PREVALENCE, RISK FACTORS AND COMPARATIVE DIAGNOSTIC STUDY BETWEEN IMMUNOFLUORESCENCE ASSAY AND ORDINARY STAINING TECHNIQUES IN DETECTION OF *BLASTOCYSTIS HOMINIS* IN FECAL SAMPLES

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Abstract

Blastocytis hominis (*B. hominis*) is a prevalent protozoon parasite. Difficulties in diagnostic approach to detect this parasite are still present. Moreover, the data on its prevalence are missing in many locations and inhabitants. This study compared between the sensitivity of immunofluorescence assay (IFA) and ordinary staining methods; Iodine, Safranine Methylene Blue (SMB), Modified Ziehl Neelsen (MZN) and Trichrome stains to identify *B. hominis* infection in fecal samples and its prevalence in Zagazig City. Full history taking and clinical examination were done for 201 cases of both sexes aged (2-50) years. Stool samples were examined using Iodine, SMB, MZN, Trichrome and IFA stains. *B. hominis* was detected in 85 cases (42.3%), of which 52 cases were *B. hominis* alone and 32 cases were asymptomatic (61.5%). The highest prevalence (49.4%) was detected among (2-15years) age group mostly males. IFA stain was taken as the gold standard. The sensitivity of trichrome, MZN, iodine, SMB stains were 91.7%, 72.9%, 60%, 55.3% respectively, and specificity was 100% for all stains.
Keywords: *Blastocytis hominis*, Prevalence, Iodine, Safranine methylene blue, modified Ziehl Neelsen, Trichrome, Immunoflurecence antibody.

Introduction

Intestinal protozoan infections considered the most prevalent infections in humans in developing countries that cause the significant morbidity and mortality (Alamir *et al,* 2013). *Blastocystis* is one of intestinal protozoon that infects a variety of vertebrates including human worldwide (Kurt *et al,* 2016). Taxonomy was based on isolated *Blastocystis hominis* from man (Lepczynska *et al,* 2016). Prevalence was between 30-50% in developing countries, 1.5-10% in developed ones and 30-40% in immunocompromised individuals (Balint *et al,* 2014).

B. hominis was in different forms; vacuolar, avacuolar, multivacuolar, cystic, amoeboid and granular. Cyst is the infective stage transmitted fecal-orally, and via contaminated food or water (Sadaf *et al,* 2013). Although, *B. hominis* was found in asymptomatic persons as a commensal parasite (Roberts *et al,* 2012), but was associated with different gastrointestinal disorders as diarrhea, abdominal pain, fatigue, constipation, flatulence and irritable bowel (Kurt *et al,* 2016).

Diagnosis of *B. hominis* by examination of the stool samples, is a routine diagnostic method in many laboratories, by ordinary methods, e.g. simple saline smear method, formaline-ether method, and Trichrome stain (Nithyamathi *et al,* 2016). But, its diagnosis was somewhat difficult due to its irregular shedding from day to day, morphological variation, the large sized form might be confused with fat cells, white blood cells and/or yeasts (Stensvold *et al,* 2010). Dogruman-Al *et al.* (2010) reported that Immunofluorescense antibody stain (IFA) gave better sensitivity. El-Marhoumy *et al.* (2015) found that IFA was a sensitive alternative, rapid and did not need skilled technician.

The current study compared between the sensitivity of immunofluorescence assay (IFA) and ordinary staining methods; iodine, Safranine Methylene Blue (SMB), Modified Ziehl Neelsen (MZN) and Trichrome stains to identify *B. hominis* infection in fecal samples and its' prevalence in Zagazig City, the

Capital of Sharkia Governorate.

Subjects and Methods

Study design and data collection: The cross sectional study was carried out from March 2015 to January 2016, on 201 cases (120 males & 81 females) with aged from 2 to 50 years, from Outpatient Clinics, Zagazig University Hospitals and Primary Health Cares with or without gastro-intestinal troubles. All were subjected to full history taking included age, sex, residence (rural or urban), personal hygiene, sources of food and drinking water supply. Those on parasitosis treatment, immunosuppressive drugs, chemotherapy or radiotherapy were excluded.

Stool collection and examination: Three fresh stool samples on three alternative days were collected in clean labeled covered containers. All samples were examined by concentrated Formol-ether sedimentation (Cheesbrough, 2000), Sheather's sugar floatation concentration (Elmarhoumy et al, 2015). Fecal samples were preserved in 10% formalin until used for stained by: a- Iodine: Lugol's iodine was applied to non-fixed smears (Fleck and Moody, 2015), b- modified Ziehl-Neelsen (MZN): Smears fixed with methanol for 30 seconds, stained with Kinyoun's carbol-fuchsin for 1 min, decolorized by acid alcohol for 2 min and then counterstained with malachite green for 2 min (Somoskövi et al, 2001), c- Safranine methylene blue (SMB): 3% HCl in 100% methanol for 3-5 min was applied to the fixed fecal smears; then staining with 1% aqueous safranine for 60 seconds followed by counterstaining with 1% methylene blue for 30 seconds (Baxby et al, 1984), d- Trichrome stain: the fecal smear immersed in 70% ethanol for 5 minutes, 5 minutes in ethanol iodine solution followed by staining with Trichrome for 10 minutes rinsed in acidified alcohol for 3 seconds, and then one dip in 95% ethanol (Cheesbrough, 1987).

Immunoflurescent antibody: IFA stain was applied using ParaFlor B reagent (Boulder, CO, USA). This reagent recognizes B. hominis directly from fecal samples in one step, using fluorescently labeled monoclonal antibodies that will react with the targeted B. hominis antigen. Steps of IFA stain were done according to manufacturer instructions. IFA stain was undertaken as the gold standard to which other used stains compared to true/false positive and true/false negative and calculate the accuracy, sensitivity and specificity of each stain. All stained slides were examined microscopically using 40x and 100x oil immersion. Criteria to assess different stains: Interpretation of morphological details, color, differentiation of the recovered B. hominis from background fecal debris (contrast) after (Moussa et al, 2008).

Ethics statement: The current study was approved by Institutional Review Board of Faculty of Medicine, Zagazig University. The study purpose was explained to all participants, and a written informed consent was obtained from all. Informed consent was taken from parents instead of their children.

Statistical analysis: Data were analyzed using SPSS version 20. So, the qualitative data represented as number and percentage. Differences between frequencies (qualitative variables) and percentages in groups were compared by Chi-square. Kappa test was used to estimate the agreement between the different stains used in this study. Positive predictive value (PPV), Negative predictive value (NPV) and accuracy were calculated. P value <0.05 was statistically significant and P<0.001 considered highly significant.

Results

Microscopically, out of 201 examined fecal samples for cases aged from 2-50 years, B. hominis was detected in 85 cases (42.3%); 52 cases (61.2%) were B. hominis as a single infection and 33 cases (38.8%) were co-infection (21.2%) as B. hominis with Cryptosporidium parvum and Cyclospora spp., B. hominis with Entamoeba histolytica (8.2%), B. hominis with Entamoeba coli (5.9%) and B. hominis with Giardia lambilia (3.5%). The 116 cases (57.7%) were B. hominis free. The prevalence of B. hominis (49.4%) in age group 2-15 years

was highly significant difference, as well as among male (69.4%) compared to female (30.6%). The highest prevalence of *B. hominis* was in Summer (44.7%), followed by Autumn (24.7%), Winter (22.2%) and then Spring (8.2%) with significant variation.

Clinically, *B. hominis* was detected alone in 32/52(61.5%) infected asymptomatic cases. The commonest symptom among 20 (38.5%) symptomatic ones in descending suffered from abdominal pain (53.1%), weight loss (34.3%), flatulence (21.9%), constipation (9.4%) and diarrhea (6.3%), some showed more than one symptom. As to risk factors, *B. hominis* infection was higher in rural areas 91.8% than urban ones. Positive cases were among those consumed tap water (77.6%), eating outdoors (83.5%) and bad personal hygiene (63.5%).

Blastocystis infection by IFA was 85/201 (42.3%) compared to Trichrome, MZN, iodine & SMB that showed 38.8%, 30.8%, 25.4% & 23.4% respectively, with significance between IFA and all other stains.

Using IFA stain as the gold standard, the accuracy of Trichrome stain was 96.5%, but MZN, iodine and SMB were 88.6%, 83.1% & 81.1% respectively. The sensitivity of Trichrome stain, MZN, iodine & SMB stain were 91.7%, 72.9%, 60% & 55.3% respectively, and specificity was 100% for all. NPV for Trichrome, MZN, iodine & SMB was 94.3%, 83.4%, 77.3%, & 75.3% respectively, and PPV was 100% for all stains.

With iodine stain, vacuolar, cyst forms were refractive, rounded or ovoid with variation in size. Central vacuole was yellowish surrounded by a thin band of cytoplasm containing deeply stained brown nuclei, easily distinguished by the grayish yellow background. Cyst was smaller than other forms, refractive yellow with a well-defined cyst wall with one or two deeply stained nuclei. With SMB stain, only the cyst form was identified as blue rounded or oval bodies, with faintly-stained center containing one or more deeply stained nuclei, background appeared pale blue. *C. parvum* and *Cyclospora* spp. were also detected in some *B. hominis* cases. With MZN stain, vacuolar, cystic, amoebic and multi-vacu-lar forms of *B. hominis* were identified. Cyst appeared rounded or oval, small in size with faintly stained center containing one or two dark stained nuclei and dark stained cell wall and amoebic form with extension pseudopodia. The multi vacuolar form contained multiple central vacuoles and a peripheral band of cytoplasm containing deeply stained nuclei. Vacuolar form appeared rounded and moderately greenish blue in color on a pale greenish blue background. The central vacuole stained pale greenish blue surrounded by a dark greenish blue rim of cytoplasm containing multiple deeply stained nuclei. *C. parvum* and *Cyclospora* were also detected by MZN stain in some *B. hominis* positive cases. With Trichrome stain, vacuolar form rounded, varied in size, central vacuole pale greenish surrounded by a thin band of cytoplasm containing deeply stained red nuclei. Cyst appeared as blue rounded or oval bodies, with faintly-stained center containing one or more deeply stained nuclei.

Details were given in tables (1, 2, 3, & 4) and figures (1, 2, 3, & 4).

Table 1: Prevalence of *B. hominis* regarding age, sex and seasons.

Variable	Total (n=201)		Positive (*n*=85)		Negative (n=116)		χ^2	P
	No	%	No	%	No	%		
Age:2 - 15	78	38.8	42	49.4	36	31		
16 – 30	60	29.9	17	20	43	37.1	9.08	<0.01*
31 - 50	63	31.3	26	30.6	37	31.9		
Sex: Male	120	59.7	59	69.4	61	52.6		
Female	81	40.3	26	30.6	55	47.4	5.77	<0.02*
Season: Summer	48	23.9	38	44.7	10	8.6		
Autumn	38	18.9	21	24.7	17	14.6		
Winter	49	24.4	19	22.4	30	25.9	56.76	<0.001**
Spring	66	32.8	7	8.2	59	50.9		

* Significant P < 0.05, ** highly significant P < 0.001, +ve = positive infection, -ve =negative infection.

Table 2: Prevalence of *B. hominis* regarding risk factors

Variable	Total (n=201)		+ve (*n*=85)		-ve (*n*=116)		χ^2	P
	No	%	No	%	No	%		
Residence: Rural	132	65.7	78	91.8	54	46.6	4.48	<0.001**
Urban	69	34.3	7	8.2	62	53.4		
Water: Tap water	139	69.2	66	77.6	73	62.9	4.98	<0.03*
Filterer or boiled water	62	30.8	19	22.4	43	37.1		
Food sources: Outdoor	147	73.1	71	83.5	76	65.5	8.1	<0.004**
Indoor	54	26.9	14	16.5	40	34.5		
Hand washing: Yes	102	50.7	31	36.5	71	61.2	12.01	<0.001**
No	99	49.3	54	63.5	45	38.8		

Table 3: Detection of *B. hominis* (n=201) by different stains

Techniques	Number	%	Kappa	P
IFA: +ve	85	42.3	---	---
-ve	116	57.7		
Trichrome: +ve	78	38.8	0.93	<0.001**
-ve	123	61.2		
MZN: +ve	62	30.8	0.76	<0.001**
-ve	139	69.2		
Iodine: +ve	51	25.4	0.63	<0.001**
-ve	150	74.6		
SMB: +ve	47	23.4	0.59	<0.001**
-ve	154	76.6		

** Highly significant (P < 0.001),

Table 4: Validity of direct stains to detect *B. hominis* compared to IFA

Variable	Trichrome	MZN	Iodine	SMB	IFA
True +ve	78	62	51	47	85
False +ve	0	0	0	0	0
True −ve	116	116	116	116	116
False -ve	7	23	34	38	0
Sensitivity	91.7	72.9	60	55.3	-
Specificity	100	100	100	100	-
PPV	100	100	100	100	-
NPV	94.3	83.4	77.3	75.3	-
Accuracy	96.5	88.6	83.1	81.1	-

Positive predictive value=PPV; Negative predictive value =NPV.

Discussion

B. hominis is the commonest intestinal protozoan identified in the stools of humans and many animals worldwide with prevalence rates ranging between 3% & 60% in different countries (Kurt *et al*, 2016). There was a controversy concerning the pathogenicity of *B. hominis*. Whether pathogenic or not, the parasite is capable of establishing chronic infections. Patients infected with *B. hominis* might remain asymptomatic, or suffer from gastrointestinal symptoms such as the abdominal pain, diarrhea, nausea, vomiting, bloating and anorexia (Dogruman-Al *et al*, 2010).

Diagnosis of *B. hominis* mainly depend on microscopy but it is difficult and most of *B. hominis* were not detectable in stool samples due to polymorphic nature of the parasite, variable shedding or use of unsuitable insensitive methods to detect the parasite (Stensvold *et al*, 2010).

In the present study, frequency of *B. hominis* infection was 42.3% (85/201), this high prevalence in Zagazig City, Sharkia was in accordance with other studies in Egypt. In Shebin El Kom, Menoufia Governorate the prevalence rate among 250 food handlers were 46.6% (Abd El Wahab and Selim, 2007). In Cairo the prevalence rate was 34.5% and it was higher 54.2% in the iron deficiency anemia patients (El Deeb and Tanta City, prevalence rate of *B. hominis* among preschoolchildren (2-5 years) of both sexes were 53% (El-Marhoumy *et al*, 2015).

Abroad, Khan and Alkhalife (2005) in Saudi Arabia reported 8.50% people's positive for *B. hominis*, in 2.4% of food handlers, *B.*

hominis was the only parasite, whereas in 1.9% & 4.2% of the cases were associated with pathogenic and non-pathogenic parasites respectively. El Safadi *et al.* (2014) in Senegal reported 100% infection among the people in low socio-economic areas. Dagaci *et al.* (2014) in Turkey reported that 94/617 (15.24%) were positive by microscopic examination and inoculation in Jones medium. Also, El Safadi *et al.* (2016) in France reported a prevalence rate of 18.1%. Nithyamathi *et al.* (2016) in Malaysia found the prevalence of *B. hominis* among 1760 school children was 10.6%

In the present study, the high prevalence was attributed to some risk factors especially in rural residence (91.8%) as consumption of tap water (77.6%), eating outdoors (83.5%) and bad personal hygiene mainly no handwashing (63.5%). These results agreed with Dagaci *et al*. (2014) who reported that patients with daily habits such as less hand washing especially before meals (15.9%), more eating outdoors (28.9%) and consumption of tap water (25.1%) were more exposed to *B. hominis*. A higher prevalence was detected among children aged 4-5 years in a rural area due to bad personal hygiene that facilitate feco-oral transmission, most of those children used unfiltered water (El-Marhoumy *et al*, 2015). Nithyamathi *et al.* (2016) reported *B. hominis* prevalence was higher in rural areas 13.7% than the urban ones 7.2%.

In the current study, *B. hominis* alone was (61.2%) followed by the co-infection with *C. parvum* and *Cyclospora* spp. (21.2%) *B. homin- is* with *E. hitolytica* (8.2%), *B. hominis* with *Entamoeba coli* (5.9%) and *B. hominis* with *G. lambilia* (3.5%). The results nearly agreed with El Marhoumy *et al.* (2015) who reported that *B. hominis* was alone in (91.2%) cases followed by *B. hominis* with *E. histolytica* (6.9%) and then *B. hominis* with *G. lambilia* (1.9%).

Also, Abd El Wahab and Selim (2007) reported that *G. lamblia, E. histolytica* and *C. parvum* mixed with *B. hominis*. But, Roberts

et al. (2011) reported that *Endolimax nana* was commonest protozoan parasite associated with *B. hominis* followed by *G. lamblia* and *E. histolytica*. Elghareeb *et al.* (2015) who reported that *G. lamblia* was frequent associated with *B. hominis*.

In the current study, high prevalence was among age group (2-15) years and males (69.4%) were the majority. This may be due to inadequate toilet training and hygiene practices of school-children and cross-transmission through close personal contact. Also, they are more active, playing with their colleagues outdoors and eating contaminated food. Also, they did not wash hands before meals with bad personal hygiene and consumption of tap water outdoors. Females they were less active outdoors and less in contact with contaminated materials. This agreed with Khoshnood *et al.* (2015) who reported that highest prevalence rate of *B. hominis* was in <15 year age (4.51%). El Safadi *et al.* (2016) who reported that *B. hominis* (26.3%) was among aged (1-14) years. But, Dagaci *et al.* (2014) reported that *B. hominis* was among aged 20-19 years was high (28.9%). Nithyamathi *et al.* (2016) reported that *B. hominis* rate among male (12%) was higher than in female (9%). Also El-Marhoumy *et al.* (2015) reported that *B. hominis* in males (59.8%) was high than in females (44.9%). But, Martin-Sanchez *et al.* (1992) recorded a high prevalence in female than male.

In the present study, *B. hominis* was high in Summer (44.7%), due to more consumption of vegetables and fruits, drinks with ice cubes and ice creams. Common water-based recreational activities may be involved, as human fecal contamination was clearly correlated with *B. hominis* load in many water sources suggesting a great risk. Trips in summer holidays particularly in overcrowded areas represented other risk factor. *B. hominis* has a wide variety of reservoirs, and these make people more vulnerable to the infection (Beyhan *et al*, 2015). Also, El Safadi *et al.* (2016) reported that *B. hominis*

infection in stool samples was significantly higher in summer. But, Amin (2002) reported that infection was lowest in May and highest in September and November.

In the present study, the pathogenicity of B. hominis infection alone (52 cases), there was an evidence of increased number of asymptomatic 32 cases (61.5%) more than symptomatic (20 cases) one (38.5%), this might be due to genetic variations or subtypes of isolates that predominate in the studied areas, and host factors as age, genetic variations and degree of the immune protection. This agreed with Amin (2002) who reported that asymptomatic *B. hominis* was 60% among American populations. Dagaci *et al.* (2014) found that 78/94 patients (82.9%) only infected with *B. hominis* had symptoms. El-Marhoumy *et al.* (2015) reported that infection was significantly high in symptomatic patients than in asymptomatic ones.

In the present study, *B. hominis* was detected in well-formed stool samples due to its asymptomatic nature, which was in contradiction of others. Dagaci *et al.* (2014) reported that abdominal pain and diarrhea were the leading symptoms in the patients. Elghareeb *et al.* (2015) reported that the commonest symptom of *B. hominis* infection was diarrhea either acute or chronic. El Safadi *et al.* (2016) reported that the most frequent symptom in symptomatic cases was bloating followed by abdominal pain. Nithyamathi *et al.* (2016) reported that abdominal pain was the commonest symptom.

In the present study, iodine was the most rapid, simple, and cheap and identified all forms, but *B. hominis* was confused with fat cells, white blood cells or yeasts and was not a permanent stain. Elghareeb *et al.* (2015) reported that iodine-stained smear was difficult to identify the organism in wet mounts due to its variation in size and shape. MZN and SMB stains gave bad contrast as the parasite and background had the same color. MZN stain was significantly more sensitive than iodine and SMB stains, being cheap, simple and different forms. This agreed with El-Marhoumy *et al.* (2015). Trichrome was better and more sensitive than MZN, iodine and SMB identified different forms, but time consuming, which agreed with Elghareeb *et al.* (2015). Tan (2008) found that Trichrome was more sensitive to detect intestinal protozoa than iodine-stained wet mounts.

IFA stain was more sensitive detected 85/201 (42.3%) in a short time with a clearity of positive and negative cases without any cross reactivity. All samples positive by other methods were also positive by IFA, without false positivity. IFA detected positive cases that were false negative by other stains and even IFA detected more *B. hominis* cases than Trichrome, which was time consuming and unsuitable for epidemiologic survey, but IFA gave high sensitivity and more suitable in central hospitals and public health laboratories. This agreed with Dogruman-Al *et al.* (2010) who reported that IFA detected more cases than either Trichrome or iodine stain. El-Marhoumy *et al.* (2015) reported that IFA was the most sensitive method followed by MZN, iodine and SMB stains respectively.

Conclusions

Blastocystis hominis is a causative agent of human disease in patients with recurrent symptoms. The results showed nonpathogenic *B. hominis* potential due to high asymptomatic cases compared to symptomatic ones as evidence of the genetic subtypes' isolates. Hygienic education for risky groups and periodic examination is a must.

Conflicts of Interest: Authors declared no conflict of interest regarding this work.

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Explanation of figures

Fig. 1: Detected parasites among *B. hominis* positive cases

Fig. 2: Symptoms of *B. hominis* single infected cases

Fig.3: Different stains; (1) Iodine fecal smear showed vacuolar form of *B. hominis* (x400). (2) Iodine fecal smear showed *B. hominis* cysts (x400). (3) SMB fecal smear showed vacular form of *B. hominis* (long arrow) and *C. parvum* (short thick arrow) (X1000). (4) SMB stained fecal smear showed *B. hominis* cyst (long arrow), *Cyclospora* (arrow head) and *C. parvum* (short thick arrow) (x1000). (5) MZN stained fecal smear showed multiple *B. hominis* cysts (long arrow) and amoebic form (arrow head) (x400). (6) MZN fecal smear showed *B. hominis* multi-vacular form (long arrow), *C. parvum* (short thick arrow) and *Cyclospora* (arrow head) (x1000). (7) MZN stained fecal smear showed *B. hominis* vacular form (long arrow), *C. parvum* (short thick arrow) and *Cyclospora* (arrow head) (x1000). (8) Trichrome fecal smear show-ed vacular form of *B. hominis* (long arrow) (x1000). (9) Trichrome fecal smear showed *B. hominis* cyst (x1000).

Fig. 4: IFA fecal smear showed brightly fluorescent rounded *B. hominis* cells (x400) (10) and brightly fluorescent rounded *B. hominis* cells (x1000) (11).

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