Isolation and Identification of *Blastocystis hominis* isolated from Irritable Bowel Syndrome Patients using Phenotypic and Genotypic Methods

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Abstract — The current study was tried to detect the role of Blastocystis hominis in the pathogenicity of irritable bowel syndrome (IBS) with their's coexistence by using the phenotypic methods by microscopic and culture and genotypic methods by PCR. The present study was conducted on 127 patients (62 males and 65 females) attended to Al-Hussein Teaching Hospital in Al-Muthanna Province from the begging of November 2014 to the end May 2015. The patients were diagnosed clinically by physician whom suffering from IBS; Patients were interviewed directly by using anonymous questionnaire form which age, gender, type of water and residence area.Inaddition to that acontrol group of 40 apparently healthy individuals (18 males and 22 females) whom were without any history of disease included in this study. Fecal specimen were collected in suitable , clean , dry container , all sample was divided into three parts, the first for microscopic examination and second for culture and final part were quickly frozen for detection B. hominis using PCR. Microscopic examination by wet mount used saline and iodine and trichrome stain then demonstrated the B. hominis and culture method on Jones media for Blastocystis hominis and Detection of virulence gene is Cysteine protease (CP gene) of B. hominis by Polymerase Chain Reaction (RCR) where detect the virulence gene. Out of 127 samples, 43 (33.86%) were found positive for B. hominis by microscopy, 56 (44.09%) by culture and 58 (45.67%) using PCR. The parasite B. hominis represent one of the main causes of IBS.

Index Terms — Blastocystis hominis, irritable bowel syndrome, Microscopy, Culture, PCR.

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1. Introduction

Irritable bowel syndrome (IBS) is a functional bowel gastrointestinal in which abdominal pain or discomfort is associated with a change in bowel habit changing with features of changing in stool habit ,Because there are no biological markers to define this disorder, investigators attempted to define IBS using symptom-based criteria, derived from epidemiological investigations (Cayley , 2005).

Although several studies considered *Blastocystis hominis* as a commensal many recent studies characterized this organism as an intestinal parasite and a causative agent of diarrhea however, its detection in both symptomatic and asymptomatic individuals suggested that its pathogenic potential is controversial (Kaya *et al*., 2007). According to update literature, it noted that prevalence rate of *B.hominis* in developed countries varies from (1.5-10) % and it reaches up to 50% in developing countries .Besides causing mild to moderate diarrhea, bloating and flatulence .

This suggested a link between *B.hominis* and IBS which is a highly prevalent gastrointestinal motility disorder with a prevalence rate ranging from (3- 20)%. The chronic nature of IBS symptoms often negatively affect the patient's quality of life and activity level and place substantial economic burden on the patients and the healthcare system. Infection with *B.hominis* appears to be common and more severe in immunocompromised patients (Tasova *et al*., 2000). Currently, the detection of human infection with *B. hominis* is

usually based on microscopic examination of fecal samples either using wet mount preparations and or trichrome-stained smears (Surcsh and Smith, 2004). Kukoscke et al., (1990) compared the effectiveness of microscopy and culture for the identification of *B. hominis* and found that culture had no benefit over microscopy. On the other hand, others reported that Short term *in vitro* cultivation would increase the sensitivity of such detection. However, Clark and Diamonds (2002) proposed that the current diagnostic techniques lack sensitivity (many infections are missed), and in the clinical laboratory setting, cultivation of the organism plays a minor role especially as results are available only after 48-72 hours. *Blastocystis hominis* was considered to be a member of normal intestinal flora in the past; recently it has been accepted as a controversial pathogen. Infection with *B. hominis* has a worldwide distribution and occurs in both children and adults and is anaerobic unicellular protozoan frequently found in the human gastrointestinal tract. *B. hominis* is now getting acceptance as an agent of human intestinal diseases with potentially disabling symptoms (diarrhea, nausea, flatulence, and abdominal pain ….etc.). IBS was defined as a functional bowel disorder, and in Iraq, today the syndrome considered as one of the most common cases caused by different enteropathogenes. So, the study tried to detect the role of *B. hominis* in the pathogenicity of irritable bowel disease with their coexistence with the most common agents of gastroenteritis especially *Escherichia coli* and *Candida albicans* by using the gene expression of their virulence genes data via Real-Time PCR. The study was aimed to detect the role of *Blastocystis hominis* in the pathogenicity of irritable bowel disease (IBS) with their coexistence by using the phenotypic methods by microscopic and culture and genotypic methods by PCR. To achieve this goal, the following objectives were conducted:

1- Isolation and identification of *Blastocystis hominis* from patients suffering from irritable bowel syndrome by using morphological and molecular techniques.
2- Detection of virulence gene is Cysteine protease (CP gene) of *B. hominis* by Polymerase Chain Reaction (RCR).

2 . Material and Methods

2.1 materials

- Distilled water
- Ethanol
- Acetic acid
- Agarose
- Chloroform
- Chromotrope
- Dextrose
- Diethyl pyrocarbonate water
- Ethidium Bromide
- Ethyl acetate
- Formalin
- Iodine solution
- Iodine crystals
- Isopropanol
- Potassium Diphosphate
- Light green
- Loading dye buffer
- Disodium hydrogen phosphate
- Sodium chloride
- PCR water
- Phosphotungstic acid
- Proteinase k
- Normal Saline
- TBE buffer(Tris-borate-EDTA)
- Xylene

2.2 Instruments and Equipments

- Containers
- Oven
- Applicator sticks

- Autoclave
- Centrifuge
- Compound light microscope
- Cover slide
- Digital camera
- Eppendorf tubes
- Exispin vortex centrifuge
- Incubator
- Micropipettes (different volumes)
- Nanodrop
- PCR Thermocycler apparatus
- Sensitive balance
- Slide
- Catton Swab
- Test tube
- Vortex
- Water bath

2.3 Sample collection and Dignosis method

Inclusion criterion for selection of the study population was to have history ofsymptoms suggestive ofIBS according to the Rome II criteria (Thompson *et al.,* 1999). The later is based on the presence of abdominal discomfort or pain for at least 12 weeks or more (consecutive or not), in the preceding 12 months. These symptoms have two of three features: relief with defecation; onset associated with a change in frequency of stool; onset associated with a change in form of stool. Fecal specimens were collected in suitable, clean, dry container, all samples were divided into three parts, the first part was subjected to microscopic examination using direct smear stained with Lugol's iodine. Formalin ethyl acetate concentration technique (FECT) was performed immediately after collection, followed by staining with trichome stain (Garcia, 2003). The second part to be cultured by using Modified Jones' Medium was incubated at 37 °C for 48-72h. (Note: The presence of bacteria in the sample will be enough for creating the anaerobic environment needed for *B. hominis to* grow). Subculturing was done by transfer about 50-100 uL of the sediment from each culture into 3 ml fresh Jones' medium and Subculture every 3-4 days (Zaman *et al.,* 1994). The final part were quickly frozen for detection *B. hominis* using PCR, shipped on dry ice and kept at –20 ^{o}C to be used in DNA extraction for PCR. DNA extraction from stool specimens was done using AccuPrep®Genomic DNA extraction kit (Bioneer. Korea). The quantity of extracted genomic DNA was estimated by using Nanodrop spectrophotometer (THERMO. USA), in addition to measurement the purity of DNA through reading the absorbance in at (260 /280 nm). The PCR detection primer was designed by using NCBI- Gene Bank data base and Primer design online, and supported from Bioneer Company, South Korea where used *CP* gene to *B.hominis*. A Forward primer (GGAGAAGAGGCCGTTGTGAA) and reverse primer (AATCCCAGGCAACAGAGCTC) were used in PCR amplification using automated thennocycler. PCR amplification was performed according to the method of Parker et al (2007). PCR master mix reaction was prepared by using AccuPower PCR PreMix Kit and this master mix done according to company instructions. After that, these PCR master mix reaction components that mentioned above, placed in standard PCR tubes containing the PCR PreMix as lyophilized materials containing all other components needed to PCR reaction such as (Taq DNA polymerase, dNTPs, Tris-HCl pH: 9.0, KCl, MgCl2,stabilizer, and tracking dye). Then the tube placed in Exispin vortex centrifuge for 3 minutes. Then transferred in Mygene PCR thermocycler. PCR thermocycler conditions for gene was done by using convential PCR thermocycler system. Where 32 cycle with initial denaturation at 95°C for 5 min, denaturation at 95°C for 30sec, annealing at 58°C

for 30sec, extension at 72°C for 45sec and final extension at 72°C for 6min. Gel was visualized using ultraviolet trans-illumination after ethidium bromide staining for B. hominis DNA specific band at 265-basepair.

2.4 Statistical analysis

Data were analyzed using SPSS program version 16 and Microsoft Office Excel 2007. Numeric variables were expressed as mean ±SD while nominal variables were expressed as number and percentage. Independent sample t-test was used to study difference in mean between any two groups while chi-square was used to study association between any two variables. P-value was considered significant when it was less than or equal to 0.05.

3 RESULTS

By using the microscopic examination technique of stool samples the following parasites were isolated:

- *Blastocystis hominis* was seen in 43 patients (33.86%) and was not seen in any of the control subjects.
- *Giardia lamblia* was seen in 12 patients (9.45%) and also was absent in control subjects.
- *Entamoeba histolytica* was identified in 11 patients (8.66%) and was negative in control group.
- Eggs of helminthes were identified in 3 patients (2.36%) but none of control subjects had any.

Blastocystis hominis was the only parasite to be significantly higher among patients in comparison with control subjects (P<0.001). These findings are outlined in table (1).

Table(1): Types of parasites detected in both groups

Parasite		Control (n = 40)		IBS (n = 127)		P-value
		No.	%	No.	%	
Blastocystis hominis	*Positive*	0	0.00	43	33.86	<0.001
	Negative	40	100.00	84	66.14	
Giardia lamblia	Positive	0	0.00	12	9.45	0.072
	Negative	40	100.00	115	90.55	
Entamoeba histolytica	Positive	0	0.00	11	8.66	0.068
	Negative	40	100.00	116	91.34	
Egg of helminthes	Positive	0	0.00	3	2.36	1.000
	Negative	40	100.00	124	97.64	

Microscopic techniques employed remain the main method for diagnosis of *B. hominis* including wet preparation, concentration and stained smear by trichrome stain for the detection and identification of *B. hominis* in stool shown as in figure (1). The role of *B. hominis* as an etiological agent of IBS is inconclusive due to the controversial nature of B. *hominis* as a human pathogen. In this study, *B.hominis* detected in 43 (33.86%) out of 127 samples from patients with IBS by using microscopy, this high percent of detection may due to *B.hominis* consider normal flora in intestine and become opportunistic.

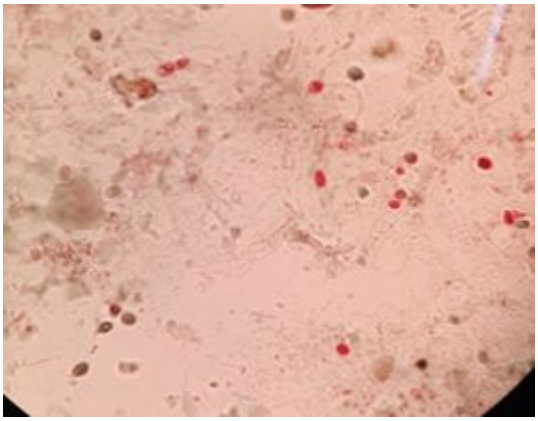

Figure (1): *B. hominis* staining by trichrome stain under micros-

copy

Other method was used to detect *B. hominis* and this included culture, where cultured in modified jones media and after incubated for 48 hours and work subculture then incubated for 72 hours, the positive rate of detection by culture was higher than light microscopic examination (44.09%), as shown in table (2). *Blastocystis hominis* was the parasite to be significantly higher among patients in comparison with control subjects (P<0.001). These findings are outlined in table (2).

Method		IBS (n = 127)		Control (n = 40)		P-value
		No.	%	No.	%	
Light Microscope	Positive	43	33.86	0	0.00	<0.001
	Negative	84	66.14	40	100.00	
Culture	Positive	56	44.09	0	0.00	<0.001
	Negative	71	55.91	40	100.00	
PCR	Positive	58	45.67	0	0.00	<0.001
	Negative	69	54.33	40	100.00	

Table 2: Methods of detection of *B. hominis*

Culture method was used to detect *B. hominis* as shown in figure (2) the positive rate was higher than light microscopy (44.09%).

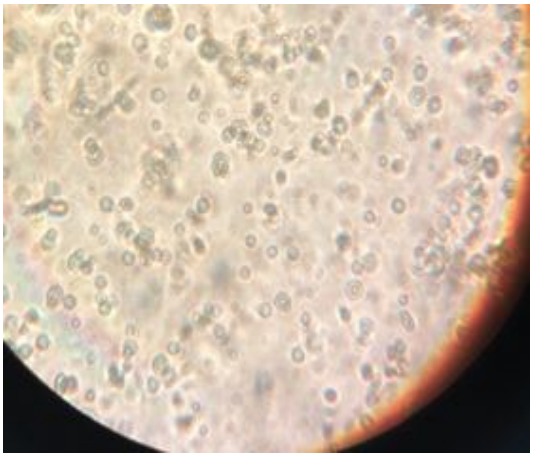

Figure (2): *B. hominis* growth on Jones modified medium

The DNA of all specimens of stool was extracted and purified using genome DNA purification Kit. The results were detected by using measurement of DNA quality and purity by using Nanodrop spectrophotometer. The results of evaluating and estimating the DNA extraction was measured by Nanodrop spectrophotometer at wavelength (260-280) nm. It gave an optimal concentration of DNA for amplification range from (69.0-9.2) ng/ µl and purity ranged from (1.16-2.25) shown as table (3).

Table (3): Values of DNA concentration and purity of selected samples of extracted DNA from stool

Sample no.	Concentration ng/ µl	Purity 260/280 nm
1	31	1.95
2	18.7	1.23
3	24.3	2.02
4	43	1.92
5	37.3	1.66
6	37.8	1.97
7	42.3	1.16
8	31.7	1.49
9	51.6	1.60
10	25.8	1.91
11	35.4	1.92
12	40.4	1.66
13	17.4	1.99
14	22.3	2.25
15	26.2	1.67
16	24.5	1.84
17	34.5	2.03

18	**69.0**	**1.26**
19	**34.7**	**1.77**
20	**9.2**	**2.03**

Based on the standard values of DNA concentration for PCR amplification, the values of the present study are considered an efficient values and suitable for the establishment of the DNA extracted with target primers or sequences amplification (Applied Biosystems, 2008). The result of amplification was performed on the DNA extracted from all studied specimens and confirmed by electrophoresis analysis. By this analysis, the strands of DNA resulted from the successful binding between specific primers of target gene (Legumain precurrsors for cyateine protease) and specimen extracted DNA. The successful binding appeared as single compact bands under UV. Light using ethidium bromide as specific DNA stain. The electrophoresis was also used estimate DNA molecular size depending on DNA marker (1500-100bp DNA ladder) and the result of this estimation revealed that the amplified DNA(PCR product) was 265bp for *Blastocystis hominis* showed as figure (3).

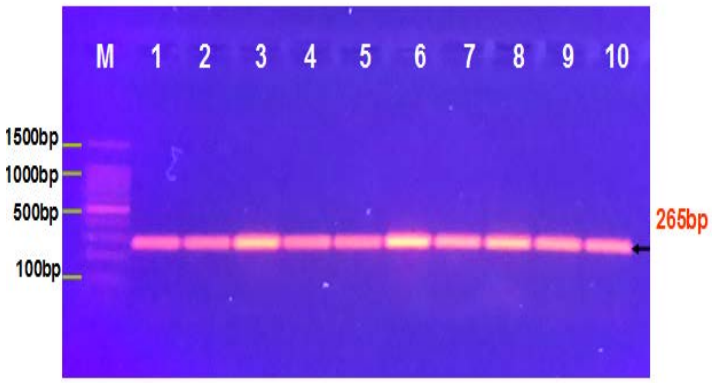

Figure (3): Agarose gel electrophoresis image that show the PCR product analysis of legumain gene (Virulence factor gene) in *Blastocystis hominis* .Where M: marker (1500-100bp), lane (1-10) positive virulence factor gene at (265bp) PCR product

The results by using PCR appear higher than microscopy and culture (45.67%) for detect *B.hominis*, as shown in table (2).The sensitivity of light microscopy was far less than that of PCR (74.14%). Nevertheless the specificity of light microscopy was 100%. On the other hand culture method was highly sensitive (96.55%) and perfectly specific (100%), as demonstrated in figure (4). In this study there was a higher incidence of *Blastocystis hominis* present in the IBS group compared to the control group this was considered statistically significant ($p<0.001$). A previous study also found a higher incidence of *B.hominis* in the IBS group compared to the control group (Alfellani *et al.*, 2013a).

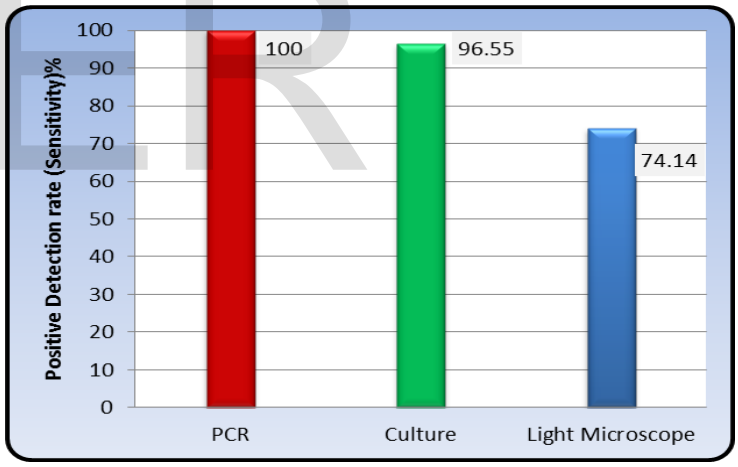

Figure 4: Comparison of positive detection rate (sensitivity) among the three methods

4 Discussion

Microscopic techniques employed remain the main method for diagnosis of *B. hominis* including wet preparation, concentration and stained smear by trichrome stain for the detection and identification of *B. hominis* in stool shown as in figure (1). The role of *B. hominis* as an etiological agent of IBS is inconclusive due to the controversial nature of B. *hominis* as a human pathogen. In this study, *B.hominis* detected in 43 (33.86%) out of 127 sam-

ples from patients with IBS by using microscopy, this high percent of detection may due to *B.hominis* consider normal flora in intestine and become opportunistic. This is in agreement with results from another study in which *B. hominis* was isolated from 32% of 95 lBS stool samples using microscopic examination (Yakoob *et al.,* 2004), and the study agreement with results from another study in which *B.hominis* was isolated from 25(30,1%) of 83 samples from patients with IBS by using microscopic examination (Amany and Mohames, 2008). Culture method was used to detect *B. hominis* as shown in figure (3-2) the positive rate was higher than light microscopy (44.09%). This is in agreement with results from another study in which *B. hominis* was isolated from 46 % of 95 lBS stool samples using culture (Yakoob *et al.,* 2004), and other study agreement was isolated from 41% from 83 sample of IBS patients (Amany and Mohames, 2008). There have been many studies which use Jones medium successfully as the medium of choice for xenic culture growth of *B.hominis* (Leelayoova *et al.,* 2002, Parkar *et al.,* 2007).

The results by using PCR appear higher than microscopy and culture (45.67%) for detect *B.hominis*, as shown in table (3-2). The results agreed with results from another study in which *B. hominis* was isolated from in 37 (44 .6%) out of 83 samples from patients with IBS (Amany and Mohames, 2008).PCR was considered the gold standard method and the sensitivity of other methods was compared to culture and microscopic examination.

5 CONCLUSIONS

The parasite *B. hominis* represent one of the main causes of IBS.The use of molecular technique is very important in detection of *B.hominis* in addition to morphological features.It noticed that *B.hominis* has a virulence gene (CP) which plays a vital role in the pathogenicity of this parasite.

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