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Original paper

Comparison of diagnostic methods (wet mount, trichrome staining, formol-ether, PCR, and xenic *in vitro* culture) for the detection of *Blastocystis* in stool samples in Urmia educational hospitals, the Northwest of Iran

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ABSTRACT. *Blastocystis* spp. is known as a common intestinal protozoan parasite in human and animals. The parasite has a worldwide distribution and is frequently detected in faecal samples in clinical parasitology laboratories. The goal of the study was to compare the sensitivity and specificity of formol-ether technique (FECT), trichrome staining, xenic *in vitro* culture (XIVC), microscopy of faecal smears, and polymerase chain reaction (PCR) methods for detecting *Blastocystis* spp. in human stool samples. The prevalence of the parasite in the stool samples referred to educational hospitals was also determined. A total of 575 cases were assessed to detect the parasite. After collecting from patients referring to Urmia educational hospitals, the samples were examined by microscopy of faecal smears, trichrome staining, FECT, XIVC using Jones' medium, and PCR, to evaluate the presence of *Blastocystis* spp. Microscopy of faecal smears, trichrome staining, FECT, and PCR technique detected 94, 100, 96, and 44 positive cases, with the sensitivity of 71.3%, 74.4%, 74.4%, and 80.4% and the specificity of 99.6%, 99.1%, 100%, and 93.1%, respectively. XIVC method identified the highest number of positive cases (129 cases) among the other methods. Our findings indicates that XIVC technique is more sensitive method for the detection of *Blastocystis* spp. in human stool, as compared to direct smear, trichrome staining, FECT, and PCR methods.

Keywords: *Blastocystis* spp., xenic *in vitro* culture, PCR, formol-ether

Introduction

Blastocystosis is a disease caused by protozoan parasite, namely *Blastocystis*. This parasite can colonize in human or animal (such as birds, rodents, fishes, and domestic animals) gastrointestinal tract [1]. As one of the most frequent species of *Blastocystis*, *Blastocystis hominis* is isolated from the stool samples and infects humans. Various it's morphological forms include vacuolar, granules, and amoeboid [1–3]. Abdominal discomfort, diarrhea, anorexia, and flatulence are several symptoms described in patients infected with this parasite; however, some patients do not show any clinical symptoms [4]. According to small subunit ribosomal RNA-based analysis, 13 subtypes, numbered from ST1 to ST13, have been identified to date, but ST10–13 subspecies have not been detected in human samples so far [5,6]. Infection with *Blastocystis* has a global distribution, and the rate of the infection is high worldwide, particularly in less developed countries. The prevalence rate of *Blastocystis* in developed countries is estimated to be 0.5–23.1%, whereas in developing countries, this rate ranges 22.1–50.0% [7]. The prevalence of *B. hominis* in Iran has been reported to be about 3.0%. Emerging evidence has disclosed that the severity of infection has a direct link with the level of hygiene

culture, the extent of animal contact, and nutritional habits [6,8–10]. The relationship of *Blastocystis* infections with human diseases is a controversial issue; nonetheless, this organism is considered as a pathogenic parasite and the primary cause of enteritis in immunocompromised patients [11].

For the detection of the *Blastocystis* parasite in the stool samples, there are various techniques, including direct smear preparation of the samples, formol-ether technique (FECT), trichrome staining, xenic *in vitro* culture (XIVC) of the parasite, and polymerase chain reaction (PCR) [1,12–14]. However, each method has some limitations in terms of specificity and sensitivity, and there is always a probability of false-negative and false-positive results. Therefore, the selection of the most appropriate method for the determination of the parasite with high specificity and sensitivity and minimum false-negative or -positive results seems to be necessary. The current study aimed to compare different detection methods of *Blastocystis* spp. and also to evaluate the prevalence of this parasite in patients referred to the educational hospitals in Urmia city.

Materials and Methods

Sample collection

A total of 575 stool samples were collected from the patients referred to the educational hospitals in Urmia city. The samples with inadequate volume to perform different experiments were discarded, and those contaminated with the urine or other materials were eliminated due to the destruction of the trophozoite structure. All the samples were utilized immediately for the experiments, otherwise they were kept in a refrigerator (at 4–8°C).

Direct smear test

To detect the trophozoite form of *Blastocystis* spp., we prepared direct smears by utilizing Lugol's stain (Merck, Germany) and normal saline. The slides and suspicious cases were then evaluated by 10× and 40× microscopic objective lenses, respectively [15].

Formol-ether technique (FECT)

The test was performed following the preparation of a suspension by adding ~1 gr of the stool sample to 10 ml of formalin (10%; Merck). A funnel was put into a centrifugation tube, and four-layer gauze was placed inside the funnel. The suspension was passed through the gauze until 7 ml of the suspension was transferred to the tube. Next, 3 ml of ether (Merck) was added to the tube and shaked vigorously for 30 seconds and then centrifuged at 700×g for 5 minutes. After transferring on a glass slide, one drop of the pellet was assessed using 10× and 40× microscopic objective lenses, respectively [16].

Trichrome staining method

Wet slides of the specimens were prepared using an applicator. The slides were first placed in Schaudinn's fixative (Merck) for at least 30 minutes and then in ethyl alcohol 70% (Merck) for 5 minutes. In the next step, the slides were transferred to iodine alcohol solution (ethyl alcohol 70% containing anionic iodine) for 2–5 minutes and dehydrated in a 70% alcohol solution, two times (the first time for 5 minutes and the second time for 2–5 minutes). After transferring to trichrome stain (Merck) for 10 minutes, the slides were floated in acetic acid containing ethyl alcohol (0.5 % v/v; Merck) for three seconds and then for one second in absolute ethyl alcohol (Merck) and finally dehydrated two times in absolute ethyl alcohol and two times in xylenol (Merck) for 2–5 minutes [17].

Jones' medium preparation

At first, the chemical powders acquired from Merck, including Na_2HPO_4 (1.244 g), KH_2PO_4 (0.397 g), and NaCl (7.087 g), were prepared and dissolved in 131.25 ml, 43.75 ml, and 787.50 ml of distilled water, respectively. The final volume of the solution was 962.5 ml, which 15.2 ml was removed; the final volume reached 950 ml. A volume of 100 ml of the solution was mixed with the yeast extract (QUE Lab, Canada), and then the whole mixture was added to the stock solution (950 ml). The resultant solution was aliquoted into the tubes of 100-ml volume and autoclaved for 15 minutes. After cooling the solution temperature, 10 ml of inactivated horse serum was added to 90 ml of the sterile solution. All the media were kept in a refrigerator and warmed up to 37°C before the inoculation of the stool samples. For culturing, 5 ml of the culture media and 100 mg of the stool samples were utilized and incubated at 37°C for 72 hours. Thereafter, one drop of the sediments was examined under a 40× microscopic objective lens to identify *Blastocystis* spp. The negative tubes were incubated for a longer period (six days) and examined daily for the presence of the parasites [18].

Table 1. Frequency distribution of positive and negative cases using different detection methods of *B. hominis* in the stool samples

Detection methods	Positive cases n (%)		Negative cases n (%)		Total samples	95% CI for the prevalence of positive cases
Direct smear	94	16.3	481	83.6	575	0.17±3%
Formol-ether	96	16.69	479	83.3	575	0.17±3%
Trichrome staining	100	17.39	475	82.6	575	0.18±3%
In vitro culture	129	22.4	446	77.56	575	0.22±5%
PCR	44	22	156	78	200	0.23±3%

Polymerase chain reaction (*PCR*)

To perform PCR technique, we randomly selected 200 samples. The DNA of the parasite in the stool samples was extracted by the aid of a commercial extraction kit (Yekta Tajhiz Azema, Tehran, Iran) according to the manufacture's instruction. The forward and reverse primers, F: 5'-GGA GGT AGT GAC AAT AAA TC-3' and R: 5'-TGC TTT CGC ACT TGT TCA TC-3' [19], were designed respectively for the amplification of SSU rRNA gene of *Blastocystis*.

For each test, 12.5 µl of master mix (ama*R* one PCR^{TM}) and 1 µl of reverse primer (both were diluted 1:10 before use, 10 pmol), as well as 1 µl of forward primer, 0.12 µl of Taq DNA polymerase, 8.38 µl of double distilled water, and 2 µl of extracted DNA (the final volume of 25 µl) were added to a micro-tube. The experiment was performed under the following condition: hot star at 94°C for (one cycle), initial denaturation at 95°C, annealing at 58°C (30 cycles), extension at 72°C, all for one minute, and final extension at 72°C for 5 minutes (one cycle). The PCR product was loaded on 1% agarose gel and electrophoresed using Tris-Borate-EDTA (TBE) 1% buffer for 20 minutes [2,20].

Statistical analysis

In this study, XIVC method was considered as the gold standard, and other techniques were compared with this technique. The specificity, sensitivity, negative and positive predictive values, and accuracy of the detection methods were analyzed using, ANOVA, and student's t-test with the SPSS software ver16 [21].

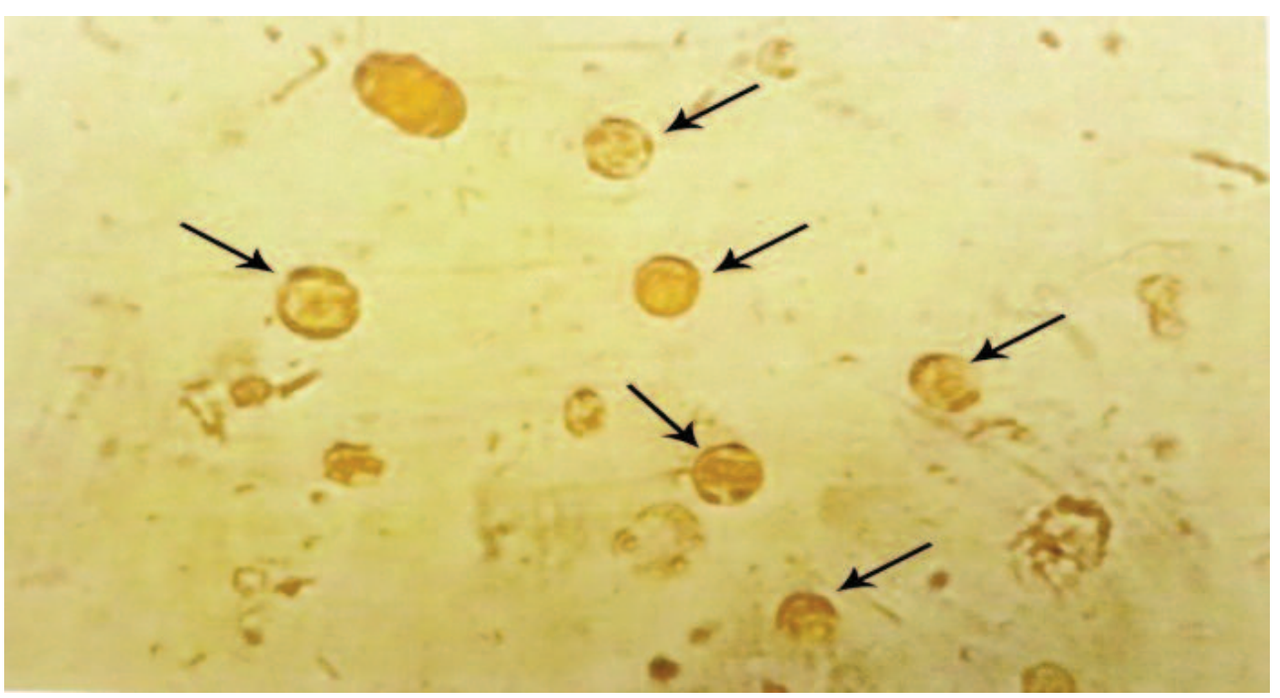

Figure 1. *Blastocystis* on direct smear test method (40×)

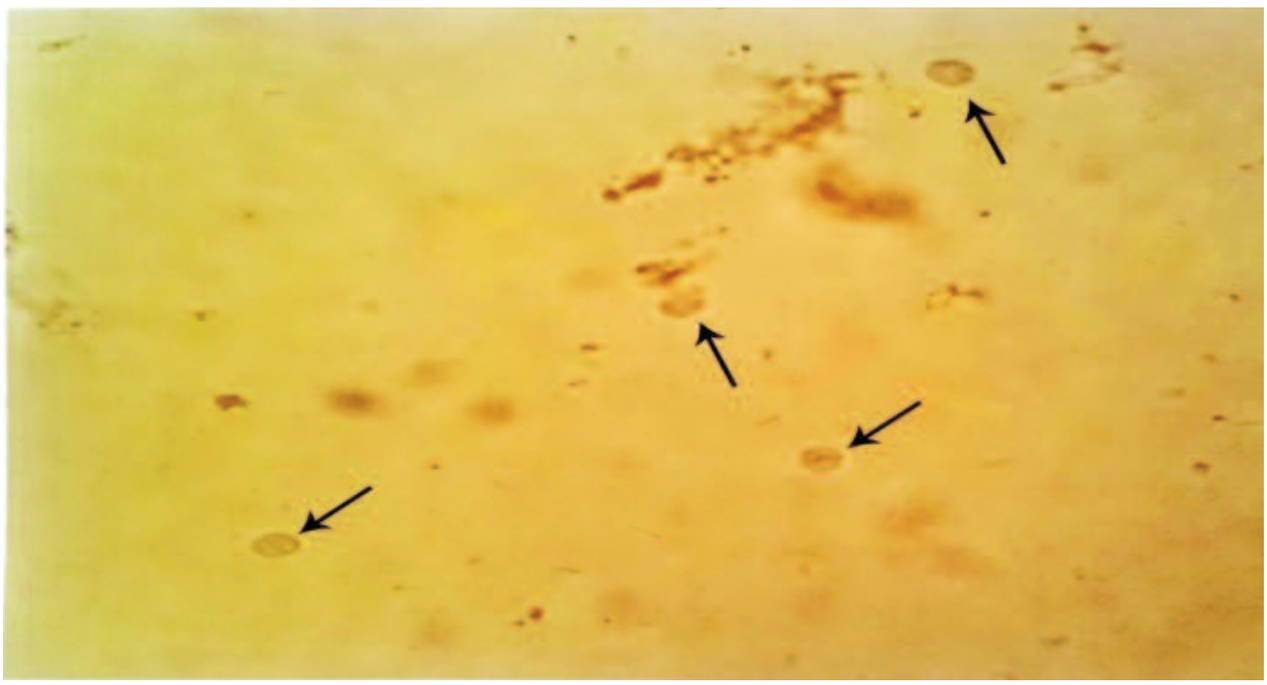

Figure 2. *Blastocystis* on FECT method (40×)

Ethical considerations

The study was approved by the Ethics Committee of the Urmia University of Medical Sciences, and all the experiments were performed in accordance with the ethical standards of this Committee (ethical code: IR.wumsu.rec.1395.31).

Results

Blastocystis frequency

A total of 575 stool samples were evaluated for the presence of *Blastocystis* spp. by direct smears, FECT, trichrome staining, and *in vitro* culture methods of the parasite. The frequency distribution of the parasite in Urmia city was estimated as 22.4%

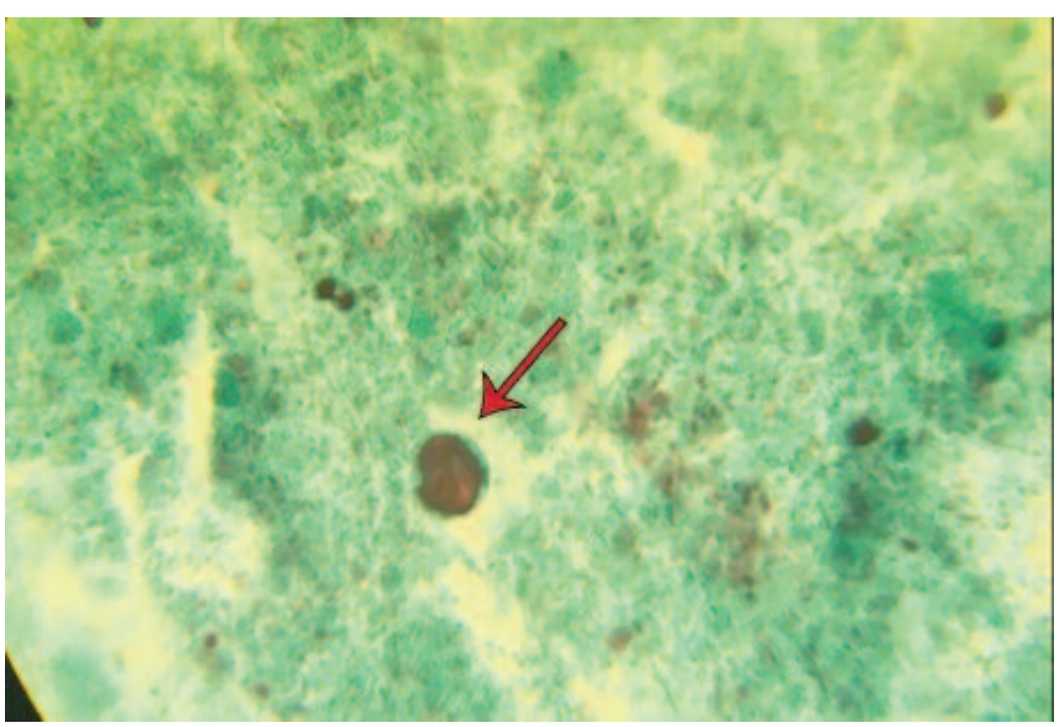

Figure 3. *Blastocystis* on trichrome staining method (100×)

Table 2. The comparison of sensitivity, specificity, positive/negative predictive value and accuracy of different detection methods

	Direct smear	Formol-ether	Trichrome	PCR
Sensitivity	71.3%	74.4%	74.4%	80%
Specificity	99.6%	100%	99.1%	93.1%
Positive predictive value	97.8%	100%	96%	75%
Negative predictive value	92.3%	93.1%	93.05%	94.8%
Accuracy	93.2%	94.2%	93.5%	90.5%

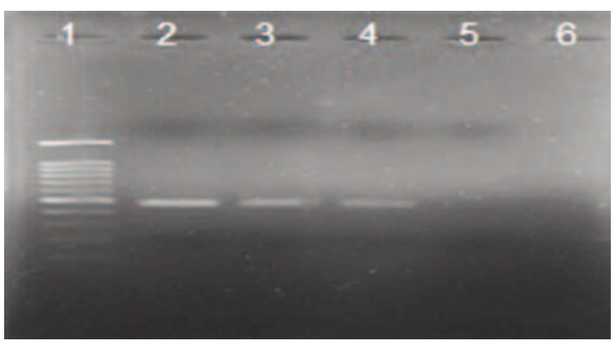

Figure 4. *Blastocystis* detection by PCR method. The first column was loaded with DNA ladder and column 2–4 were loaded with 3 positive cases. Column 5 and 6 were loaded with a negative case and negative control, respectively

using the XIVC technique. The distribution frequency of both positive and negative cases is summarized in table 1.

Microscopy of faecal smears

Among 575 stool samples examined by the direct smear test, only 94 positive cases (16.3%) were detected; however, no *Blastocystis* was observed among 481 samples (Fig. 1).

FECT

All the faecal samples were examined for the presence of *Blastocystis* spp., but only 96 positive cases (16.69%) were detected utilizing FECT (Fig. 2).

Trichrome staining method

Trichrome staining technique detected 100 positive cases (17.39%) among the total samples the number of cases identified by this method was higher than that detected by direct smear and FECT (Fig. 3).

PCR assay

In this study, 200 random samples were chosen and subjected to PCR assay. The test identified parasites in 44 out of 200 samples (22%), which was the highest number of positive cases compared to direct smear, FECT, and trichrome methods (Fig. 4).

XIVC

In the current study, culture method was used as the gold standard, and the samples were cultured in 5 ml of the Jones' medium. The highest number of positive cases (22.4%) was detected utilizing the XIVC method (Fig. 5).

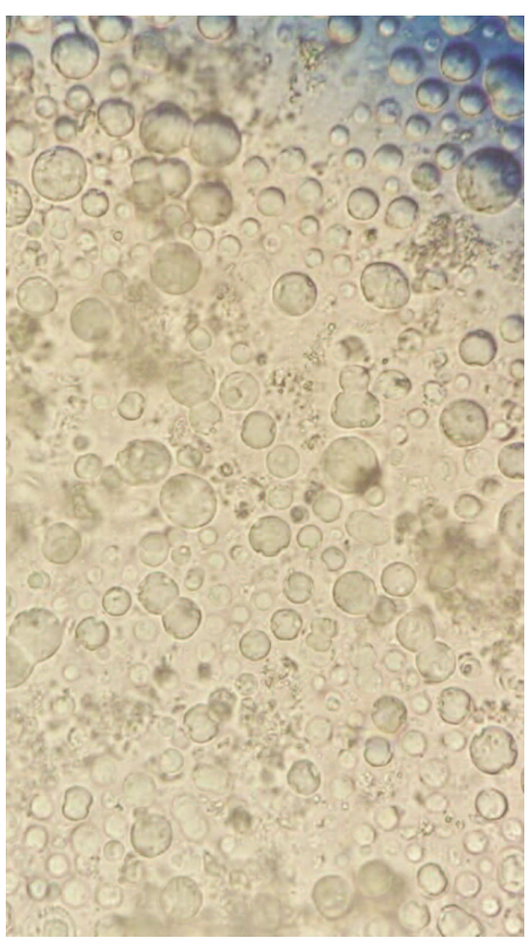

Figure 5. *Blastocystis* detection by XIVC method (40×)

Specificity and sensitivity of the methods

The specificity and sensitivity of different methods in comparison with the *in vitro* culture technique were examined in 575 stool samples. The obtained results are represented in table 2.

Discussion

Blastocystis spp. is a widespread protozoan parasite. For decades, scientists and researchers were in doubt about the pathogenicity of the parasite. Many people recover without any medication, while some others need treatment, depending on the severity of the clinical symptoms [22,23].

In spite of the high rate of *Blastocystis* infection in the Northwest of Iran, particularly in Urmia city, there are currently no reliable detection methods due to their limitations. Tappeh et al. [24] utilized direct smear and FECT and demonstrated the prevalence rate of 16.2% among the staffs of the rehabilitation centers. In the present study, XIVC technique displayed that the incidence of the parasite in Urmia city was 22.4%, which is much higher than the rates reported in Northeastern Iran by Salehi et al. [25] and in Tehran city of Iran by Barati et al. [26], who showed the prevalence rates of 8.1% and 7.6% for *Blastocystis hominis*, respectively. The reasons for such high infection rate in our study may be related to the zoonotic nature of the parasite and also the abundance of animal husbandry jobs in Urmia city. On the other hand, the consumption of raw vegetables is common in this region, which can be another contributing factor to the elevation of parasitic infection [27].

In clinical diagnostic laboratories, microscopy of faecal smears is frequently used to detect *Blastocystis* spp.. Although rapid results can be achieved by applying the direct smear method, this method has numerous limitations, and also false-negative results are unexpected [28]. In the current study, 94 positive cases (16.3%) were detected by applying the microscopy of faecal smears, and the sensitivity and specificity of the method were 71.3% and 99.6%, respectively. These results are in line with those published by Santos and Rivera [12]. They used *in vitro* culture as the gold standard and indicated that direct smear method had the least sensitivity, but the culture method could detect the most positive cases.

In our study, 96 positive cases (16.69%) were identified using FECT with the sensitivity and specificity of 74.4% and 100%, respectively. A rationale behind such low sensitivity could be the destruction of the parasite's structure, instability of ether, or using inappropriate volume of the samples. Elghareeb et al. [15] emphasized that the *in vitro* culture of the parasite had the most sensitivity among the other methods, including direct smear, iodine staining, FECT, trichrome, and *in vitro* culture methods, which affirms our results. They also reported 274 positive cases (22.8%) by applying the culture method.

According to the obtained results, 100 positive cases (17.39%) were detected by utilizing trichrome staining method. The sensitivity and specificity of the method were 74.4% and 99.1%, respectively. Our findings illustrated that trichrome staining had higher sensitivity compared to direct smear and FECT methods. Similarly, Mine and Rosa [29] exhibited that trichrome staining is a more appropriate method relative to direct smear technique.

Molecular methods such as PCR have been utilized extensively to detect microorganisms in various human samples. We employed PCR technique as a detection method whereby 44 positive cases were detected among 200 samples (22%). Our results disclosed that PCR has less sensitivity compared to the culture method. Possible explanations for such low sensitivity could be the quality of the DNA extraction kit and the primer sequences, as well as the presence of PCR inhibitors in faecal samples that affects PCR results. Owing to these shortcomings, some subtypes were not detectable with PCR. Yoshikawa et al. [30] evinced that using various commercial DNA extraction kits may lead to different results with multiple sensitivities. Also, a study by Roberts et al. [31] uncovered that applying varying primers could cause significant alterations in PCR sensitivity. Our results are in conformity with those reported by Eida and Eida [32] who introduced PCR technique as a less sensitive method compared to the culture method, for the detection of *Blastocystis* spp. However, the results of Stensvold et al. [33] survey were different from ours. By using PCR method against the FECT and culture techniques, they evaluated 43 clinical specimens. Their result proved 100% specificity and a significantly higher sensitivity of the applied method than the FECT. Accordingly, they recommended PCR method for screening clinical specimens of *B. hominis* infection

and for use in prevalence studies.

In the present work, a modified culture medium (Jones' medium) was applied. The parasite proliferated properly, and the highest number of positive cases (22.4%) were detected using the culture technique. Regarding the culture method, our results are similar to those reported by Zhang et al. [34] who indicated that the in vitro culture method had higher sensitivity and specificity compared to other methods. Our results also resembled to those published by Dogruman et al. [35] who explored that the culture method had the highest sensitivity among the other techniques in case of *Blastocystis* spp. detection. Furthermore, our findings confirm the results reported by Mohammad et al. [36], who observed that the culture technique could detect the highest number of positive cases. Of course, we should not forget that it is possible that *Blastocytis*-positive samples may be culture-negative because some subtypes are not likely grown in the culture. Another reason could be microbial and/or yeast contaminations [37]. Molecular methods have also revealed the inadequate sensitivity of traditional parasitological methods, such as XIVC (ranging from 52% to 79%) and microscopy of trichrome stained faecal smears [38,39]. Despite advantages, the cultivation method suffers from several drawbacks. Moreover, there are number of issues involved in the culture of protozoan parasites that make these procedures highly complex and are subjected them to many variables, some of which are known, and some other are still undefined. Certainly, protozoan parasites have complex life cycles and involves tremendous number of variables, including comprising parasite stage, parasite location in host body, host body temperature, parasite species and/or strain, and parasite-protective mechanisms [40].

In conclusion, based on the results achieved in our and other studies, for promoting the quality of the parasite detection and subsequently the treatment of the patients, it is preferable to use XIVC technique, as a reliable and sensitive method for the detection of *Blastocystis* spp.

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