

Original Research Article

<http://dx.doi.org/10.20546/ijcmas.2016.507.101>

***Blastocystis hominis* Detection among Gastrointestinal Disorders' Patients in Kirkuk Province Using Three Different Laboratory Methods**

**Abdulrazzaq Mohammed Hammood<sup>1</sup>, Batool Ali Ahmed<sup>2</sup> and Yahya Jirjees Salman<sup>3\*</sup>**

<sup>1</sup>College of Science/Kirkuk University, Iraq

<sup>2</sup>College of Health & Medical Technology/ Middle Technical University/ Baghdad, Iraq

<sup>3</sup>College of Dentistry/ Kirkuk University, Iraq

\*Corresponding author

**A B S T R A C T**

Blastocystis infection has been reported to be associated with irritable bowel syndrome (IBS), inflammatory bowel disease (IBD) and chronic diarrhea. The availability of data on the subtypes of *Blastocystis hominis* found in these patient groups would be of interest in understanding the significance of *Blastocystis* infection in chronic illness. This study is a trial to identify *Blastocystis* subtypes among patients with IBS, IBD, chronic diarrhea and a symptomatic individual in Kirkuk/ Iraq. The results revealed that diarrhea due to *Blastocystis* infection were highly recorded among patients with IBS and IBD aging from 20 to 29 years using direct microscopy examination. In-vitro culturing reveal 104 of *Blastocystis hominis* stages (only 80 samples) were pure and harvested for DNA extraction. Whereas Conventional PCR employee from stool culture reveal positive for *Blastocystis hominis* bearing genomic masses ranged from 486 to 512 bps. An attempt for detecting the parasite subtypes using subtype 1, 3, 5, 7 was performed, and the following results were recorded: high rate 25 % of 53 positive samples from total of 80 samples was belongs to subtype 3 followed by 18.75 % for subtype 1. While for each subtype 5 and 7 only 11.25 % were recorded, P>0.05. Subtypes 3 and 1 where dominant among elderly patients with IBS and IBD. In a symptomatic individual no intestinal parasites including *Blastocystis* and no an excess Candidiasis were recorded. Relationship between patients' gender and *Blastocystis* distribution among gastrointestinal disorder (GID) patients was not significant; controversy to patient's ages which show high frequencies of *Blastocystis hominis* among patients aging from 41 to 80 years. *Blastocystis hominis* mostly coexisted with *Giardia lamblia* among patients with IBS, whereas *Entamoeba histolytica* was highly found among patients with IBD than in IBS patients. Conclusions: Direct microscopy is useful method for screening of *Blastocystis hominis* in stool samples of patients with GID, while culturing of feces has had great role in confirming positive cases of direct microscopy, parasite harvesting for molecular studies and drug monitoring. Polymerase chain reaction (PCR) is more accurate and precise technique for detecting subtypes of *Blastocystis hominis*. Further studies should be carried on in the same province using the remaining subtypes for detecting real etiology for blastocystiasis among GID patients.

**Keywords**

*Blastocystis hominis*, GID, IBS, IBD, Subtypes, *Giardia* & *Entamoeba*.

**Article Info**

Accepted: 25 June 2016  
Available Online: 10 July 2016

## Introduction

Intestinal parasitic infections has had remarked impact on public health, particularly among peoples living under standard levels of sanitation and poor hygienic conditions (Alfellani *et al.*, 2013). *Blastocystis hominis* is the most common intestinal parasite in humans and many other animals (Windsor *et al.*, 2002). *Blastocystis*, an unusual anaerobic, single-celled stramenopile, is a remarkably successful intestinal parasite of a vast array of host species including humans (Stensvold, 2013). Infections with this organism are spread worldwide and it is often the most frequently isolated protozoan in parasitological surveys (Boorom *et al.*, 2008; Chandramathi *et al.*, 2010; Roldan *et al.*, 2009). In developing countries, *B. hominis* has higher prevalence (30 to 50 %) in comparison with developed countries (1.5 to 10 %) (Li *et al.*, 2007). The pathogenicity of *B. hominis* still has been debated. A report supporting the pathogenic potential of this parasite (Ok *et al.*, 1999); that can be found in patients with or without gastrointestinal symptoms. Some of the symptoms associated with *Blastocystis* infection include abdominal pain, constipation, diarrhea, alternating diarrhea and constipation and others (Qadri *et al.*, 1989). Irritable bowel syndrome (IBS) is a highly prevalent gastrointestinal disorder of unknown cause with the same above symptoms (Wilson *et al.*, 2004). Inflammatory bowel disease (IBD) is a disease of unknown cause associated with diarrhea and colonic lesions that are identified by endoscopy (Sands 2006) . However other studies state an opponent view point and it is believed that other factors probably are the causing agents of these symptoms (Kaya *et al.*, 2007; Hussein *et al.*, 2008). Morphologically the parasite has four phases: vacuolar, granular, amoebic

and cystic phase. The later phase has been considered a dominant phase found in environment (soil and water) so, it acts as vehicle for transmitting the parasite into the host. Human to human and human to animal transmission was not obvious (Yoshikawa *et al.*, 2004). Diagnosis of *B. hominis* overlap with other causatives of diarrhea specially the size of the cysts that measures 3 to 10  $\mu\text{m}$  which is close to oocysts of *Cyclospora*, *Entamoeba histolytica* and other protozoan parasites (Tan, 2004). Routinely direct microscopy by preparing of wet preparation of Lugol's iodine, fecal smear staining with trichrome stain can demonstrate *B. hominis* (Tan, 2008), but *Blastocystis* poses considerable challenges for the diagnostic laboratory. Firstly, the uncertain pathogenesis of the parasite discourages many clinicians from considering *Blastocystis* to be the etiological agent of disease. Secondly, the polymorphic nature of the organism in wet mounts can result in confusion with yeast, *Cyclospora* sp., or fat globules (Stenzel *et al.*, 1994). For these reasons alternative methods such as serology particularly ELISA using copro-antigen kit was invited to detect *B. hominis* in stool samples rather than direct microscopy (Stenzel *et al.*, 1997). Information about *B. hominis* in Iraq was not clear, except the study was carried out in south part of Baghdad by Raof and Abdul-Raham (2011) and in Kirkuk karyaghdi (2013) when they carried out diagnostic study on some intestinal parasites, via which *B. hominis* contributed 3.6 %. In 2015, Salman in Kirkuk city / Iraq who carried on a comparative study using direct microscopy and ELISA-copro antigen test for detecting *Blastocystis hominis* rate among peoples with irritable bowel syndrome, he found the all rate of Blastocystosis 58.22% This rate was contributed 59.44% and 58.99% for direct microscopy finding and ELISA testing. Statistically the differences between

two methods was not significant. Fecal Deoxyribonucleic acid (DNA) analysis by nucleic-acid based methods in particular has led to significant advances in *Blastocystis* diagnostics and research over the past few years enabling accurate identification of carriers and molecular characterization by high discriminatory power (Clark, 2013). Moreover, *Blastocystis* comprises a multitude of subtypes (STs) (arguably species) many of which have been identified only recently and molecular epidemiological studies have revealed a significant difference in the distribution of STs across host species and geographical regions (Stensvold, 2007). Having a cosmopolitan distribution, the parasite is a common laboratory finding in the stools of individuals with and without intestinal symptoms across the entire globe and while the parasite remains extremely difficult to eradicate and isolate in culture, appropriate molecular tools are now available to resolve important questions such as whether the clinical outcome of colonization is linked to ST and whether *Blastocystis* is transmitted zoonotically (Stensvold, 2013). *Blastocystis hominis* was not concerned and researched in Iraq obviously, only few studies were carried out in Baghdad and Kirkuk city (Raof and Abdul-Rahim in 2011, Karyaghdi, 2013 and Salman, 2015). So the current study was conducted to as the first molecular study in Kirkuk city-Iraq for detecting *Blastocystis hominis* among peoples with Irritable bowel syndrome (IBS) using conventional Polymerase Chain reaction (PCR) technique and to find the parasite subtypes altering the IBS.

## Materials and methods

### Study design

A cross-sectional study was performed from the 21 of December/ 2014 till the 21 of

December /2015 a total of 608 stool samples were collected from patients attending into gastroenterology and endoscopy unit in Azadi teaching Hospital, Private clinics and Ibn-Nafees private medical laboratory in Kirkuk city. They were diagnosed under the supervision of consultant physician. Samples were examined in Department of basic science labs, Faculty of Dentistry, Kirkuk University and Ibn-Nafees private medical laboratory. Those patients included males and females of different ages ranged from 1 year to over than 70 years. According to the physical examination; patients were suffering from irritable bowel syndrome (472) or gastroenteritis such as inflammatory bowel disease or other disorder gastric disease (59). For each patient special questionnaire was filled with complete information.

## Laboratory investigations

### Direct Stool Examination

### Macroscopic examination

It was done by naked eyes to determine consistency, color, odor and presence of blood or mucous.

### Microscopic examination

Direct wet mount smear, (WHO, 1991) Iodine-stained smear, (King, 1975) Formal-ether concentration technique (Cheesbrough, 1987) and Stool culture on Modified Boeck and Drbohlav's culture medium (Leelayoova *et al.*, 2002)

After one or two subcultures, *Blastocystis* suspensions were centrifuged at 12,000 g for 1 min. The resulting pellet was stored at -20°C until the DNA was extracted.

### DNA extraction from stool samples

According to The E.Z.N.A. ® Stool DNA Kit instructions from Omega bio kit company –German DNA from *Blastocystis hominis* was extracted

Total of 145 extracted DNA elutes were checked for purity using Thermo Scientific Nano DropTM 2000c spectrophotometer manual protocol, USA manufactured

### *Blastocystis hominis* DNA amplification and sequences

Only 80 *Blastocystis hominis* DNA extracts from a total of 145 samples on Locke-egg (LE) media were chosen according to purity range (1.6 -1.8) for DNA amplification. Briefly, the PCR were conducted in a volume 25 $\mu$ l and contained pure Taq (Bioneer- Korea) .Each containing 1.5 units of Taq DNA polymerase,10 mM Tris-HCl, pH 9,50 mM KCl,1.5 mM MgCl<sub>2</sub> , 200 $\mu$ M of each dNTP and stabilizers, including bovine albumin, 2 $\mu$ l of genomic extract and 0.5  $\mu$ M of the following primers:

Foreword: 5, GGA GGT AGT GAC AAT AAA TC-3, (Bohme)

Reverse: 5, TAA GAC TAC GAG GGT ATC TA-3, (Stensvold)

The PCR involved denaturation at 94 C° for 7 minutes ,35 cycles at 94 C° for 60 seconds,56C° for 45 seconds ,followed by a final extension at 72 C° for 7 minutes. Amplicons from 486 to 512 bps observed after electrophoresis on agarose gel were considered a positive result that proceeded for detecting *Blastocystis hominis* sub-types (1, 3, 5 and 7).

### Genotyping

To identify *B. hominis* genotypes, four pairs of Sequences-tagged site (STS) primers

[SB83, SB227, SB336, and SB155] were used with PCR technique (*Table 1*). PCR was performed as following: DNA of 80 isolates was amplified by specific primers. The reaction was carried out within 50  $\mu$ L final volumes including the following materials: 10  $\mu$ L DNA, 5 PG of each pair primers, 2.5 mM Cl<sub>2</sub>Mg, 5  $\mu$ L PCR buffer pH = 8.3, and 0.5 UI Taq DNA polymerase [Bioneer]. This combination was entered the cycle described as follows: denaturation at 94°C for three minutes, and 30 Cycles denaturation at 94°C for 30 sec, annealing at 56°C for 30 sec, extension at 72°C for 30sec, and final extension at 72°C for five min. The product of PCR was flowed on 1.5 % agarose gel containing ethidium bromide with 100 -1000 bps DNA ladder.

### Statistical Analysis

The statistical analysis was performed using statistical analysis system (SPSS); version 16. (SPSS Inc. Chicago IL. USA). Frequency and percentage were used with qualitative data. Z-test and Chi-square were used to compare frequencies.

### Results and Discussions

#### Direct Investigations

#### Demographical picture of the studied groups

Table 2 showed that the mean of patients' ages was [(36.09±16.36) and (34.14±15.96) years for Irritable Bowel Syndrome (IBS) and Inflammatory Bowel Diseases (IBD) respectively] while it was (31.66 ±14.21 years) for healthy control with non-significant differences between groups (P =0.111).

Furthermore, the males was noticed to be predominant among the studied groups:

healthy control group (HC) [2.86:1 males to females ration] so as for (IBS) and (IBD) patients [(1.58:1), (1.944:1) M: F ratio for IBS and IBD respectively] although there was no significant differences between these groups ( $P= 0.178$ ).

According to the main clinical features, and in comparison with healthy control group it was noticed that there were highly significant differences between the IBS and IBD groups in comparison with control group ( $P = 0.001$ ). Table 2 revealed that there was higher frequency of IBS patients 186(39.4%) were complaining from diarrhea and 131(27.8%) suffering from constipation whereas 140(29.7%) of IBS were noticed to had alternative cycle of both diarrhea and constipation. In comparison with disease control group (i.e. IBD group) frequent percentage of them 27(34.2%) were suffering from diarrhea while 22(27.8%) have constipation and comparable frequency 16(20.3%) have both diarrhea and constipation. Unusual 4(7.0%) healthy volunteers was observed suffering from diarrhea and 7(12.3%) suffering from constipation while only one (1.8%) was noticed to have alternative cycle of both diarrhea and constipation.

Interestingly, according to the mean of BMI, IBS patients were almost obese ( $26.1919\pm3.63\text{Kg/m}^2$ ), while IBD were overweight ( $25.58\pm7.12\text{Kg/m}^2$ ) while the healthy control individuals were within the normal weight ( $24.56\pm3.40\text{ Kg/m}^2$ ).

#### **The distribution of the studied groups according to age groups**

The distribution of patients according to age groups for both spouses under study summarized in (Table 3).

It was found that the majority of IBS patients 226(47.9%) were at age group of (20-40) years. It is clear from this table that this age group form the highest constituents of the studied groups not for IBS but also for IBD and HC groups [36(45.6%), and 29(50.9%) for IBD and HC groups respectively]. However, there was no significant differences between the different age groups ( $P= 0.711$ )

#### **Determination the etiological agents for IBS**

In addition to *Blastocystis hominis* there were other pathogens participate in triggering of IBS and IBD such as bacteria, *Giardia lamblia* and *Entamoeba histolytica* beside fungal infections particularly *Candida albicans*. Table 4.3 showed the distribution of IBS and IBD patients according to the laboratory findings. This table revealed that *Blastocystis hominis* was form the second etiological agent among IBS patients (33.7%) and IBD (41.8%) while the other etiological agents involved (62.1%) of IBS cases and (25.3%) of IBD cases.

On the other hand, in addition to *Blastocystis hominis*, the other mentioned causative agents particularly bacteria is the most etiological agent among IBS patients (26.1%). Fungi, particularly *Candida albicans* was noticed in (7.5%) of IBS cases as a sole agent and in (1.5%) in conjugation with bacteria whereas no case was recorded among HC with bacterial infection as a causative agent for diarrhea. On the contrary, *Blastocystis hominis* shared lab-finding among healthy control group. The results of this investigation showed highly significant differences in comparison with the studied groups.

### ***Blastocystis hominis* PCR results**

Table 5 was exerting purity mean of extracted *Blastocystis* DNA from 104 positive for *Blastocystis hominis* as  $1.98 \pm 0.178$ . Also showing positive bands to *Blastocystis hominis* parasite were extended from 486 to 512 bps  $\pm 1.56$ . While figure 1 exerting mean genomic mass  $505 \pm 3.25$  bps using the aid of electrophoresis.

From 104 positive stool samples only 80 samples were harvested on Locke-egg (LE) media, only 80 samples were retested by conventional PCR technique for getting *Blastocystis hominis* genome sequences, for this purpose only the following subtypes (1,3,5 and 7) were amplified. The result was showed high rate of sub-type 3 as 25% followed by 18.75% for sub-type 1. On the other hand the rate 11.25 % was recorded for each of sub-type 5 and 7, table 6.

Table 7 was clarifying the relationship between *Blastocystis hominis* subtypes distributions in regard of patients' age. Via which statistical analysis reveal no differences between parasite distribution genome sequences and gender although the rates of all subtypes in samples from females were higher than in male samples particularly the subtypes 3 , $P < 0.05$ .

Considering IBS patients age and frequencies of *Blastocystis hominis* subtypes, patients in age group from 61 to 80 years, there samples show high frequency of subtypes than other age groups. The rate was 36.25% distributed in 29 samples,  $p < 0.05$ . While patients aging from 41 to 60 years were comes in second category as 23.75% was recorded in 19 patients. In spite of variances in subtypes sequences frequencies between males and females in relation to patients ages, but statistically the differences was not significant.

Despite of the intestinal parasites acts vital role in causing diarrhea for any community, but other external factors such as poverty, famines, refuges and internally displaced peoples also had role in diarrhea etiology. For these reasons the demand for applying accurate laboratory method for detecting intestinal parasites including *Blastocystis hominis* was required to assess blastocystiasis as one causes of diarrhea , gastro-intestinal distress particularly irritable bowel syndrome and bowel inflammatory diseases . *Blastocystis hominis* (B. hominis) is a parasite of uncertain role in human disease particularly in diagnosis of irritable bowel syndrome. It may be identified during a workup for gastrointestinal symptoms, usually in stools (Ustun and Tugay, 2006). Also this parasite is not widely studied or tacked in consider by the physician or by laboratory technician in Kirkuk city, so this is the first study that carried out in this Province on patients suffering from diarrhea and those diagnosed as IBS. The overall rate of *B. hominis* 13.5 % was high, this rate was contributing 12.74 % among IBS compare to 0.76% among patients with IBD. These finding were reflecting poor hygienic condition, low level of sanitation and high degree of environmental contamination with the cystic stage of this parasite (Leelayoova *et al.*, 2008). Another explanation to this high rate might be attributed to period in which the study was carried on from 21th of December 2104 to 21th December of 2015, via which Iraqi peoples suffered from war that most of peoples in Tikrit, Mosul and Ramadi Provinces were migrate from their place to Kirkuk city as displaced peoples. Migrated peoples habitat in camps or on street without any bases such as healthy water, lack of food, electricity and etc., all of these factors can explain the rail cause to this high rate of *B. hominis* infection. The overall rate in current study was not in agreement with those recorded in Kirkuk by

Karyaghdi (2013) who recorded 3.6 % and with that carried on in a Turkish university hospital by Ozçakir *et al.* (2007); who found 12.2% of *B. hominis*. Moreover the rates of infection in neighboring and in some Arab countries were higher than that recorded in current study; as in Turkey Culha and Ozar (2008) found 19.8% in rural area of Antakya. While in Hamedan west of Iran, Taherkhani *et al.* (2008) were reported 21% of *B. hominis*. In Naples southern of Italy, 52.7 % of *B. hominis* was recorded among immigrants (Gualdieri *et al.*, 2011). Variance in the rates might be due to size of samples, habit of food and water consumption or to types of laboratory methods usages particularly double methods employ in current study.

The etiological agents of gastro-intestinal disorders in current study involve 4 microorganisms as *B. hominis*, *Giardia lamblia*, *Entamoeba histolytica* and an excess colonization by *Candida* species .Collectively they contribute 65.3% among IBS followed by 24.2 % among IBD and 10.5 % among health control. The rates of *B. hominis* and *Giardia lamblia* were high among IBS patients, the following rates 12.4% and 5.3 % were recorded respectively .Whereas in IBD the rate of 1.7 % was found for both parasites equally. On the other hand 6.7% of *Entamoeba histolytica* was recorded among IBD compare to the 0.2 % among IBS .This variation in the rates might be attributed to synergism or commensalism between *Giardia* and *Blastocystis* as both of them habitat the same region in small intestine (Tan, 2004) .While *Entamoeba histolytica* habitat large intestine and it has invasive pattern; these finding was highlighting that *Entamoeba histolytica* can be taken in consider among IBD. Controversy to *B. hominis*, *Giardia lamblia* which plays role among IBS. The most common approaches for the detection of

*Blastocystis* sp. [Stenzel and Boreham, 1997; Stensvold *et al.* 2007a; Tan, 2008] consist of direct smear examination by light microscopic or xenic in vitro culture. However, given the occurrence of different forms of *Blastocystis* sp. (especially the hardly recognizable cystic form), deterioration caused by environmental conditions or drug treatment and the fact that *Blastocystis* sp. can be confused with other microorganisms, this method seems to have largely underestimated this parasite in the context of enteric parasite diagnosis.

Moreover, culturing this parasite is time consuming and can bias subsequent genotyping due to the different ability of isolates to grow in selective medium [Roberts *et al.*, 2011]. Therefore, to overcome these limitations, several molecular polymerase chain reaction (PCR)-based diagnostic approaches using feces directly or after culture of fecal specimens have been described [Santin *et al.*, 2011; Yoshikawa *et al.*, 2004; Scicluna *et al.*, 2006 Roberts *et al.*, 2011]. Studies comparing the relative performances of these various diagnostic methods [Suresh and Smith, 2004; Parkar *et al.*, 2007; Stensvold *et al.*, 2007a] showed that the PCR approach was as sensitive as the culture approach. More recently, Poirier and colleagues reported a highly sensitive real-time quantitative PCR (qPCR) assay developed to detect *Blastocystis* sp. in stool samples [Poirier *et al.*, 2011]. Therefore conventional PCR technique was applied for detecting *Blastocystis* in current study and sequences of *Blastocystis* DNA extracts from stool samples were checked to positive cases by using culture and direct microscopy in order to obtain the parasite subtypes. High rate 25 % of positive cases were with subtype -3 this finding referring to specific human subtype and pointing to probability of human to human transmission in Kirkuk

community. This finding was compatible with that recorded by Yoshikawa *et al.*, 2004) who refer to the following records: 92.5% in Bangladesh, 70 % in Japan and 52

% in China. Also agreed with that recorded in Egypt by Abaza *et al.*, 2014 whom they found this subtype in feces of patients with IBS as 56.1%.

**Table.1** Primer sequences used in the study.

Primer sub-types	Product size, bps	Sequences of Forward (F) and Reverse (R) primers(5'-3')	Gene bank Acc.NO
SB 83 Sub1	351	F:GAAGGACTCTCTGACGATGA R:GTCCAAATGAAAGGCAGC	AF166086
SB 227 Sub III	526	F:TAGGATTGGTGTGGAGA R:TTAGAACAGTGAAGGAGATGGAAG	AF166088
SB336 Sub V	317	F:GTGGGTAGAGGAAGGAAAACA R:AGAACAAAGTCGATGAAGTGAGAT	AY048751
SB155 Sub VII	650	F:ATCAGCCTACAATCTCCTC R:ATCGCCACTTCTCCAAT	AF166087

**Table.2** Demographical picture for the studied groups

Parameters	Patients Group IBS	Disease Control IBD	Healthy Control	P value Pearson Chi-Square
Mean of Age ± SD	36.09±16.36	34.14±15.96	31.66 ±14.21	P=0.111 (NS)
Mean of BMI ± SD (Kg/m <sup>2</sup> )	26.1919±3.63	25.58±7.12	24.56±3.40	P=0.001 (HS)
M:F ratio	289:183=1.58	43:36=1.944	40:17=2.86	P=0.178 (NS)
<b>Clinical Features:</b> No (%)				
1. Diarrhea	186 (39.4%)	27 (34.2%)	4 (7%)	P= 0.001 (HS)
2. Constipation	131 (27.8%)	22 (27.8%)	7 (12.3%)	
3. Alternative Diarrhea & Constipation	140 (29.7%)	16 (20.3%)	1(1.8%)	
4. Normal	15 (3.2%)	14 (17.7%)	45(78.9%)	
<b>Lab-Findings:</b> No (%)				
1. Nil.	20(4.2%)	26(32.9%)	50(87.7%)	P= 0.001 (HS)
2. <i>Blastocystis hominis</i>	159(33.7%)	33(41.8%)	7(12.3%)	
3. Others	293(62.1%)	20(25.3%)	0(0.0%)	
Total	472	79	57	608

**Table.3** Distribution of the studied groups according to age groups

Age groups (years)	Studied Groups			Pearson Chi-Square
	HC	IBD	IBS	
(<20)	11	16	72	P= 0.711 (NS)
	19.3%	20.3%	15.3%	
(20-40 )	29	36	226	
	50.9%	45.6%	47.9%	
(41-60)	15	23	136	
	26.3%	29.1%	28.8%	
( 61-80	2	4	38	
	3.5%	5.1%	8.1%	
Total	57	79	472	590
	100.0%	100.0%	100.0%	100.0%

**Table.4** Distribution of the studied groups according to the causative agents

Laboratory Findings No.(%)	Studied Groups			Total	Pearson Chi-Square
	HC	IBD	IBS		
Nil	50(87.7)	26 (32.9)	20 (4.2)	96 (15.79)	P value = 0.001 (HS)
<i>Blastocystis hominis</i>	7 (12.3)	33 (41.8)	159 (33.7)	199 (32.73)	
Others	0(0.0)	20(25.3)	293 (62.1)	313(51.48)	
Total	57 (100.0)	79 (100.0)	472 (100.0)	608 (100.00)	

**Table.5** *Blastocystis hominis* DNA purity assessment by Nonono-drop2000 spectrophotometry using different two wave lengths with mean absorbance

Purity assessment levels	Mean ± S.E
230/260 nm	1.898 ± 0.157
260/280 nm	2.08 ± 0.223
Mean of 230/280	1.98 ± 0.178
Mean genomic mass(bps)	505 ± 3.25

**Table.6** Frequency of different genotypes of 80 *Blastocystis hominis* isolates

Genotypes	No. positive	Percentages Positive	P-value
1 SB 83(Sub-type,1)	15	18.75	
3 SB 227(Sub-type,3)	20	25.00	
5 SB 336(Sub-type,5)	9	11.25	
7 SB 155(sub-type,7)	9	11.25	
Total	53	66.25	0.633( NS)

\*NS= P>0.05

**Table.7** Frequencies of *Blastocystis hominis* sub-types (1, 3, 5, 7) and total rate among 80 positive cases by direct microscopy in relation to genders.

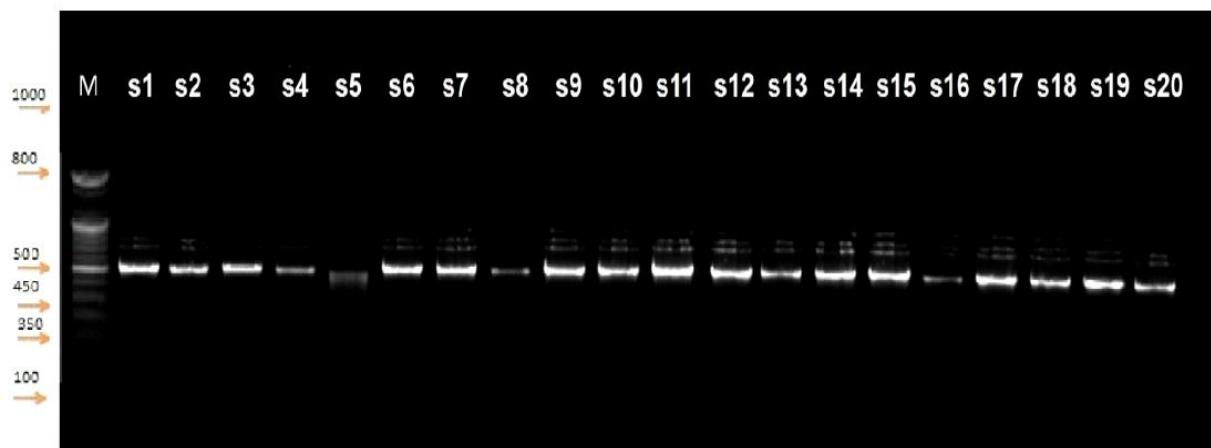
Genders	Exam No. *	<i>Blastocystis hominis</i> sub-types					Total
		Sub-type 1	Sub-type-3	Sub type-5	Sub-type-7		
		Positive No. (%)	Positive No. (%)	Positive No. (%)	Positive No. (%)		
Males	46	9 (19.59)	9 (19.59)	4 (8.69)	4 (8.69)	26 (56.52)	
Females	36	6 (17.64)	11 (33.33)	5 (14.70)	5 (14.70)	25 (69.44)	
Total	80	15 (18.75)	20 (25.00)	9 (11.25)	9 (11.25)	53 (66.25)	
Obtained P-values		0.708	0.609	0.435	0.435	0.633	

\*positive for direct microscopy. P>0.05

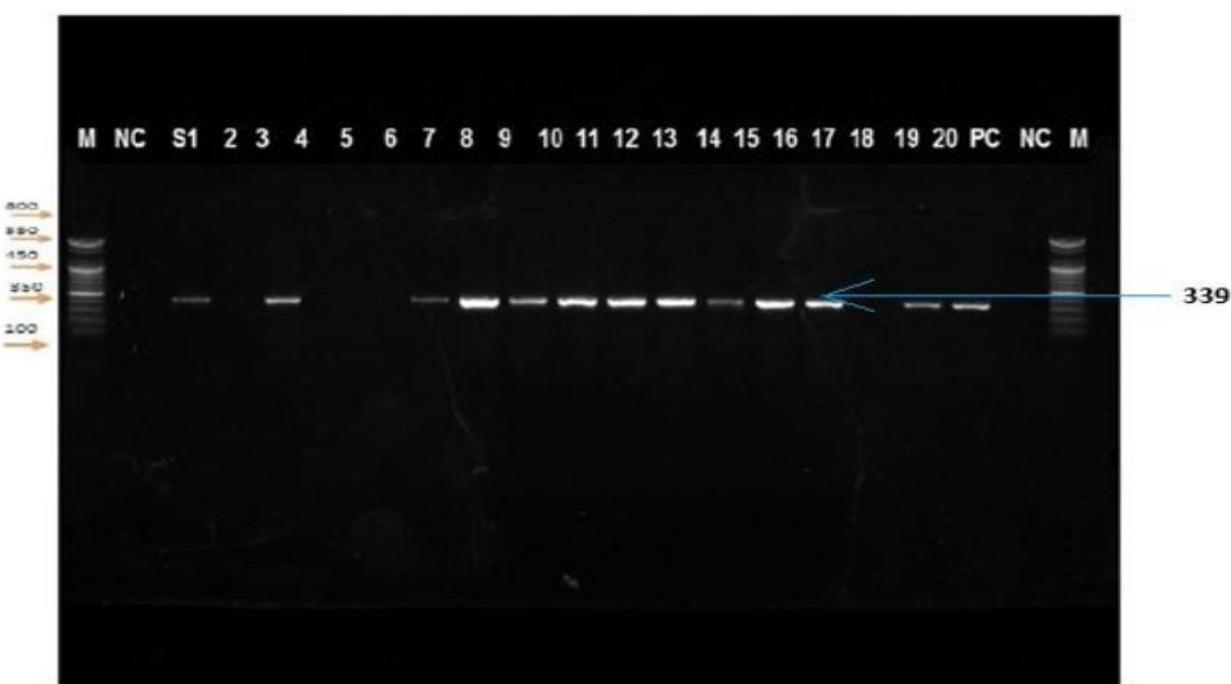
**Table.8** Distribution of *Blastocystis hominis* according to sub-types (1, 3, 5, and 7) in relation to patient's ages.

Genders	Males				Females				
Sub-types	SB 83	SB 277	SB 336	SB 155	SB 83	SB 227	SB 336	SB 155	Total
Age groups/year	No.%	No.%	No.%	No.%	No.%	No.%	No.%	No.%	No. %
< 20	1(1.25)	1(1.25)	0(0.0%)	0(0.0)	0 (0.0)	0(0.0)	0(0.0)	0(0.0)	2 2.50
21 - 40	0 (0.0)	0 (0.0)	1 (0.0)	0 (0.0)	0(0.0)	1(1.25)	1(1.25)	0(0.0)	3 3.75
41-60	5(6.25)	4(5.00)	2 (2.5)	0 (0.0)	1(1.25)	3(3.75)	0(0.0)	4(5.0)	19 23.75
61-80	3(3.75)	5 (6.25)	4 (5.00)	2 (2.50)	5(6.25)	9(11.25)	3 (3.75)	0(0)	29 36.25
Total	9(11.25)	10(12.5)	7(8.75)	2(2.5)	6(7.5)	10(12.5)	4(5.0)	5(6.25)	53 66.25

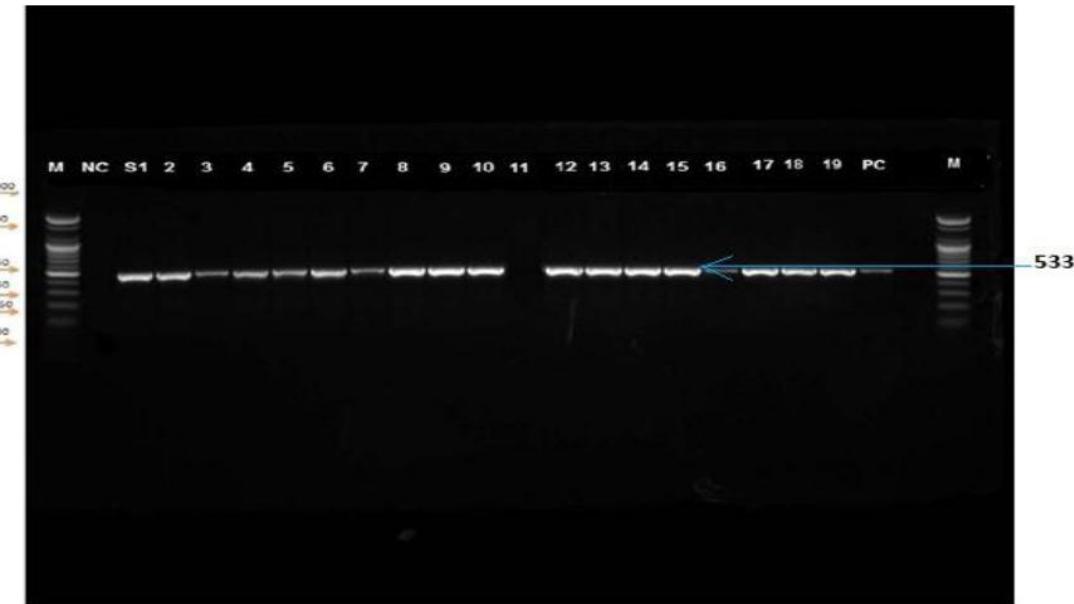
**Fig.1** *Blastocystis hominis* DNA extracts from 20 sample on Locke-egg (LE) media amplified by using electrophoresis showing positive bands compare to standard DNA marker in the first lane (from 100 to 1000 bps) on 1.5% of agarose gel; showing positive bands to *Blastocystis hominis* parasite were extended from 486 to 512 bps  $\pm$  1.56 with mean of  $505 \pm 3.25$ .



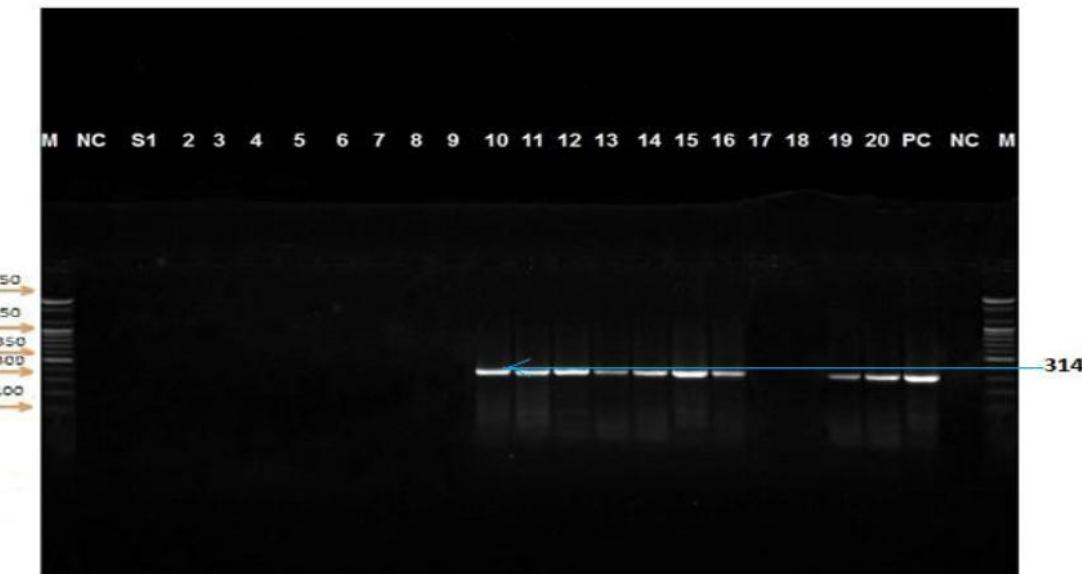
**Fig.2** *Blastocystis hominis* DNA extracts from Locke-egg (LE) medium amplified by using primer sub-type 83(subtype-1) showing positive and negative bands compare to standard DNA marker in the first and the last lanes. Specific bands to *Blastocystis hominis* parasite were extended from 346 to 356 bps  $\pm$  1.56 with mean of  $339.6 \pm 1.44$ .



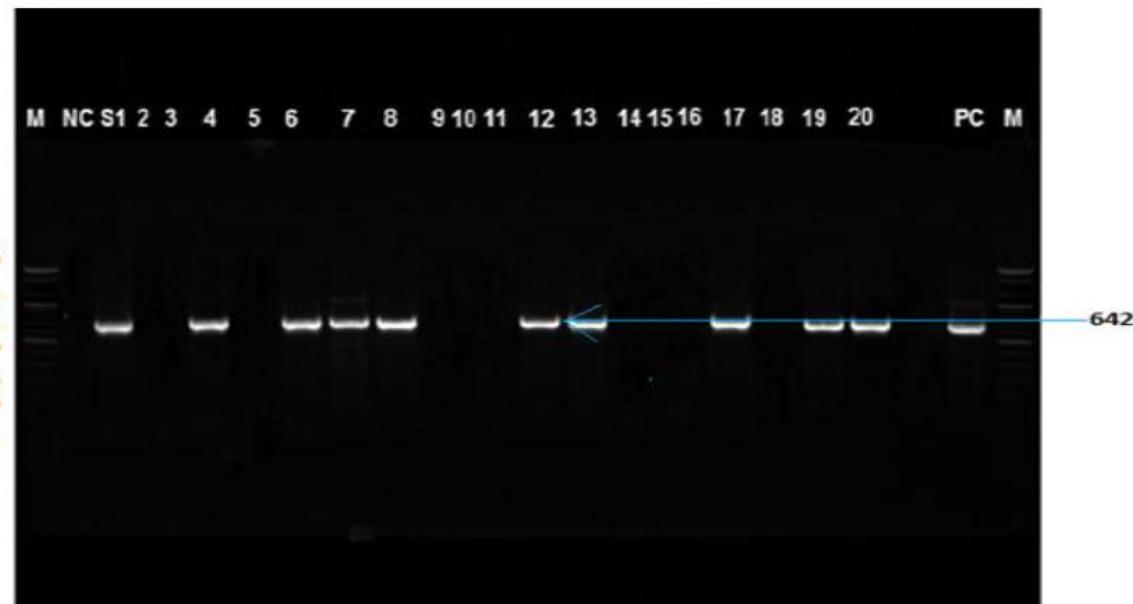
**Fig.3** *Blastocystis hominis* DNA extracts from Locke-egg (LE) medium amplified by using primer sub-type 227(subtype-3) showing positive and negative bands compare to standard DNA marker in the first and the last lanes. Specific bands to *Blastocystis hominis* parasite were extended from 526 to 546 bps $\pm$ 1.53 with mean of 533.16 $\pm$  2.21.



**Fig.4** *Blastocystis hominis* DNA extracts from Locke-egg (LE) medium amplified by using primer SB 336(subtype-5) showing positive and negative bands compare to standard DNA marker in the first and the last lanes. Specific bands to *Blastocystis hominis* parasite were extended from 312 to 318 bps $\pm$ 1.50 with mean of 314.88 $\pm$  1.57.



**Fig.5** *Blastocystis hominis* DNA extracts from Locke-egg (LE) medium amplified by using primer SB 155 (subtype-7) showing positive and negative bands compare to standard DNA marker in the first and the last lanes. Specific bands to *Blastocystis hominis* parasite were extended from 636 to 640 bps  $\pm$  2.8 with mean of  $642.11 \pm 1.38$ .



In the present study, ST1 was the second dominant variant detected (18.75%). ST1 also came second after ST3 in Iran (48%) (Moosavi *et al.*, 2012; Australia (31%) (Robert *et al.*, 2013), France (25.6%) (Souppart *et al.*, 2009), China (24.5%) (Li, 2007), and the Netherlands (22%) (Bart *et al.*, 2013). In Egypt, as detected by Hussein *et al.*, 2008 and Souppart *et al.*, 2010 , ST1 shared the second position with both ST6 (18.2% each) and ST2 (19% each), respectively. The present ST distribution in the Lebanese population was reported as ST3 (33.3%), ST2 (33.3%), ST1 (30.6%), and ST4 (2.8%), with ST1 significantly more prevalent among symptomatic patients of this population (El-Safadi *et al.*, 2013).

Regarding subtypes 5 and 7 in current study which were found as 11.25 % ,this can be explained by the role of animals in transmitting ; particularly subtype 5 can be transmitted by dogs and cats (Yakoob, *et al.*,

2010).While subtype 7 by the chickens (Yan *et al.*, 2007).

Considering patient gender and age in relation to subtype's distribution in spite of statistical analysis revealing no significant differences in regard of gender, but high records of subtypes 3, 5, 7 in females than in males. Versus to high rates of subtypes 1 and 3 in males than in females were reflecting mixed infection in females (zoonotic) while in males it may pure human to human transmitting.

Whereas high rate finding of *Blastocystis hominis* among patients aging from 41 to 60 years and from 61 to 80 years with dominance of subtype 3 and 1 respectively was highlighting the bad condition of IDPs in general and in particular elderly patients. Other explanation to this finding might be attributed to diminishing of immune state among these groups of study populations in

addition to malnutrition due to displacing may had impact on increasing the parasitic infections.

In conclusion, four types of microorganisms were acts as causative agents of GID, with dominance of *Blastocystis* than other three. GID including IBS and IBD were higher among patients aging from 20 to 29 years. Direct microscopy reveal high rate of *Blastocystis* than culture method, while PCR technique exert more accurate results in revealing dominance of subtype 3,1 in feces of patients than subtypes 5 and 7.further studies should be carried on for demonstrating role of subtypes 2,4, and 6 in blastocystiasis among patients with GID in Kirkuk city.

### Ethics statement

Before the study began, the study objectives were explained to the participants and if they were children objectives were explained to their mothers. Oral consent was obtained. The study protocol was reviewed and approved by the ethical review board of the Faculty of Dentistry, Kirkuk University.

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#### How to cite this article:

Abdulrazzaq Mohammed Hamood, Batool Ali Ahmed and Yahya Jirjees Salman. 2016. *Blastocystis hominis* Detection among Gastrointestinal Disorders' Patients in Kirkuk Province Using Three Different Laboratory Methods. *Int.J.Curr.Microbiol.App.Sci*. 5(7): 883-900.  
doi: <http://dx.doi.org/10.20546/ijcmas.2016.507.101>