International Journal of Current Research and Academic Review

ISSN: 2347-3215 Volume 3 Number 8 (August-2015) pp. 290-299

www.ijcrar.com

A Study of *Blastocystis hominis* Infection in Sulaimani Pediatric Teaching Hospital

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KEYWORDS

Blastocystis hominis,
Stool sample,
Culture,
PCR,
Sulaimani,
Iraq

A B S T R A C T

This study aimed to investigate three aspects the 1st includes an investigation for *Blastocystis hominis* infection in children attending Pediatric Teaching Hospital in Sulaimani city, the 2nd was to culture the parasite, and the 3rd was to find the frequency of different subtypes using polymerase chain reaction (PCR). The study started from 1st May to 30th October 2012.Atotal of 307 stool samples were collected from children of six months to 12 years old from both genders who attended the hospital. Stool samples were examined and identified by direct wet mount method using normal physiological saline and logol's iodine solution. The results revealed that the total prevalence rate of infection with *B. hominis* was 22.15%. The prevalence rate of infection in females was higher (23.8%) than that in males (20.9%) with no significant difference between both genders. In rural area the prevalence rate was lower (20.55%) than the urban area (22.64%), and the rate of infection in males of rural area was higher (25%) than females(15.15%), while in urban area the rate in males was lower (19.7%) than in females (26.85%) with no significant differences between both genders in both locations. Also the results showed that the higher prevalence rate was in children of (10-12) years of age in both gender, while the lowest rate was recorded in 6 months to 3years of age with no significant difference of infection rate between both age groups and genders. Culturing of *B.hominis* in Liver infusion medium showed great ability of growth within14 days, also *invitro* cultures for PCR amplification of *B.hominis* genotype gave better results than those of frozen stool samples. Subtype 1 (ST1) was detected by sequencing method for the first time in molecular genotyping of human Blastocystis infection in Sulaimani city and Iraq.

Introduction

Blastocystis is a prevalent enteric protist that infects a variety of vertebrates. Asymptomatic and symptomatic infection in humans have described, the infection with *Blastocystis* is termed blastocystosis that has been associated with abdominal pain, diarrhea, constipation, fatigue, skin rash, and other symptoms (Boorom *et al*., 2008). *Blastocystis* is the most common parasite isolated from human stool samples in developing and developed countries. Rates of infection vary from 3.3% in developed countries to 53.8% in developing countries (Roberts *et al*., 2011).

Accumulating epidemiological, *invivo* and *invitro* data strongly suggest that *Blastocystis* is a pathogen (Tan, 2008). With the advent of molecular data (on small-subunit ribosomal DNA and other genes), these organisms are now thought to belong to the stramenopiles (Coyle *et al.,* 2012). Four different morphological forms of the parasite have been reported; vacuolar, granular, ameboid, and cystic stage most commonly involves in transmission of the infection (Aguilar and Lucı´, 2009). The most common form of the transmission of *Blastocystis* is the fecal–oral route (Roberts *et al*., 2012).

Based on small-subunit ribosomal DNA analysis, the genus comprises at least 13 subtypes (STs), nine of which have been found in humans, it is very likely that each subtype represents a separate species, in human ST3 appears to be the most common subtype, followed in prevalence by ST1, ST2, and ST4 (Stensvold *et al*., 2012).

Materials and Methods

Stool specimens: Atotal of 307 stool samples were obtained randomly in patient and out patients attending the Pediatric Teaching Hospital in Sulaimani City from 1st of May to 30thOctober 2012. Before analyzing the fecal samples the special questionnaire form was prepared to denote full information from each patient which was relevant to various epidemiological factors that might be responsible for parasite infection which included patient name, age, gender, region, period of infection, treatment, type of parasite form. The stool samples were collected in clean universal screw cap bottles from patients of 6 months to 12 years of age , tabled with number and name of the patients complaining of diarrhea and abdominal pain. The stool samples were divided into two parts: one portion was fixed and preserved in %10 formalin for direct wet mount using saline and iodine, and another amount of same samples without formalin were stored in deepfreeze at -20 ˚C for DNA extraction.

Culture of *B. hominis* isolates

B.hominis was isolated from 10 symptomatic patients with gastrointestinal problems (abdominal pain, diarrhea and vomiting). Stool samples were examined for *B.hominis*. Approximately 50 mg of feces were inoculated into a 15-ml screw-capped tube containing 5 ml of liver infusion medium.

All inoculated tubes were tightly closed, placed in a rack and incubated at 37 °C. The presence of *Blastocystis* was observed daily by placing 1 drop of cultured sediment on a glass slide, which was covered with a cover-slip and examined under light microscopy (100X and 400X objectives). Parasites were harvested from liver infusion agar medium by centrifugation for 5min at 500 rpm and washed twice with 3ml sterile phosphate buffered saline (PBS) (pH 7.4) (Tan *et al*., 2006).

Genomic DNA preparation

The genomic DNA extraction of *B. homonis* from stool samples was done by using I-genomic Stool DNA Extraction Mini Kit (Bioner, Korya, and Cart Number 17451), according to the manufacturers' protocol.

Polymerase chain reaction for detection of *B. hominis* 16S-like ribosomal RNA gene

At first the amplification of six DNA from frozen stool samples was carried out by PCR thermocycler and *GoTaq® Green Master Mix kit* (Promega, U.S.A) *according to manufacturer's instructions in final volume 25µl reactions.* Two pairs of different primers were used for detection of 1770 bp and 1100 bp 16S-like ribosomal RNA gene of *B.hominis*, the first pair included the forward BH1 (5 ´GCTTATCTGGTTGA TCCT GCCAGT3́) and the reverse BH2 (3 ´TGATCCTTCCGCAGGTTCACCTAC 5́). This pair of primers have been established by Silberman, *et al.* (1996) and also used by Init, *et al*. (2007), the PCR conditions consisted of one cycle denaturing at 94°C for 5 min, 35cycles including annealing at 54°C for 1 s, extending at 72°C for 1 s, denaturing at 94°C for 30 s, and additional cycle with a 5-min chain elongation at 72°C. the second pair of primers included the forward BH3 (5 ´ GGAGGTAGTGACAATAAATC 3 ´) and the reverse BH4 (3 ´ CGTTCATGATGA ACAATTAC 5´) which was used for Nested PCR as Böhm-Gloning *et al*. (1997) done, the PCR conditions consisted of one cycle denaturing at 94°C for 4 min, 35cycles including annealing at 54°C for 30 s, extending at 72°C for 30 s, denaturing at 94°C for 30 s, and additional cycle with a 5-min chain elongation at 72°C.The PCR products were electrophoresed in 1% agarose gel with Tris-boric-EDTA buffer (BioBasic, Canada), the fragments were visualized by UV light and fragment sizes were confirmed with bands of a DNA length standard (DNA 10Kb size marker (Promega, U.S.A).

Sequence analysis

DNA sequence analysis was performed on five PCR-positive samples. The PCR products were purified using the Gel/PCR DNA Fragments Extraction Kit (Vogelstein and Gillespie, 1979) as per the manufacturer's instructions. Purified PCR products were then sequenced at Koya University (Kurdistan, Iraq). The 16S-like ribosomal RNA gene of *B.hominis* sequences were then compared with those available in Gene Bank using the BLASTN program run on the National Center for Biotechnology Information (NCBI).

Statistical analysis

Both SPSS (V. 17) and Statigraphic (V.4) software were used for statistical analysis depending on t- test and Chi-Square (X^2). $P<0{\cdot}05$ was considered statistically significant

Results and Discussion

A total of 307 stool samples among children aged between 6month to 12years were shown a prevalence rate of 22.15 % (68/307) of infection with *B.hominis* (Table 1). From the results it was clear that the prevalence rate of infection with *B.hominis* in males and females were 20% (37/177) and 23.8 % (31/130) respectively, with no significant difference between both genders (Table 2). In rural area the rate of infections with *B. hominis* according to gender showed that male's infection was higher (25%) than females (15.15%), while in urban region females were higher (26.8%) than males

(19.7%) infection, with no significant differences between the number and rates of infection with *B.hominis* according to gender in rural and urban locations (Table 3). The highest prevalence rate was found in children of 10-12 years of ages, and the rate was higher(56.52 %) in females than males (41.17) of the same age group, and then followed by the other age groups (7-9) years and (4-6) years respectively, however, the lowest rate was recorded in (0.5-3) years of age in both genders (12.72 % in males and 11.39 % in females). There were no significant differences in positive rate between age groups and genders (Table 4).

Detection of *Blastocystis* using in vitro amplification

The dominant morphological form in the cultures of liver infusion media was the vacuolar form, followed by the granular forms especially when the isolates were cultured for a long time (in old stool culture tubes) (Figure, 1). During the incubation period, various stages of binary fission were detected for reproduction of *B.hominis* (Figure,2).

Detection 16S-like rRNA gene of *B.hominis* by using PCR

Amplifications of 16S-like ribosomal RNA gene of *B. hominis* for the all six *Blastocystis* isolates from positive frozen stool samples stored at -20˚C was negative for both primers(Figure, 3), while genomic DNA of all five *Blastocystis* isolates from fresh stool cultured were successfully amplified approximately 1770 bp PCR amplicon using the primer pair (BH1/ BH2) (Figure, 4).

Fragments of approximately 1100 bp of the 16S-like rRNA gene of *B.hominis* were amplified by nested PCR from the same five specimens with primers (BH3 and BH4) as shown in Figure (5).

The Aligned 1770 bp 16S rRNA gene of the *B. hominis* local isolates showed Subtype 1 that was determined by an exact match of 99% similarity as a result of DNA sequencing. The 16S rRNA gene sequences obtained in this study has been deposited in Gene Bank under accession numbers U51151 (Yoshikawa, *et al*., 2000).

From the results it was found that the total prevalence rate of infection with *B.hominis* was (22.15%), it was similar to Al-Sheikly (2002) (21.36%) and Raof and Abdul-Rahman, (2011) (24.6%) in Baghdad city, but it was higher than those rates recorded by Al- Ougelli (2007) in Baghdad (6 %) and Ozçakir *et al*. (2007) in a Turkish university hospital (12.2%), while it was less than those recorded by Gualdieri *et al*. (2011) among immigrants (52.7%) in the Naples city in Italy. This may be attributed to the differences in the number of samples, geographical distributions, age group used, socioeconomic and immunologic status, personal hygiene, differences in temperature at different seasons and close contact with animals. Also the present study revealed that there was no significant difference in the prevalence rate of infection with *B.hominis* between male and female groups, this may returned to practicing the same activities and habits, this finding was similar with the results recorded by Yaicharoen, *et al*., (2006) and Leelayoova, *et al*., (2008) and the results slightly increased incidence in female than male groups; this probably due to the low level of individual and public hygiene conditions or their contact with animals that causes manypathogen infections especially parasitic infection. The result was the same as the result of Carbajal, *et al*. (1997) in Valencia, and different with Taherkhani, *et al*. (2008) in Iran and Nimri

(1993) in Jordan. The prevalence rate of infection with *B.hominis* in rural area was lower than in the urban area this may be due to the differences in sample sizes, patients in rural area may be treated in their inhabitant's hospitals for it is too far to reach the city hospitals, the result agrees with Wang, *et al*. (2002) who found a relationship between urban and rural places in the infection rates with *B. hominis*, but it was different with Lee *et al*. (2012) who found that *Blastocystis* was prevalent among rural children because of their close contact with animals. The results showed that in rural areas the male's prevalence rate was higher than in females, while in urban the prevalence rate of infection in males was lower than in females, with no significant difference between both genders in the infection with *B. hominis* in both places, this may be return to practicing habits of both genders in both places, this finding was similar to the results found by Wang,*et al*. (2002); Ali and Mohammed (2010) also there were no significant differences in the prevalence rate of *Blastocystis* infection according to age and genders. The finding is consistent with the results of Leelayoova, *et al*.,(2008) and Inititho, *et al*., (2012). From the results the highest prevalence rate of infection was found among children aged (10-12) years, while the lowest prevalence rate of infection was recorded in both genders of (0.5-3) years old this may be because less chances to be infected with *B.hominis*, this result was similar to Al-Sheikly (2002) but the results were different with Yaicharoen, *et al*. (2006) and Nimri (1993), this may be because of contaminated water sources, and these children were fed formula milk in addition to mother's milk.

Culturing of *B. hominis* in the liver infusion medium was the favored medium for growing of this parasite in which vascular form followed by granular, this was similar to the results got by Yamada and Yoshikawa, (2012) and Al-sheikly (2002).No cyst and ameboid stage were noticed from fresh and cultured samples. Different stages of binary fission inside cultured and fresh stool sample were detected; this was similar to that found by Zhang, *et al*. (2007) and Yamada and Yoshikawa (2012) it may related to that binary fission is the only accepted form of reproduction of *B.hominis* in culture and in stool demonstrated by both light microscopy and transmission electron microscopy.

Table.1 The prevalence rate of *B.hominis* infection in Sulaimani Pediatric Teaching Hospital

No. of Examined samples	Positive		Negative	
	No.	%	No.	%
307	68	22.15	239	77.85

Table.2 The prevalence rate of B.hominis infection according to gender in Sulaimani Pediatric Teaching hospital

Gender	Examined samples		Positive for *B.hominis*		P-value
	No.	%	No.	%	
Male	177	57.65	37	20.9	0.522
Female	130	42.34	31	23.8	
Total	307	98.99	68	44.7	

Table.3 Relationship between number and rate of infection with *B. hominis* in gender according to rural and urban inhabits

Gender	Rural			Urban			P-value
	Examined samples	Positive for *B.hominis*		Examined samples	Positive for *B.hominis*		
	No.	No.	%	No.	No.	%	
Male	40	10	25	137	27	19.7	0.0635
Female	33	5	15.15	97	26	26.85	
Total	73	15	20.55	234	53	22.64	

Table.4 Relationship between age groups and genders infected with *B.hominis*

Ages	Males			Females			P-value
	Examined samples	Positive for *B.hominis*		Examined samples	Positive for *B.hominis*		
	No.	No.	%	No.	No.	%	
0.5-3	110	14	12.72	79	9	11.39	0.3105
4-6	34	11	32.35	18	5	27.27	
7-9	15	5	33.33	11	3	27.27	
10-12	17	7	41.17	23	13	56.52	
Total	176	37	100	131	30	100	

Figure.1 Photomicrographs for *B.hominis* forms in cultured media (Magnification 400X). A: Vacuolar form, B: Granular form

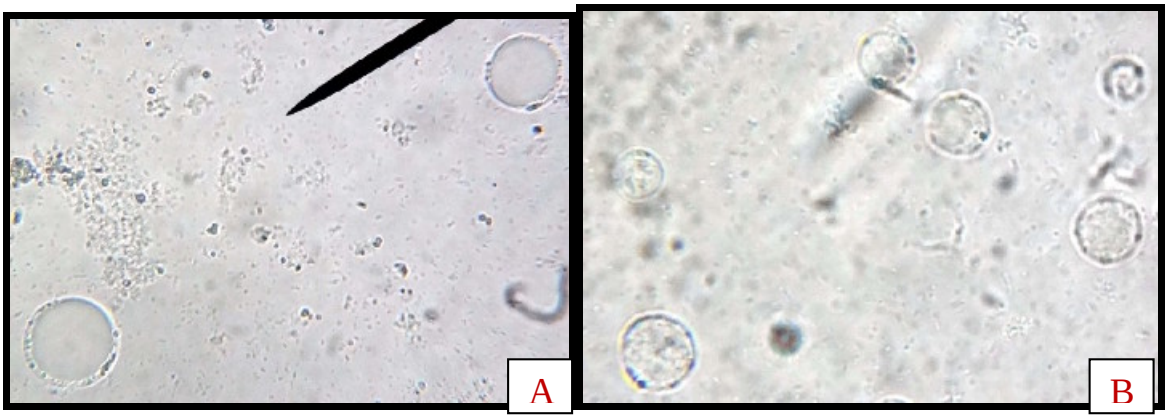


Figure.2 Different stages of *B.hominis* division by Binary fission in the cultured Media (Magnification 400X)

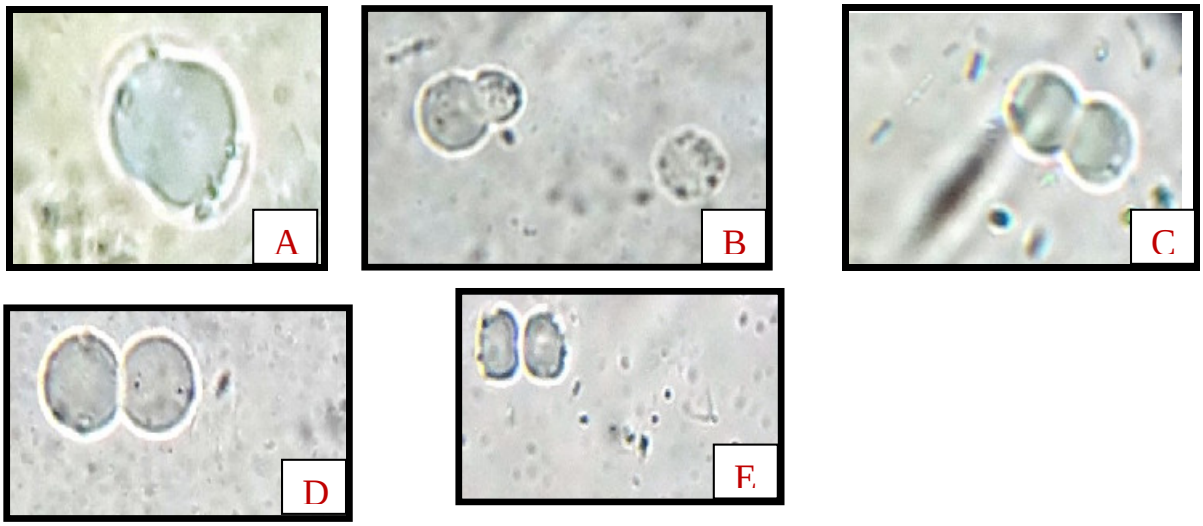


Figure.3 Gel electrophoresis of the amplified PCR product of the 16S rRNAgene of *B.hominis.* Lanes 1-6: No amplification using primer pair BH1/BH2.Lanes 7-12: No amplification using primer pair BH3/BH4 (Nested PCR). Lane (M1):1000 bp DNA Marker Lane (M2):1500 bp DNA Marker.

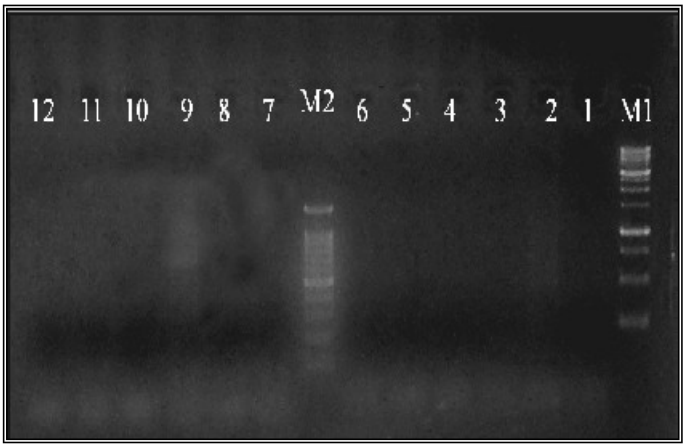


Figure.4 Gel electrophoresis of the amplified PCR product (1770bp) of the 16S rRNA gene of *B.hominis* for all five isolates of *B. hominis*. Lane 1-5: 1770 bp PCR products. Lane M: 10kb DNA Ladder Marker.

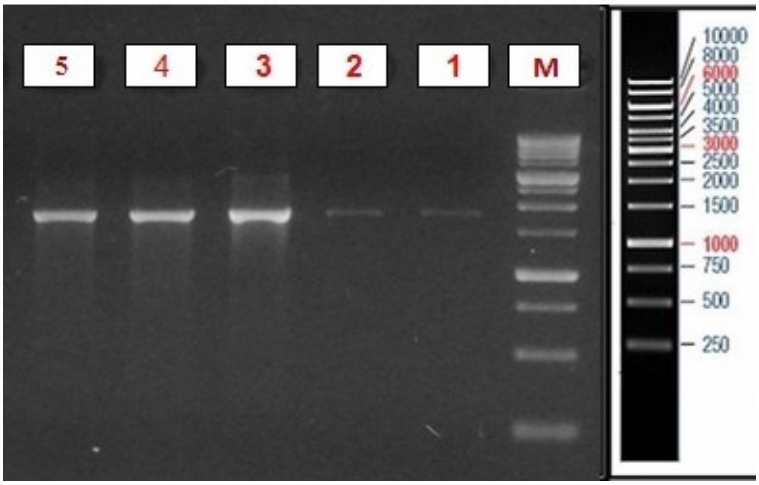
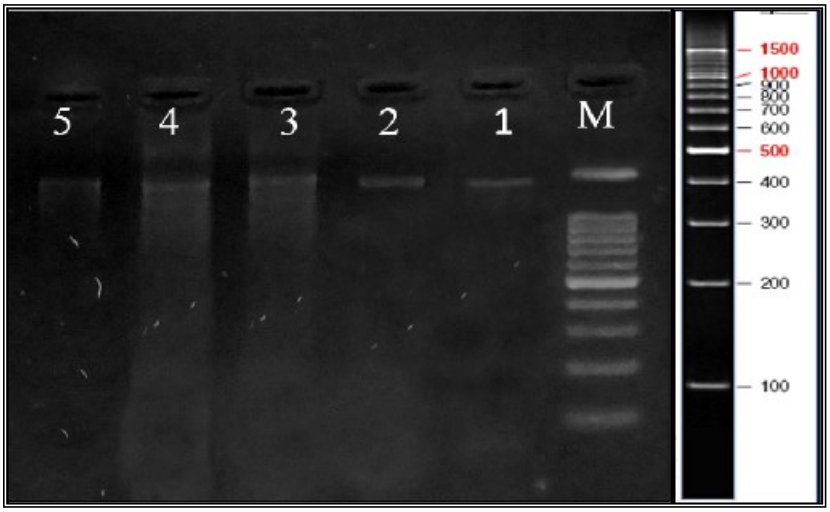


Figure.5 The corresponding PCR product was analyzed by nested-PCR using primers BH3, BH4. Lane (1-5) 1100 bp 16S rRNA gene of five isolates of *B. hominis*, Lane M: 1500 bp DNA Marker



The results of DNA extraction from frozen stool samples at (-20˚C) and performing PCR amplification to determine genotyping of isolates with two different primers pairs were not successful was due to sort of kits used for stool DNA extraction, PCR inhibitors in the stool specimen, high concentration of nucleases that can interfere with the detection processes and the low number of *B.hominis* in stool sample for DNA extracting kit that may complicate PCR testing. The finding results were similar to Termmathurapoj, *et al*. (2004), but were different with Parker *et al*. (2007).PCR amplification of five cultured genomic DNA using (BH1and BH2) primers was successfully produced an amplicon of 1,770 bp of 16S-like ribosomal RNA gene of *B.hominis*, this may be due to the specificity of these primers which was similar to the previous findings of Init, *et al*., (2007). Nested PCR reaction amplified an approximately 1100 bp with BH3 and BH4 primers for the same five PCR positive products which confirmed the finding result for *B.hominis* genotypes of Bohm-Gloning,*et al*. (1997). From the finding results of 1770bp for detecting 16S-like ribosomal RNA gene, subtype 1 of *B.hominis* was isolated from human stool and when compared with the gene bank accession number U51151, it was noted that the similarity between them was 99%. The finding result was supported by Noel, *et al*. (2005).

Conclusion

It was concluded that *B.hominis* is prevalent in Sulaimani city at a rate of 22.15% .The prevalence rate in females was higher than males, and in urban was higher than rural but the rate of males of rural was higher than females while in urban the rate was higher in females with no significant difference between both genders in both areas, also the highest prevalence rate was found in children of 10-12 years of age, and the subtype 1(ST1) of the parasite was recorded for the first time in Sulaimani exclusively and in Iraq as a whole through this study.

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