitivity and specificity in the accurate diagnosis of *Blastocystis* sp.
Our findings showed notable difference in the detection of *Blastocystis* in stool isolates using the three diagnostic methods. The presence of this parasite was recorded in 70% of samples by microscopy, in 85.7% by *in vitro* culture and in 91.4% by conventional PCR.
Laboratory diagnosis of *Blastocystis* sp. can be challenging and the prevalence data can be influenced depending on the method of diagnosis, making choosing the accurate method an important task in diagnosing *Blastocystis* (Suli *et al*., 2018).
Blastocystis sp. is highly polymorphic it has variation in size and shape (Stensvold *et al*., 2007; Tan, 2008). Our microscopic data showed that the vacuolar and granular forms were mostly detected using microscopic examination. This finding is in agreement with previous results since these forms are easily distinguished from other protozoa (Zhang *et al*., 2007; Vassalos *et al*., 2010). On the contrary, other studies indicated the presence of vacuolar, granular, amoeboid and cyst forms of *Blastocystis* sp. in the microscopic detection (Coyle *et al*., 2012; Elghareeb *et al*., 2015). Thus, relying on using microscopic examination only in diagnosis is controversial and many studies underestimated it (Tan, 2008; Termmathurapoj *et al*., 2004).

Additionally, the low microscopic sensitivity recorded in our study may be according to some researches due to the Lugol's staining method which shows less sensitive than cultivation in Jones' medium (Padukone *et al*., 2018; Dogruman *et al*., 2009).

On the other hand, our data showed that *in vitro* culture failed to detect the parasite in 6 positively proved cases by PCR. This results may be explained either because they were disintegrated prior to culturing or for some conditions that affected its growth and hence detection in culture (Eida and Eida, 2008; Leelayoova *et al*., 2002).

Despite its high cost, PCR is considered as a gold standard detection assay with no time consuming, in comparison with *in vitro* culture method that is time consuming, yet microscopy detection needs experience but with low cost.

Our diagnosis results are consistent with previous studies that indicated that molecular assays and *in vitro* culture are superior over the direct microscopic examination in the detection of *Blastocystis* spp. from human stool isolates (Padukone *et al*., 2018; Rene *et al*., 2009; Stensvold, 2015). However, some studies suggested that *in vitro* culture is superior to direct PCR assay (Termmathurapoj *et al*., 2004; Santos and Rivera, 2013).

False-negative results using PCR were detected in two positively confirmed isolates by microscopy and culturing techniques. Even though, there is no clear explanation for such results, low concentration of DNA, the presence of PCR inhibitors in some specimens or degradation of parasite material during storage may be the cause (Eida and Eida, 2008; Parkar *et al*., 2007).

Remarkably, the majority of our samples (65.3%) showed the presence of *Blastocystis* sp. alone, while co-infection with other intestinal parasites was detected in 34.7%. This finding strongly indicates the importance of considering *Blastocystis* sp. in laboratory diagnosis. It also agrees with that our patients showed enormously different symptoms, making it hard to associate *Blastocystis* presence with specific gastrointestinal symptoms and emphasize the importance of recognizing it as a pathogen agent.

Conclusion

This study is the first in Syria to highlight the importance of *Blastocystis* sp. diagnosis using molecular method. We strongly recommend considering this parasite in laboratory reports, diagnosis, and treatment especially when no coinfection is present. The molecular methods are an excellent tool for the accurate detection and identification of *Blastocystis* sp. in stool samples. But if not feasible for a diagnostic laboratory, it is recommended to use at least

two different diagnostic techniques; such as microscopy in parallel with *in vitro* culture in Jones' medium for accurate diagnose of this parasite.

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