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BLASTOCYSTIS SPP. INFECTION AMONG IBS PATIENTS: VARIOUS DIAGNOSTIC METHODS AND EPIDEMIOLOGICAL STUDY

By

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Abstract

This study determined *Blastocystis* spp. infection in patients with different classes of IBS. It also detected the sensitivity and specificity of microscopy, Jones' medium and PCR in detecting *Blastocystis* spp. in IBS patients' stool samples.

This cross sectional study was carried out during the period from December 2014 to November 2016. The 100 IBS patients were 57 males and 43 females. Stool samples were collected from IBS patients and were examined by direct wet mount, different stains, cultured on Jones' medium and PCR to detect *Blastocystis* spp. *Blastocystis* spp. infection was common in the age group (15-30) "41.5%" and in rural areas "80.5%".The percentage of *Blastocystis* spp. positive cases detected by direct smear, Giemsa stain, modified trichrome stain, culture on Jones' medium and PCR were 69%, 77%, 80%, 82% and 54% respectively. *Blastocystis* spp. infection was more prominent in IBS- C class "56.1%". *Blastocystis* spp. infection was either single or mixed with other infections.

The study showed that *Blastocystis* spp. infection was common in IBS patients especially IBS-C class. Modified trichrome stain and culture on Jones' medium were recommended to be the best methods used for its laboratory diagnosis.

Keywords: *Blastocystis* spp.; Irritable bowel syndrome, Direct smear; Modified trichrome stain; Jones' medium, PCR.

Introduction

Blastocystis spp. is one of the emerging parasites which characterized by being anaerobic, cosmopolitan, eukaryotic, and enteric protozoa inhabiting the intestinal tract of human (Nissapatorn *et al*, 2007; WHO, 2008).

Blastocystis spp. prevalence in humans often exceeds 5% in industrialized countries and can reach as high as 76% in developing countries; it also varies within communities of the same country (Tan, 2008; Souppart *et al*, 2009; Alfellani *et al*, 2013). This prevalence variation can be explained by poor hygienic measures, animal contact and consumption of polluted food or water (Lee *et al*, 2012; Nagel *et al*, 2012).

Ingestion of food or water contaminated with the cyst form of the parasite is the most acceptable pathway (Mungthin *et al*, 2001; Yoshikawa *et al*, 2004). The infective cysts could survive in chlorinated water at standard concentrations (Leelayoova *et al*, 2008).

Cystic, vacuolar, avacuolar, multivacuolar, granular and amoeboid forms of *Blastocystis* spp. have been recognized which have important implications on diagnosis (Stenzel and Boreham, 1996; Vdovenko, 2000; Leelayoova *et al*, 2008).

Pathogenesis was account to be due to interface between parasite products and enterocytes that influence host inflammatory and immunological responses (Boorom *et al*, 2008).

The most common complaint of blastocystosis in patients is severe abdominal ache accompanied by diarrhea or/ and constipation as well as anorexia, blood and mucus in stool (Zierdt, 1991).

Irritable bowel syndrome (IBS) is the most common functional gastrointestinal disorder in clinical practice, it is not a disease but it is a syndrome composed of a number of symptoms with similar manifestations (Kim and Camilleri, 2000; Akehurst and Kaltenthaler, 2001). No specific diagnostic measures identify IBS, because the essential pathophysiology is unknown, thus, the diagnosis remains reliant on symptoms and exclusion-of diseases (Singh *et al*, 2003).Rome com-

mittee for the classification of functional gastrointestinal (GIT) disorders has classified IBS on the basis of abdominal and bowel symptoms that occur with sufficient frequency in affected patients (Dorn *et al,* 2009). There were Rome II criteria (Thompson *et al,* 1999) and Rome III criteria (Longstreth *et al,* 2006) used to diagnosis.

Post-infectious IBS (PI-IBS) is known as the onset of IBS following a gastrointestinal infection (Thabane and Marshall, 2009). Several parasites as *Entamoeba histolytica, Giardia* spp. and *Blastocystis* spp. have been plamed as causative factors of IBS, though the relationship is less distinct (Spiller and Garsed, 2009; Wensaas *et al,* 2012) .

Blastocystis spp. have been linked to IBS and are responsible for the irritable form of the disease and considered as reemerged parasites in immune-compromised host (Graczyk *et al,* 2005). So this indicated that the stools of all patients presenting with IBS should be examined (Vogelberg *et al,* 2010).

The aim of this study was to find out the relationship between *Blastocystis* spp. infection and the various classes of irritable bowel syndrome: alternating pattern or pain-predominant (IBS-A), constipation-predominant (IBS-C) and (diarrhea-predominant (IBS-D), Also, to measure sensitivity and specificity of microscopy, culture on Jones' medium and PCR in detecting *Blastocystis spp.* in IBS patients' stool samples.

Subjects and Methods

Type of the study: The study was a cross-sectional study, and was conducted in the period from December 2014 to November 2016 in Department of Parasitology, Faculty of Medicine, Minia University, Egypt.

Source of stool samples suspected to be infected with *Blastocystis* spp.: Stool specimens of 100 IBS patients were collected and examined for the presence of *Blastocystis* spp. The patients were clinically diagnosed to suffer from IBS. They were 57 males and 43 females and their age was classified as > 15 years old; 15-30 years old and > 30 years old. They had been enrolled from inpatient wards and outpatient clinics of the Tropical Medicine Department of Mina University Hospitals, Egypt, from December 2014 to November 2016.

Patient`s related demographic and clinical data were recorded using a prepared questionnaire including; name, age, residence, onset of colonic symptoms, colic, constipation, diarrhea.

Samples were collected into dry clean sterile wide mouth plastic containers containing no preservatives with tight fitting lids and were directly transferred to the Parasitology Department, Faculty of Medicine, Minia University.

Ethical considerations: Verbal consent was obtained from all patients included. All procedures were conducted according to the ethical standards approved by the Institutional Human Ethics Committee, Faculty of Medicine, Minia University, Egypt.

Stool examination: Each stool sample was firstly examined macroscopically to detect the physical properties. Microscopical direct wet smear method was applied. All stool samples were permanently stained using Giemsa stain, Modified trichrome stain and Acid fast Kinyoun's stain.

Stool cultivation: A part of a non preserved stool samples, approximately 50 mg from each sample was cultivated in a sterile 7ml screw capped tube containing 5 ml Jones' medium supplemented with 10% horse serum (Jones, 1946) (Invitrogen, Groningen, Netherlands), 100 IU/ml penicillin and 100µg/ml streptomycin (Sigma-Aldrich, St. Louis, MO, USA). The inoculated medium was incubated at 37°C for 2-3 days. The cultures were screened for *Blastocystis* spp. by standard light microscopy every 12 hours and sub-cultured for an additional 2-3 days in fresh medium. (Saksirisampant *et al,* 2010).

PCR: Another part of the stool sample was preserved in distilled water in -80°C deep freeze to be used for DNA extraction and further PCR analysis.

DNA was extracted from all frozen stool

samples by using the QIAamp™ DNA Stool Minikit (Qiagen, Hilden, Germany) as per the manufacturer's instructions.

The extracted DNA was subjected to PCR amplification of SSU ribosomal DNA. A forward STS primer ('5-GGAATC CTC TTAGAG GGA CAC TATACA T-3') and reverse STS primer ('5-TTA CTA AAA TCC AAA GTG TTC ATC GGA C-3') was used in PCR amplification.

PCR amplification was performed by 35 cycles; each cycle consisted of denaturation at 94°C for 1 min, annealing at 60°C for 1 min and extension at 72°C for 1 min. PCR product was checked for positivity by running on 1.5% agarose gel electrophoresis and was visualized using ultraviolet trans-illumination after ethidium bromide staining for *Blastocystis* spp. DNA specific band at 310-base pair.

Statistical analysis: Data were presented as numbers and percentage using Statistical SPSS for Windows, issue 15.8. Statistical significance was determined using Z-tests (Man Whitney), Chi-square tests, and one-way analysis of variance. P value less than 0.05 was considered significant.

Results

In this study, culture on Jones' medium "82%" was the best method then the permanent stains (Modified trichrome stain "80%") and finally PCR "54%". Interestingly, Acid fast stain failed to detect *Blastocystis* spp. in any of the faecal samples (Fig. 1).

The product of PCR on agarose gel electrophoresis was visualized using ultraviolet trans-illuminator after ethidium bromide staining for *Blastocystis* spp. DNA specific band which was detected at 310 base pair (Fig. 3).

Fecal samples obtained from 57 IBS male patients and 43 IBS female patients were examined by direct smear, Giemsa stain, Modified trichrome stain, culture on Jones' medium and PCR for detection of *Blastocystis* spp. There was no significant difference between male and female patients in the percent of *Blastocystis* spp. positive faecal samples. Regarding the age of male and female IBS patients, although the two age groups (15-30 years and >30 years) showed the highest positive percent and the age group (< 15 years) showed the lowest positive percent, these results were statistically insignificant as shown in table (1).

Regarding the residence of IBS patients, 82 of them were from rural areas and 18 of them were from urban areas. Examination of their faecal samples by various techniques for detection of *Blastocystis* spp. revealed that the highest percent of positive faecal samples was from rural areas while the lowest percent of positive faecal samples was from urban areas with statistical significant (Tab.1).

The sensitivity, specificity, positive predicative value and negative predicative value of direct smear, Modified trichrome stain and PCR were estimated in comparison to culture on Jones' medium. Direct smear method showed the highest sensitivity 95.7%and PCR had the highest specificity 83.3% (Tab. 2).

In the current study, culture on Jones' medium succeeded in detection of *Blastocystis* spp. in 16 cases failed to be diagnosed by microscopy while microscopy detected *Blastocystis* spp. in 3 cases failed to be diagnosed by culture on Jones' medium (Tab. 3).

In the present study, PCR failed to detect *Blastocystis* spp. in 25 cases diagnosed by microscopy while microscopy failed to detect *Blastocystis* spp. in 10 cases diagnosed by PCR (Tab. 4).

In the current study, out of 82 *Blastocystis* spp. positive faecal samples detected by culture on Jones' medium, 51 of them were also diagnosed by PCR but 31 samples could not be diagnosed by PCR. On the other hand, out of 54 *Blastocystis* spp. positive faecal samples detected by PCR, only three were negative by culture on Jones' medium (Tab. 5).

The present study estimated percentage of positive fecal samples for *Blastocystis* spp. by culture on Jones' medium in the three

classes of IBS patients (class A, C & D). The parasite was more common in class C without significant differences (Tab. 6).

This study revealed that 16 patients were infected by *Blastocystis* spp. alone while 64 patients were suffering from mixed parasitic infections which include *Blastocystis* spp. with either *Cryptosporidium* spp., *Giardia lamblia* or *Entamoeba* spp. These data were obtained from examination of the fecal samples by modified trichrome stain (Fig. 4).

Table 1: Incidence of *Blastocystis* spp. among IBS patients and its relation to personal data by laboratory diagnostic methods

IBS patients / Personal data		No (%)+ve direct smear	P	No (%)+ve Giemsa stain	P	No (%)+ve modified trichrome stain	P	No (%)+ve culture on Jones' medium	P	No (%) +ve PCR	P
Male	57	41 (59.4%)	.09	44 (57.1%)	0.1	46 (57.3%)	0.08	47 (57.3%)	0.08	29 (53.7%)	0.56
Female	43	28 (40.6%)		33 (42.9%)		34 (42.5%)		35 (42.7%)		25 (46.3%)	
Total	100	69 (100%)		77 (100%)		80 (100%)		82 (100%)		54 (100%)	
Age											
< 15 years	18	10 (14.5%)	1.2	13 (16.9%)	.76	14 (17.5%)	.83	12 (14.6%)	0.17	10 (18.5%)	.08
15-30 years	41	32 (46.4%)		31 (40.2%)		32 (40%)		36 (43.9%)		27 (50%)	
>30 years	41	27 (39.1%)		33 (42.9%)		34 (42.5%)		34 (41.5%)		17 (31.5%)	
Total	100	69 (100%)		77 (100%)		80 (100%)		82 (100%)		54 (100%)	
Residence											
Urban	18	16 (23.2%)	.04	13 (16.9%)	.001	14 (17.5%)	.001	16 (19.5%)	.001	13 (24.1%)	.001
Rural	82	53 (76.8%)		64 (83.1%)		66 (82.5%)		66 (80.5%)		41 (75.9%)	
Total	100	69 (100%)		77 (100%)		80 (100%)		82 (100%)		54 (100%)	

P value less than 0.05 = significant.

Table 2: Sensitivity, specificity, positive and negative predictive values of direct smear, Modified trichrome stain and PCR in comparison with culture on Jones' medium for detection *Blastocystis* spp. in IBS patients

	Culture on Jones' medium			
	Sensitivity	Specificity	+ve predictive value	-ve predictive value
Direct smear	95.7%	48.4%	80.5%	83.3%
Modified Trichrome stain	95%	70%	92.9%	77.8%
PCR	62.1%	83.3%	94.4%	32.6%

Table 3: Comparison between direct microscopy and culture on Jones' medium to detect *Blastocystis* spp. in IBS patients

Direct microscopy	Culture on Jones' medium		P value
	Positive	Negative	
Positive	66 (80.5%)	3 (16.7%)	< 0.001*
Negative	16 (19.5%)	15 (83.3%)	
Total	82 (100%)	18 (100%)	

Table 4: Comparison between direct microscopy and PC in detection of *Blastocystis* spp. in IBS patients

Direct microscopy	PCR		Chi-square	P value
	Positive	Negative		
Positive	44 (81.5%)	25 (54.3%)	8.5	0.003
Negative	10 (18.5%)	21 (45.7%)		
Total	54 (100%)	46 (100%)		

P value less than 0.05 = significant.

Table 5: Comparison between culture on Jones' medium and PCR in detection of *Blastocystis* spp. in IBS patients

culture on Jones' medium	PCR		Chi-square	P value
	Positive	Negative		
Positive	51 (94.4%)	31 (67.4%)	12.3	<0.001*
Negative	3 (5.56%)	15 (32.6%)		
Total	54 (100%)	46 (100%)		

P value less than 0.05 = significant.

Table 6: Incidence of *Blastocystis* spp. infection in various IBS classes by culture on Jones' medium.

Class	No. of patients	No.(%) of +ve cases	Chi-square	P value
Class A	22	15 (18.3%)	4.344	0.114
Class C	52	46 (56.1%)		
Class D	26	21 (25.6%)		
Total	100	82 (100%)		

Discussion

Blastocystis spp. is an anaerobic unicellular eukaryote that can inhabit the intestinal tract of humans and many animals (Brumpt, 1912). This parasite plays a significant role in several chronic GIT illnesses such as IBS (Giacometti *et al*, 1999; Jones *et al*, 2009). IBS is an extremely widespread gastrointes-

tinal disorder characterized by abdominal pain with the diarrhea and/or constipation (Boorom *et al,* 2008). Post-infectious IBS (PI-IBS) is known as the beginning of IBS next to gastrointestinal infection, mainly infectious gastroenteritis (Rhodes and Wallace, 2006; Thabane and Marshall, 2009). The prevalence of PI-IBS following infectious gastroenteritis ranges from 4% to 31% (Thabane *et al,* 2007).

The present results revealed that culture on Jones' medium (82%) was the best method in diagnosis of *Blastocystis* spp. in IBS patients followed by microscopy (direct smear and different stains) then PCR (54%). This was in agreement with the results of many other studies (Barua *et al,* 2015; Elghareeb *et al,* 2015; Uobeed *et al,* 2015). In contrast to the result of this study, the results of studies done by Eida and Eida (2008), Das *et al,* (2016) and Mohamed *et al,* (2017) who reported that PCR assay had the highest percentage of *Blastocystis* spp. detection among IBS patients. Culture on Jones' medium was more accurate method for detection of *Blastocystis* spp. owing to that light infection with *Blastocystis* spp. is common and organisms can be simply missed by direct microscopy. It was also more sensitive than PCR and this could be due to two possibilities; the first of them is the disintegration of the DNA of the parasite during the storage process and the second is the inability of STS primer to detect some subtypes (majority of ST4, ST8 & ST9) of *Blastocystis* spp. (Stensvold, 2013).

The present study showed that the vacuolar form of *Blastocystis* spp. consisted of a large central vacuole occupied most of space, limiting the cytoplasm and other intracellular components to a thin peripheral rim and granular form structurally resembled the vacuolar form except in the presence of granules in central body and cytoplasm (Fig.2). The vacuolar form of *Blastocystis* spp. was the most common detectable form. This result agreed with Mehta *et al,* (2015) and Darabian *et al,* (2016). This result could be explained by the fact that vacuolar form of *Blastocystis* spp. is a diagnostic stage and easily distinguished from other protozoa.

The result of PCR product gave band of amplified *Blastocystis* spp. specific SSU ribosomal DNA of the expected size which was 310bp. A similar result of PCR product was obtained with Eida and Eida (2008).

In this study, the number of positive cases for *Blastocystis* spp. in IBS male patients was 47 (57.3%) and the number of positive cases for *Blastocystis* spp. in IBS female patients was 35 (42.7%). This result came in a harmony with the results of Al-Fellani *et al,* (2007) and Wang *et al,* (2002) who reported that males were more frequently infected with *Blastocystis* spp. than females. Roshandel (2006) found that females were more susceptible to *Blastocystis spp.* than males. This could be attributed to the fact that the Egyptian customs and traditions outdoor activities that made them more liable to infection than females.

The age group from 15-30 years old was the most affected age group by *Blastocystis* spp. infection 36 (43.9%). Similarly, a study done in Turkey concluded that *Blastocystis* spp. infection was common in young age (Dagci *et al,* 2014). But a second study carried out in Iraq found that the *Blastocystis* spp. Infection was more prominent in the old age group (Hammood *et al,* 2016). A third study performed in Brazil reported that the 10 year age group was most frequently affected by *Blastocystis* spp. (Nascimento and Moitinho, 2005). This result can be attributed to the more frequent exposure to sources of infections as young adults usually favor the junk food.

In the present study, *Blastocystis* spp. infection was common in rural areas 66 (80.5%) than in urban ones 16 (19.5%). This result agreed with Lee *et al.* (2012) in Nepal where *Blastocystis* spp. infections were higher in rural areas But in China, there was no difference in positive rates of *Blastocystis* spp. among patients in urban areas and rural ones

(Wang *et al,* 2002). This could be attributed to the lack of access to proper sanitary system and safe drinking water supply.

In this result the sensitivity and specificity of Jones' medium proved to be the standard for detection of *Blastocystis* spp. in IBS patients as compared with direct smear, Modified trichrome stain and PCR. These agreed with Meloni *et al,* (2011); Clark *et al,* (2013); Santos and Rivera, (2013) and Mohemmi *et al,* (2015) as they found that culture on Jones' medium was the best method for detection of *Blastocystis* spp. in human faecal samples.

In the present study, out of 82 positive samples by culture on Jones' medium, 66 (95.7%) positive samples were detected by direct smear microscopy and 16 (51.6%) samples were negative by direct smear. While culture on Jones' medium didn't detect 3 (4.3%) cases out of 69 positive cases detected by direct smear microscopy.

Barua *et al.* (2015) found that among 39 culture positive samples, 15 samples were positive and 24 samples were negative in direct smear microscopy. Stensvold *et al,* (2006) reported that out of 43 fecal samples 8 fecal samples were not detected by microscopy and 3 samples were negative by culture on Jones' medium.

This can be explained by the inability of direct smear microscopy to detect small number of the parasite, while culture on Jones' medium propagate the parasite number that make it easily to be detected.

In the present study, PCR failed to detect 25 (36.2%) cases diagnosed by microscopy while microscopy failed to detect ten (32.3%) cases diagnosed by PCR. In agreement with the present study, 14 (16.9%) microscopy negative stool specimens were positive by PCR (Eida and Eida, 2008). But PCR could amplify DNA in 37 (44.6%) stool samples from the patients with IBS, in two positive cases (2.4%) with microscopy; DNA was not amplified (Eida and Eida, 2008). This could be possibly due to either degradation of DNA during processing of the specimen or unsuccessful DNA extraction or *Blastocystis* spp. isolates were not amplified by the STS primer (Stensvold, 2013).

In this study, culture on Jones' medium failed to diagnose 3(16.7%) cases detected by PCR but PCR couldn't diagnose 31 (37.8%) cases detected by culture. Similar to the present study, culture on Jones' medium failed to detect *Blastocystis* spp. in 4 (4.8%) positive cases with PCR (Eida and Eida, 2008).

The cases that couldn't be detected by culture on Jones' medium may be due to disintegration of the parasite prior to culture or the probability of presence of some inappropriate chemical and/or physical conditions that affected parasite growth.

The present study revealed that (56.1%) of IBS patients who were infected by *Blastocystis* spp. belonged to IBS-C class, while (18.3%) belonged to class-A and (25.6%) to class-D. This may be attributed to the constipation as a common symptom in Egyptian population due to lack of healthy food. There was also another study in which IBS-C was the most frequent class detected among IBS patients with blastocystosis (64%) followed by IBS-D (24%) (Jimenez-Gonzalez *et al,* 2012). However, there were other studies which showed that *Blastocystis* spp. infection was more frequent in class IBS-D (Yakoob *et al,* 2010; Abaza *et al,* 2014).

This can be attributed to the fact that PI-IBS is considered as one of the major causes of the total burden of IBS in the community. Also, the type of the pathogen which causes the infection, its severity and duration of infection may play a role.

In the present study, *Blastocystis* spp. were found to be single infection in 16 (20%) cases and mixed infection with other parasites (as *Giardia lamblia, Cryptosporidium* spp. and *Entamoeba histolytica*) in 64(80%) cases. This agreed with Yakoob *et al.* (2010) which found that 53% of IBS patients had *Blastocystis* spp. infection mixed with other

parasitic infection.

In contradictory with this result studies conducted in Turkey, Australia and Jordan reported that single *Blastocystis* spp. infection was found in 76(80.9%), 35(74.5%) & 32(59.2%) respectively in IBS patients (Nimri and Meqdam, 2008; Roberts *et al*, 2011; Dagci *et al*, 2014). The higher percentage of mixed parasitosis in this study could be due to bad hygienic measures and the spread of infection in the environment that constituted predisposing factors for the chance of infection with multiple parasites.

Conclusion

This study showed that *Blastocystis* spp. are common intestinal parasitic infection in IBS patients which may be single or mixed other parasitic infection. *Balstocystis spp.* infection is more widespread in rural areas. Although the different diagnostic methods with exception of acid fast stain succeeded in its detection, it can be concluded that by direct microscopy, Modified trichrome stain was the most accurate stain for its laboratory diagnosis. Culture on Jones' medium was recommended as the best method for more frequent parasite detection in the clinical and laboratory fields.

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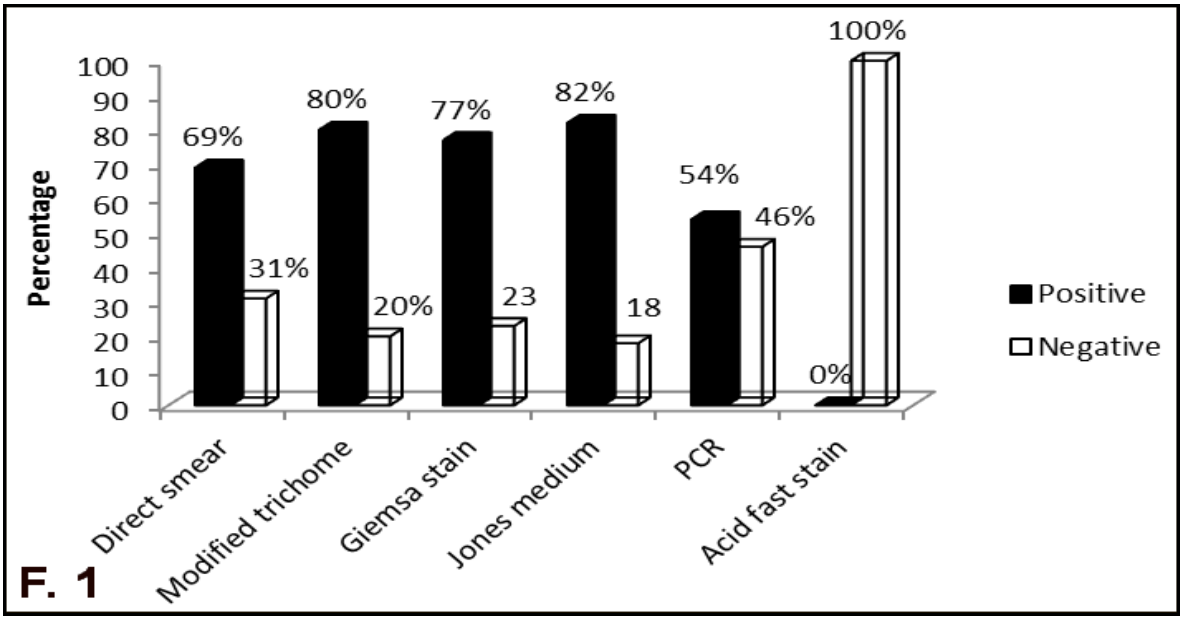
Explanation of figures

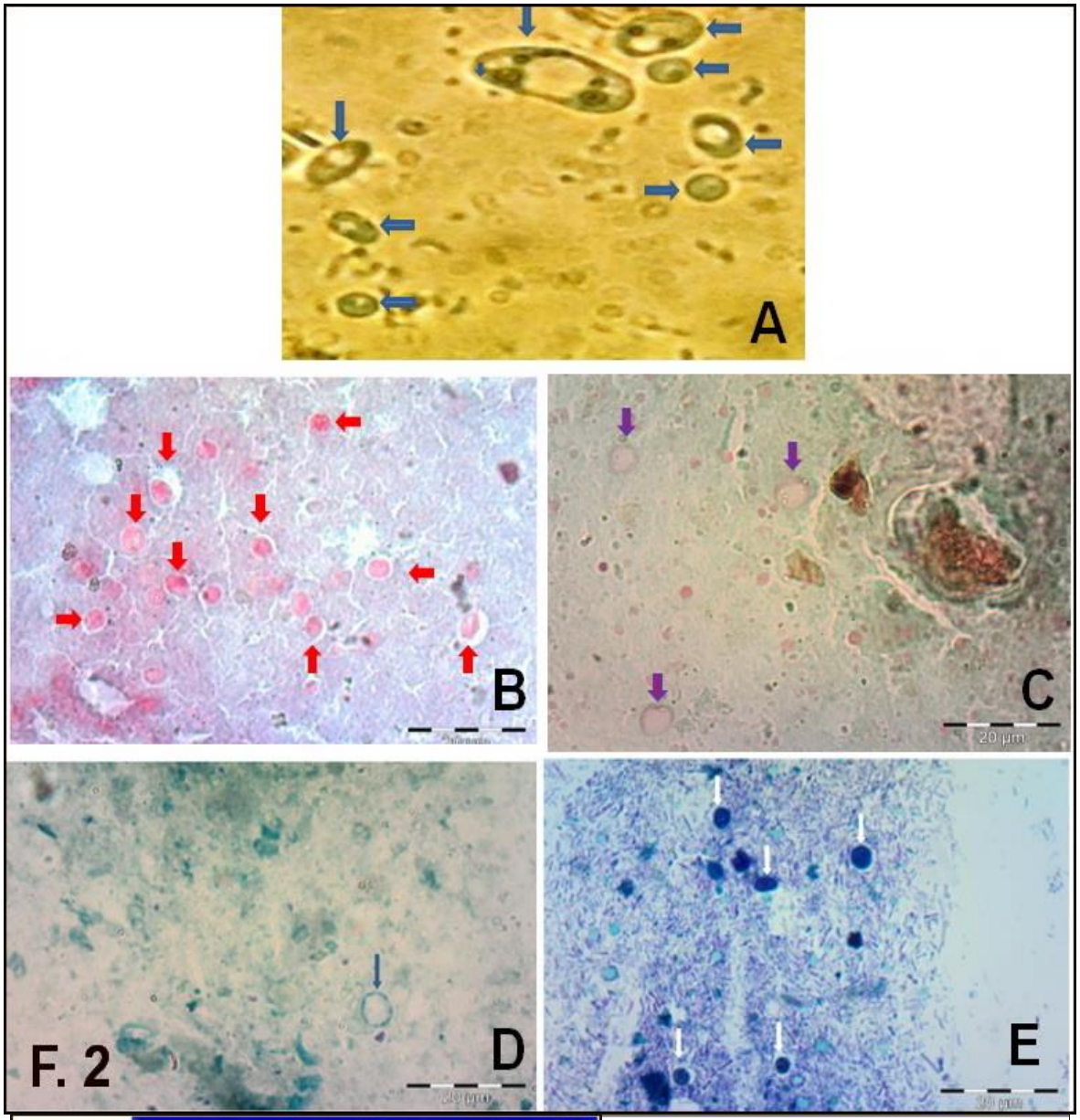
Fig. 1: Different diagnostic techniques used for detection of *Blastocystis* spp. infection in IBS patients

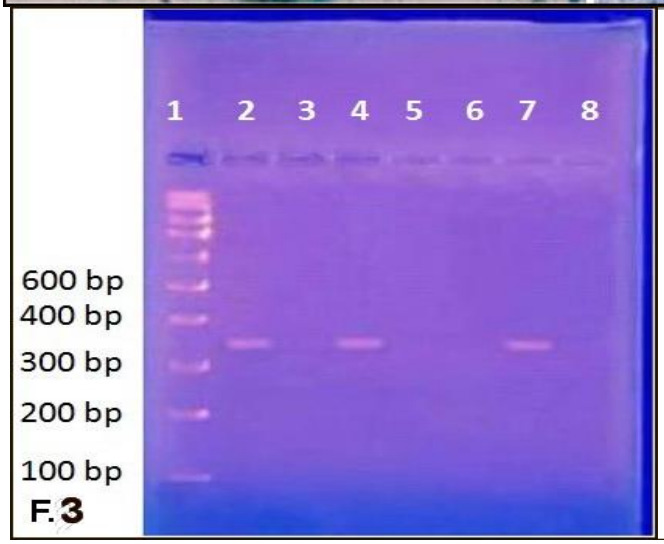
Fig 2: Light microscopy. A. direct saline faecal smear showed *Blastocystis* spp. as rounded cysts with central vacuole (vacuolar form "blue arrows"). B. Modified trichrome stained fecal smear showed (red arrows), vacuolar form with visible nuclei in peripheral cytoplasmic rim stained purple and a large central vacuole. C. Modified trichrome stained fecal smear showed (violet arrows) with granular and cystic forms. Granular forms showed multiple small granules stained purple. D. Giemsa stained fecal smear showed a large central vacuole (blue arrow). E. Giemsa stained faecal smear showed *Blastocystis* spp. granular forms with multiple small central granules *(white arrows).*

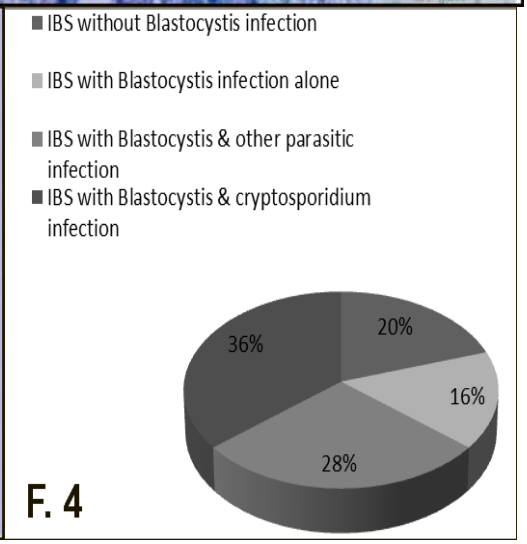
Fig 3: 1.5% agarose gel electrophoresis of PCR product, Lane1; 100 bp DNA ladder & lanes 2-8; PCR products of *Blastocystis spp.* specific SSU ribosomal DNA of representative fecal samples (2, 4 &7 positive PCR "a band of approximately 310 bp" and 3, 5, 6 & 8 negative PCR

Fig. 4: Mixed parasitic infections with *Blastocystis* spp. by using permanent stain (Modified-trichrome stain




A
B
C
20 µm
D
E
F. 2


1 2 3 4 5 6 7 8
600 bp
400 bp
300 bp
200 bp
100 bp
F.3


IBS without Blastocystis infection
IBS with Blastocystis infection alone
IBS with Blastocystis & other parasitic infection
IBS with Blastocystis & cryptosporidium infection
36%
20%
16%
28%
F. 4