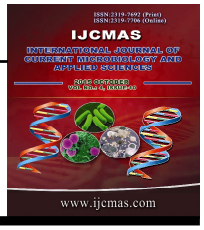
Int.J.Curr.Microbiol.App.Sci (2015) 4(10): 686-695

International Journal of Current Microbiology and Applied Sciences
ISSN: 2319-7706 **Volume 4 Number 10 (2015) pp. 686-695**
http://www.ijcmas.com



Original Research Article

Detection of *Blastocystis hominis* among Peoples in Kirkuk Province Using ELISA and Direct Microscopy

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Keywords

Blasto cystishomonis, ELISA, Direct microscopy, pH, and IBS

A B S T R A C T

Blastocystis hominis (*B. hominis*) is a common intestinal parasite of humans worldwide. Infection has been reported as asymptomatic, acute symptomatic and chronic. From 1st of November 2014 to 30th of June 2015; a total of 217 stool samples were collected from patients attending private medical labs in Kirkuk Province, whom they were suffering from Gastro-intestinal disorders including diarrhea and Irritable bowel syndrome (IBS) 21 patients. For each patient special questionnaire was prepared including all informations. Direct double wet preparations using 0.85 % of NaCl and 1 % of Lugols iodine were used for each specimen, and then after tested by ELISA using *Blastocystis* copro-antigen ELISA kit manufactured by Sayvo Company-imported from Netherlands. The overall rate of *B. hominis* by using direct microscopy and ELISA was 58.22 % distributed in 129 stool samples. This rate was contributed 59.44% and 58.99 for direct microscopy finding and ELISA testing, p>0.005. Both laboratory methods show high sensitivity and specificity; the rates were 99.73% and 99.25% in detecting *B. hominis*, P>0.05. Stool samples from patients aging from 6 months to 10 years and from 21 years to 30 years reveal high rates of 17.51% and 17.05% respectively. While lowest rates 9. 21 % and 4.60 % were recorded among patients aging from 51 to 60 years and among patients aging over 61 years respectively, P<0.05. In regard to gender; the following overall rates 50.66 % and 26.68 % were recorded in males using direct microscopy and ELISA respectively compare to 25.76% and 10.58% in females by both methods respectively, P<0.05.Correlation between type of lab methods and patients age and gender; ELISA-copro antigen test reveal 7.33 % of *B. hominis* among patients ageing from 31 to 40 years in both gender. Versus to direct microscopy method; high rate 12.44 % positive was found among males ageing from 6 months to 10 years and high positive rates5.06 % was equally recorded among females ageing from 21 to 30 years and among age group 6 months to 10 years respectively. Liquid stool samples exert high rate positivity of *Blastocystis* 77.14 %, 75.58 % and 63.33 % in brown, yellow and green liquid colors respectively. Relationship between stool color and *B. hominis* distribution was not significant while it was significant in regard of stool consistency P<0.05. According to pH of the stool, significance and high rate 63.56 % was with pH from 6.1 to 7.0, compare to 27.13 % and 9.30 % for pH 7.1 to 8.5 and from 4.0 to 6.0 respectively. Correlation among stool consistency, pH and *B. hominis* was significant, P<0.05. Stool samples belongs to irritable bowel syndrome patients was highly found 90.47 % in colorless mucoid stool with high dominancy rate 57.89 % in pH from 7.1 to 8.5 compare to other pH, P<0.05. *B. hominis*rate was high in Kirkuk city-Iraq by using direct microscopy and ELISA-copro antigen with high sensitivity and specificity. Young aged peoples with brown liquid and colorless mucoid stool in IBS are samples choice with in pH of 6.1 to7.0 for detecting *B. hominis.*

Introduction

Blastocystis hominis is the most common intestinal parasite in humans and many other animals (Windsor *et al.,* 2002). Infections with the 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 (30–50 %) in comparison with developed countries (1.5–10 %) (Li *et al.,* 2007; Vogelberg *et al.,* 2010). The pathogenicity of *B. hominis* still has been debated. There are some reports supporting the pathogenic potential of this parasite (Ok *et al.,* 1999). Clinical features related to *B. hominis* include nausea, anorexia, abdominal pain, flatulence, and acute or chronic diarrhea (Sohail and Fischer, 2005). However other studies state an opponent view point and it is believed that other factors probably are the causing agents of these symptoms (Ok *et al.,* 1999; Hussein *et al.,* 2008; Kaya *et al.,* 2007; Rossignol *et al.,* 2005). 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 µm which is close to oocysist 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.,* 1991; 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 in 2011), whom they found 24.6 % of *B. hominis*in 59 stool samples (Karyaghdi, 2013). Also in Kirkuk karyaghdi in 2013 was carried out diagnostic study on some intestinal parasites, via which *B. hominis* contribute 3.6 % (Raof and Abdul-Rahman, 2011). So current study was conducted to assess role of direct microscopy and ELISA-copro antigen test in demonstrating *B. hominis* among diarrheic patients and to some extant in stool samples of IBS patients in Kirkuk city.

Materials and Methods

Time, location and study design:

The present study was cross sectional study involved patients with abdominal discomfort, persistent of diarrhea and IBS. Whom they referred by internal medicine doctors in private clinics to Ibn-Nafies medical private laboratory in Kirkuk Province-Iraq from 1st of November 2014 to 30th of June 2015.

Stool samples collection

A total of 217 stool samples were collected from both gender whom they ageing from 6 months to over than 61 years after

completion of a special questionnaire, which containing required information necessity for searching etiology of *hominis* among enrolled patients to current study. As soon as samples have been brought, they examined for direct microscopy by preparing double wet preparations of 0.85 % of NaCl and 1 % of Lugol's iodine. Also fecal smears were prepared for staining with some routine stains. The rest of stool samples were preserved by adding sufficient amount of potassium dichromate 2.5 % for ELISA application later, all specimen were kept in refrigerator till to use.

ELISA Copro-antigen test

Briefly the kit is consist of micro-plates of 96 wells, each well was solid phase coated with immobilized monoclonal antibodies specific for *B hominis*. The first step involves removing an excess of potassium dichromate by three times washing with phosphate saline buffer (Pbs), till the supernatant becomes clear. Then after in case formed stool approximately 0.1 to 0.15 gm of specimen (about the size of small pea) was transferred in to 400µl of sample diluent. In case the stool was liquid. 150 µl of the specimen has been transferred in to 400 µl of sample diluent. The mixtures were thoroughly mixed (Vortex). The mixture was left at room temperature for about 30 minutes or centrifuged for 5 minutes at 3000 rpm to obtain good supernatant. Second step involve transferring 100 µl of the supernatant of each stool sample, positive, negative controls (duplicates) and cut-off if supported with the kit in to separate wells; the first well A1 was left without adding any reagent as blank and gently shacked for about 30 seconds. ELISA plate was covered and incubated for 1 hour at 37 C at 100 % humidity. Third step involve washing the wells by working wash solution (20 ml of concentrated wash solution in to 980 ml of deionized distill water) by adding 300µl of wash solution, gentility agitated and discarded. This step was repeated for three times. After that the strips were dried and framed by gently tapping them over clean absorbent paper. Fourth step involve adding 100µl of substrate (TMB) solution for each well involving A1 well. The plate was incubated in dark place for 15 minutes. Fifth step was by adding 100µl of stop solution (1M of H2SO4) into each well. Color intensity was determined by using ELISA reader machine after adjusting the wave length on450/629 nm. The absorbance is proportional to the number of *B. hominis* cells present in the sample. Samples that exhibit absorbance values higher than the cut-off should be considered positive. In current study the estimated and applied cut-off was.0.600 I.U/ml. This procedure was applied according the leaflet of manufactured company (Savyon Diagnostics Ltd.) Netherlands.

Some fecal smears were stained with 5 % of giemsa satin, modified Ziehl-Neelsenstain (hot method) and Chromotrope R2 stain for demonstrating the internal constituents of the parasite for detecting which phase was dominant in stool sample, specially the samples of patients with IBS. All obtained data were arranged in tables and test statistically to assess variances at probability of 0.05.

Results and Discussion

The overall rate of *Blastocystis hominis* by using direct microscopy and ELISA was 58.22 % distributed in 129 stool samples from a total of 217 stool samples. This rate was contributed 59.44% and 58.99 for direct microscopy finding and ELISA testing. Statistically the differences between two methods employ for detecting *Balstocystis* stages in stool samples was not significant,

P>0.05.For determination the efficacy of two methods in detecting *Blastocystis hominis* in stool samples, both methods show high sensitivity and specificity; the rates were 99.73% and 99.25% P>0.05 (Table 1).

Regarding *Blastocystis hominis* frequency according to patient age and gender; the result was arranged in table 2, via which it was obvious that stool samples from patients aging from 6 months to 10 years and from 21 years to 30 years reveal high rate 17.51% and 17.05% respectively. While lowest rates 9.21 % and 4.60 % were recorded among patients aging from 51 to 60 years and among patients aging over 61 years respectively, P<0.05. Stool samples examining by microscopy and ELISA in regard to patients gender show significant differences between males and females. The following overall rates 50.66 % and 26.68 % were recorded in males using direct microscopy and ELISA respectively compare to 25.76% and 10.58 % in females by both methods respectively, P<0.05.Correlation between type of lab methods and patients age and gender; ELISA-copro antigen test reveal 7.33 % of *Blastocystis hominis* among patients ageing over 31 to 40 years in both gender.

Considering stool color and consistency in relation to *Blastocystis hominis* frequency; liquid stool samples exert high rate positivity of *Blastocystis* 77.14 %, 75.58 % and 63.33 % in brown, yellow and green colors respectively. Relationship between stool color and *Blastocystis hominis* distribution was not significant while it was significant in regard of stool consistency P<0.05. The results of the current study in regard of pH of stool samples and blastocystiasis show significance and high positivity specially pH of stool samples ranging from 6.1 to 7.0, the rate was 63.56 % compare to 27.13 % and 9.30 % for pH 7.1 to 8.5 and from 4.0 to 6.0 respectively (Table 3). Correlation among stool color, consistency, pH and *Blastocystis hominis* was significant, P<0.05. Stool samples belongs to irritable bowel syndrome patients was highly found 90.47 % in colorless mucoid stool with high dominancy rate 57.89 % in pH from 7.1 to 8.5 compare to 36.84 % and 5.26 % in stool samples with pH of 6.0 to 7.0 and in samples from 4.0 to 6.0 respectively, P<0.05.

Table.1 Positive and negative rates of *Blastocystis hominis*, sensitivity and specificity of direct microscopy and ELISA copro-antigen methods

Laboratory methods	Total positive		Total negative		Sensitivity	Specificity
	No.	%	No.	%		
Direct microscopy	88	40.55	129	59.44	99.72 %	99.62%
ELISA-Copro antigen	89	41.01	128	58.99	99.74 %	99.63 %
Total	88.54	0.78	128.55	8.22	99.73 %	99.62 %

Total number examined: 217

Table.2 Distribution of *Balstocystis hominis* according to age and gender using direct microscopy and ELISA-copro antigen

Age groups/years	Examined positive No. %	Males		Females	
		Direct microscopy	ELISA	Direct microscopy	ELISA
		Total +ve No. %	Total +ve No. %	Total +ve No. %	Total +ve No. %
6 month to 10 years	38 17.51	2712.44	125.52	115.06	52.30
11 to 20	2210.13	115.06	62.76	83.68	31.38
21 to 30	37 17.05	2611.98	125.52	115.06	31.38
31 to 40	2812.90	188.29	125.52	104.60	41.84
41 to 50	2511.50	156.91	8 3.68	104.6	41.84
51 to 60	209.21	8 3.68	6 2.76	52.30	20.92
Over than 61	10 4.60	5 2.30	2 0.92	10.46	20.92
Total	17782.9	110 50.66 *	5826.68**	5625.76	23 10.58

Total number examined: 217 *, **:P<0.05.

Table.3 Frequency of *Blastocystis hominis* according to stool color, consistency and pH

Stool color and consistency	Examined No %	Positive No %	Stool samples pH ranges		
			4 to 6	6.1 to 7.0****	7.1 to 8.5
			No %	No %	No %
Yellowish liquid	29 13.36	22 75.82 *	3 10.34	14 48.27	5 17.24
Yellowish soft	21 9.67	12 57.14	2 9.52	33.33 7	3 14.28
Yellowish formed	15 6.91	5 33.33	00.00	4 80.00	1 20.00
Greenish liquid	30 13.82	19 63.33 *	210.52	1368.42	4 21.05
Greenish soft	19 8.75	8 42.10	112.50	5 62.50	2 25.00
Greenish formed	11 5.06	4 38.36	125.00	3 75.00	0 0.00
Brown liquid	35 16.12	27 77.14 *	3 11.11	18 66.66	6 22.23
Brown soft	23 10.59	10 43.47	110.00	770.00	2 20.00
Brown formed	13 5.99	23.07 3	0 0.00	3100.00	0 0.00
Colorless mucoid ***	21 9.67	19 90.47 **	1 5.26	7 36.84	11 57.89 ***
Total	217 100	129 59.44	12 9.30	82 63.56	35 27.13

*,**: P<0.05 ***Stool belongs to irritable bowel syndrome.

Blastocystis hominis (B. hominis) is a parasite of uncertain role in human disease. 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*40.78 % was high, which reflect 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 1st

November 2104 to 30th June 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) and with Leelayoova (2008) in Thailand, whom they record 3.6 % and 18.9 % of *B. hominis* infection respectively (Karyaghdi, 2013; Leelayoova, 2008). Also the rates of infection in neighboring and in some Arab countries were lower than that recorded in current study; in study carried on in a Turkish university hospital Ozçakir *et al.* (2007) found 12.2% of *B hominis* and 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 that shows high rates of sensitivity and specificity as shown in table 1.

In spite of no significance differences between direct microscopy and ELISA method in demonstrating *B hominis* in stool samples, but ELISA copro-antigen test revealing 41.01 % than 40.55% was indicating to high precision and accuracy of the former tests; because in ELISA test when any stool sample contain the parasite (antigen) will bound to specific immobilized antibody on the solid phase of ELISA microwell (Bryant, 1986). So this test becomes high sensitive and specific than direct microscopy that requires good skills and experience in stool examination for detecting the parasitic infection. Some authors (Salman and Mustafa, 2013; Maraha and Butting, 2000) considered direct microscopy is easy, simple and less time consume than other test, but in current study ELISA test was superior to direct microscopy in detecting *B. hominis*. Regarding frequency of *B. hominis* according gender and types of laboratory methods; stool specimen from males in both methods reveals high rates of *B. hominis* than in females' specimen, this can be explained by the fact, that males in nature spend most of daytimes out of doors than females. This trait will enhance them male chances to parasitic infection involving *B. hominis* than females. Number of males 161 compare to 81 in current study may have role in increasing the rate of infection in males than in females can considered as second interpretation to this elevation. This finding was not compatible with that recorded in Baghdad and Kirkuk Province by Raof and Abdul-Rahaman (2011) and by Salman Mustafa (2013), whom they not record any differences in prevalence of *B. hominis* between males and females.

Age category (Table 2) had a significant effect polluted outdoor environment insufficient education contacting infected subjects and improper toilet and food hygiene are possible predisposing factors (Gerba *et al.,* 1996; Sagebiel *et al.,* 2009) Significant impact of age on incidence of infection with *B. hominis* was noticed in samples of patients aging from 6 months to 10 years with high frequency in males using direct microscopy and ELISA tests might be due to nosocomial infection, because this age group contribute about 38 patients, most

of them were admitted patients in Kirkuk pediatric general hospital. This hospital is most often crowded because there is no other pediatric hospital in Kirkuk Province. Also high rates in this age group might be due good sampling and arrival of the specimen to laboratory within time for stool examination. Outdoor activities by the adult males may also explain the significantly higher prevalence of *Blastocystis* infections among these groups. Previous studies have found significantly higher infection rates in adults than in children with the highest prevalence rate among young adults aged between 18 and 30 years (Nimri and Batchoun, 1994; Yaicharoen *et al.,* 2005; Qadri *et al.,* 1989). In contrast, other reports found a higher prevalence rate in children and females as compared to adults and males (Martin-Sanchez *et al.,* 1992; Senay and MacPherson, 1990). Moreover, a recent study has reported a significant reduction in the *Blastocystis* infection prevalence rate in older children when compared with younger children (Pipatsatitpong *et al.,* 2012). These contradictory findings suggest that the distribution of *Blastocystis* infection shows spatial heterogeneity with respect to age or sex factors. The age and gender correlations identified in this study may not represent physiological properties intrinsic to those hosts, but rather may be caused by the variation in environmental conditions associated with age and gender.

From clinical view diarrhea can be defined in absolute or relative terms based on either the frequency of bowel movements or the consistency (looseness) of stools. According to consistency, it classified in to watery, rice water, osmotic, inflammatory, bloody and mucoid diarrhea, while the color in diarrhea mostly related to types of nutrition, physiological condition, gastro-intestinal tract infection or injuries like rectal prolapse, bleeding, fistula and hemorrhoids. According to color of stool sample, laboratory technician can predict which types of parasites will be under microscopic field. High rate of *B. hominis* in brown liquid stool can be explained by the fact, that bile storage in gall bladder mostly used for fat digestion in small intestine, so *B. hominis* causing irritation to brush boarder can lead to rapid pass of stool to colon with incomplete water absorption produce brown color (Green, 1975). The same explanation to high frequency of *B. hominis* in yellowish liquid stool specimens. While high incidence in green liquid stool mostly related to in complete digestion of biliverdin (as its nature was green) (Sood, 1989).

Irritable bowel syndrome patients stool samples revealing 19/21 positive of *B. hominis* with the rate90.47 % was highlighting an idea of "*B. hominis* is co factor or predisposing factor to IBS among patients with persistent diarrhea". Appearance of mucus and colorless stool among IBS patients was indicating to high irritation caused by the parasite to large intestine region that evoke high rate sloughing of mucosa of the intestine (Boorom *et al.,* 2008).

Normal pH of stool is ranged between 7.0 and 7.5, during intestinal parasitic infection this range was mostly changed in to acid such as intestinal amoebiasis (Salman and Mustafa, 2013). In current study 82/63.56 % of stool samples have pH between 6.1 and 7.0 followed by 35/27.13% with pH of 7.1 to 8.5. This finding was referred to that of "pH between 6.1 and 7.0 was optimum pH for surviving of *B. hominis* particularly when the case was not IBS". Moreover in case when samples stool belongs to IBS, the alkalinity might be due to changes happened due to an excess inflammation inducted by other factors in GIT, because 11/57.89 %of alkaline pH of stool samples was related to IBS patients.

In conclusion, from the results of current study the followings can be concluded: *B. hominis* rate was high among patients with diarrhea and IBS. Young aged males stool samples should be checked for *B. hominis* using ELISA-copro antigen test as confirming to direct microscopy. Correlation between *B. hominis* incidence and patients' age, gender, stool color and consistency required further study to obtain significance. Macroscopically colorless mucoid, alkaline stool samples are sample of choices for detecting *B. hominis* microscopically.

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