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ORIGINAL PAPER

Blastocystis hominis and *Dientamoeba fragilis* in patients fulfilling irritable bowel syndrome criteria

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Abstract Studies have suggested a possible role for *Blastocystis hominis* and *Dientamoeba fragilis* in the etiology of irritable bowel syndrome (IBS). We studied the prevalence of *B. hominis* and *D. fragilis* in patients with IBS-diarrhea (IBS-D). Three hundred and thirty patients were enrolled, 171 (52%) with IBS-D and 159 (48%) were controls, respectively. Stool microscopy, culture, and polymerase chain reaction (PCR) for *B. hominis* and *D. fragilis* were done. *B. hominis* was positive by stool microscopy in 49% (83/171) of IBS compared to 24% (27/159) in control ($p<0.001$). *B. hominis* culture was positive in 53% (90/171) in IBS compared to 16% (25/159) in control ($p<0.001$). *B. hominis* PCR was positive in 44% (75/171) in IBS compared to 21% (33/159) in control ($p<0.001$). *D. fragilis* microscopy was positive in 3.5% (6/171) in IBS-D compared to 0.6% (1/159) in control ($p=0.123$). *D. fragilis* culture was positive in 4% (7/171) in IBS compared to 1.3% (2/159) in control ($p=0.176$). *D. fragilis* PCR was positive in 4% (6/171) in IBS-D compared to 0% (0/159) in control ($p=0.030$). *B. hominis* is common, while *D. fragilis* was less prevalent in our patients with IBS-D. *B. hominis* and *D. fragilis* culture had a better yield compared to stool microscopy and PCR.

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Introduction

Parasitic infections afflict a wide range of populations in both urban and rural areas throughout the world. The direct impact of protozoan parasites on human is considerable in the third world countries with poor sanitation and quality of drinking water (Zonta et al. 2010). Parasitic infections such as *Blastocystis hominis* and *Dientamoeba fragilis* are associated with abdominal pain, bloating, and alteration of bowel habits resembling irritable bowel syndrome (IBS; Yakoob et al. 2004; Johnson et al. 2004). However, a diagnosis of IBS is based on ROME III criteria independently of specific stool collection and testing methods recommended for detection of parasites (Drossman 2006). Also, the methods commonly used to diagnose parasitic infections have a poor yield, and hence, these infections are never diagnosed. The prevalence of these parasites is highest in areas of poor sanitation and drinking water treatment as they are transmitted by fecal-oral route.

A significantly increased level of IgG2 levels against *B. hominis* was found in IBS patients compared with asymptomatic controls, indicating that the predominant response in these patients may be directed to carbohydrate antigens (Hussain et al. 1997). A number of studies have incriminated *D. fragilis* as a cause of IBS, allergic colitis, and diarrhea in human immunodeficiency virus patients (Johnson et al. 2004). In a recent study, a high prevalence of *D. fragilis* was reported in fecal samples collected from patients attending complementary medicine practitioners in the British Isles (Windsor et al. 2002). Approximately 25% of these stated intestinal symptoms, ranging from acute gastroenteritis to chronic intestinal symptoms, while similar in proportion did not report having intestinal symptoms. It is also noteworthy that over half of the *D. fragilis* positive samples were found in combination with *B. hominis* as

previously observed (Stensvold et al. 2007; Schuster and Jackson 2009; Stark et al. 2005; Norberg et al. 2003). *B. hominis* is a common human intestinal parasite that is commonly found in stool on examination. It can be easily demonstrated by light microscopy, culture, and polymerase chain reaction (PCR) examination of stool samples (Norberg et al. 2003; Zaman and Khan 1994). The pathogenic role of *B. hominis* is still controversial as it is frequently found not only in individuals with enteric symptoms but also in apparently healthy and asymptomatic subjects (Silberman et al. 1996). It is thought that genotypic difference exists between the asymptomatic and symptomatic *B. hominis* isolates (Stensvold et al. 2006; Yoshikawa et al. 2000). The aim of this study was to determine the prevalence of *B. hominis* and *D. fragilis* in patients with symptoms of IBS-D and compared them with healthy immunocompetent controls.

Materials and methods

Patient

A total of 330 stool samples were examined that were obtained from patients fulfilling Rome III criteria of IBS-D in 171 (52%) and 159 (48%) controls who attended the gastroenterology outpatient clinic at the Aga Khan University, Karachi between January 2008 and December 2009, respectively (Drossman 2006). The mean age of patients with IBS-D was 40±15, age range 16–83 years, and male: female ratio of 117:54. In control group, 159 (48%) were healthy volunteers with mean age 42±14. These patients underwent thorough history, physical examination, complete blood count, serum creatinine, electrolytes, and stool microscopy, culture, and PCR for *B. hominis* and *D. fragilis*. These patients had intact immunity. The study was approved by institutional ethics review committee.

All the stool specimens were processed by stool microscopy for *B. hominis* and *D. fragilis*. Stool culture was done for *B. hominis* and *D. fragilis*. DNA was extracted from the unfixed stool specimen, and it was used for PCR for *B. hominis* and *D. fragilis*. A note was made of the presence of other parasites such as *Giardia lamblia*, *Entamoeba* species, and *Cryptosporidium*, and these patients, ten in number, were excluded while no patient demonstrated *Enterobius vermicularis*. In previous study, *D. fragilis* mono-nucleated or bi-nucleated forms have been documented in the lumen of *E. vermicularis* (Johnson et al. 2004). A microbiological investigation was also performed to detect *Salmonella* spp., *Campylobacter jejuni*, *Clostridium difficile*, and *Vibrio cholerae*. However, a viral screen was not carried out on stool specimens in view of restriction of cost.

Microscopy of fecal smear

Fecal sample microscopy for demonstrating *B. hominis* was done as described before (Zaman and Khan 1994); approximately 2 mg of feces was emulsified on a glass slide in one drop of physiologic saline and covered with a cover slip. A similar preparation was made on another slide using Lugol's iodine. These preparations were examined under both the low power (×10) and high dry (×40) objectives. Specimen smear was fixed in sodium acetate/acetic acid/formalin (SAF) and then stained with modified trichrome stain to look for *D. fragilis* (Johnson et al. 2004).

Culture

Jones medium without starch was used for culturing *B. hominis* as described before (Zaman and Khan 1994). The cultures were incubated at 37°C and examined after 48 h. If no *B. hominis* were seen up to further 2 days, they were regarded as negative. The sediment was examined under both the low power (×10) and high dry (×40) objectives. *D. fragilis* culture was done in Robinson's medium as previously described (Windsor et al. 2003).

Extraction of genomic DNA

Stool DNA was extracted by using Stool DNA Extraction kit (Qiagen) according to the manufacturer's protocol. Extracted DNA was stored at −20°C until PCR was carried out for *B. hominis* and *D. fragilis*

PCR

The primers used were previously described (Table 1). The primer pair SR1F and SR1R was used to amplify a conserved region of the *B. hominis* small subunit (SSU) rRNA, while primer TRD3 and TRD5 design was based on the sequence of the of SSU rRNA gene of *D. fragilis* (Silberman et al. 1996; Yoshikawa et al. 2000; Table 1). The PCR reaction volume was 25 μl that comprised of 2.5 μl of 10× PCR buffer (Promega), 2.0 μl of 25 mM $MgCl_2$ (Promega), 0.4 μl of deoxyribo-nucleotide triphosphate mix (10 mM each dNTP, Promega), 0.5 μl (5 IU/μl) of *Taq* polymerase (Promega), 1 μl (0.25 μM) primers (IDT), and 2.0 μl of template DNA. The PCR products and molecular markers were electrophoresed in 2% agarose gel with Tris-acetate-EDTA electrophoresis buffer. The size markers were 100 bp ladder (Promega, USA). The PCR amplification for each primer pair was repeated at least thrice. Bands were visualized by the imaging system (Gel Doc 2000, Gel Documentation System, Bio Rad, UK) after being stained with ethidium bromide.

Table 1 Primer sequences used for *Blastocystis hominis* and *Dientamoeba fragilis*

		Primer sequences 5′–3′	Amplified product size, bp	PCR cycles
Blastocystis hominis				
SR1[a]	F	GCT TAT CTG GTT GATCCT GCC AGT AGT	1,800	94°C for 3 min; 94°C for 30 min, 57°C for 1 min 30 s, 72°C for 2 min (35 cycles); 72°C 7 min
SR2	R	TGA TCC TTC CGC AGG TTC ACC TA		
Dientamoeba fragilis				
TRD3[b]	F	GATCCAACGGCAGGTTCACCTACC	1,700	94°C for 5 min; 94°C for 1 min, 56°C for 1 min 30 s, 72°C for 2 min (35 cycles); 72°C 5 min
TRD5	R	GATACTTGGTTGATCCTGCCAAGG		

[a] Yoshikawa et al. (2000)

[b] Silberman et al. (1996)

Statistical method

Results are expressed as mean±standard deviation for continuous variables (e.g., age) and number (percentage) for categorical data (e.g., gender, stool culture, and diarrhea). Univariate analysis was performed by using the independent sample *t* test, Pearson Chi-square test, and Fisher exact test where appropriate. A p value of <0.05 was considered as statistically significant. All p values were two sided. Statistical interpretation of data was performed by using the computerized software program SPSS version 16.0.

Results

The age and sex of the patients were not related to the positivity of *D. fragilis* and *B. hominis* when culture was used as the diagnostic test for *D. fragilis* and *B. hominis*. One hundred ninety-three (59%) had diarrhea, and 71 (52%) had abdominal pain.

Distribution and diagnostic yield of various tests for *B. hominis*

B. hominis was positive by microscopy in 33% (110/330), culture in 35% (115/330), and PCR in 33% (108/330; Table 2). Microscopy for *B. hominis* was positive in 76% (83/110) in patients fulfilling the criteria of IBS compared to 24% (27/110) in control ($p<0.001$; Table 3). Culture for *B. hominis* was positive in 78% (90/115) in IBS compared to 22% (25/115) in control ($p<0.001$). PCR for *B. hominis* was positive in 69% (75/108) in IBS compared to 31% (33/108) in control ($p<0.001$; Table 3).

Distribution and diagnostic yield of various tests for *D. fragilis*

D. fragilis was positive by microscopy in 2% (7/330), culture in 3% (11/330), and PCR in 2% (6/330), respectively (Fig 1; Table 2). Microscopy for *D. fragilis* was positive in 86% (6/7) in IBS compared to 14% (1/7) in control ($p=0.123$; Table 3). Culture for *D. fragilis* was positive in 64% (7/11) in IBS compared to 36% (4/11) in control ($p=0.425$; Table 3). PCR for *D. fragilis* was positive in 86% (6/6) in IBS compared to none in control ($p=0.030$; Table 3).

Coinfection with *B. hominis* and *D. fragilis* in different groups

Microscopy was positive for both *B. hominis* and *D. fragilis* in 3% (5/171) with IBS compared to zero (0/159) in control ($p=0.061$; Table 2). Culture for both *B. hominis* and *D. fragilis* was positive in 2.3% (4/171) with IBS compared to zero (0/159) in control ($p=0.123$; Table 3). PCR was positive for both *B. hominis* and *D. fragilis* in 3% (5/171) with IBS compared to zero (0/159) in control ($p=0.061$; Table 3).

Comparison of culture for *B. hominis* and *D. fragilis* with other test

In patients with IBS, *B. hominis* culture was positive in 90, while microscopy for *B. hominis* was positive in 82% (74/90; $p<0.001$) and PCR in 76% (68/90; $p<0.001$). In IBS patients, *D. fragilis* was positive in seven (4%) with culture,

Table 2 Diagnostic yield of various tests for *Blastocystis hominis* and *Dientamoeba fragilis*

	Microscopy	Culture	PCR
Blastocystis hominis			
Positive	110 (33)	115 (35)	108 (33)
Negative	220 (67)	215 (65)	222 (67)
Dientamoeba fragilis			
Positive	7 (2)	11 (3)	6 (2)
Negative	323 (98)	319 (97)	324 (98)
Blastocystis hominis and *Dientamoeba fragilis*			
Positive	5 (2)	4 (1)	5 (2)
Negative	325 (98)	326 (99)	325 (98)

Table 3 Comparison of *Blastocystis hominis* and *Dientamoeba fragilis* in different groups

	IBS, *n*=171	Control, *n*=159	*P* value
Blastocystis hominis			
Microscopy			
Positive	83 (48)	27 (17)	<0.001
Negative	88 (52)	132 (83)	
Culture			
Positive	90 (53)	25 (16)	<0.001
Negative	81 (47)	134 (84)	
PCR			
Positive	75 (44)	33 (21)	<0.001
Negative	96 (56)	126 (79)	
Dientamoeba fragilis			
Microscopy			
Positive	6 (4)	27 (17)	0.123
Negative	165(96)	132 (83)	
Culture			
Positive	7 (4)	4 (2)	0.545
Negative	164 (96)	155 (98)	
PCR			
Positive	6 (4)	0 (0)	0.030
Negative	165 (96)	159 (100)	
Coinfection with *B. hominis* and *Dientamoeba fragilis*			
Microscopy			
Positive	5 (3)	0 (0)	0.061
Negative	166 (97)	159 (100)	
Culture			
Positive	4 (2)	0 (0)	0.124
Negative	167 (98)	159 (100)	
PCR			
Positive	5 (3)	0 (0)	0.061
Negative	166 (97)	159 (100)	

while microscopy for *D. fragilis* was positive in 57% (4/7; $p<0.001$), and PCR for *D. fragilis* was not positive in any patients 0/7 ($p=1$).

Discussion

This study showed *B. hominis* was significantly positive in IBS while *D. fragilis* was positive in only 4%. In patients with IBS, *B. hominis* culture was significantly positive compared to microscopy and PCR for *B. hominis*. Similarly, culture of *D. fragilis* had a better yield compared to microscopy with modified trichrome staining and PCR. Coinfection with both *B. hominis* and *D. fragilis* in IBS patients was documented in 3% with culture and 2.3% by microscopy. *B. hominis* and *D. fragilis* infections did not show any age and gender distribution. However, one third of the *D. fragilis* infections were in patients who were in their twenties. None of the patients with *D. fragilis* demonstrated concomitant infection with *E. vermicularis.*

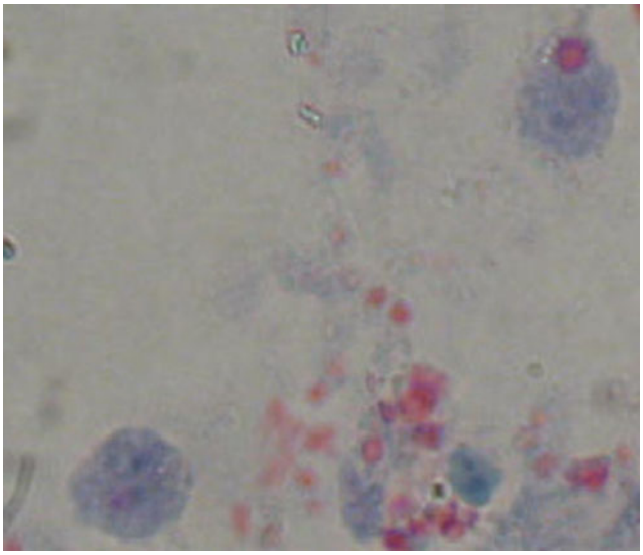

Fig. 1 *Dientamoeba fragilis* in stool specimen stained with modified trichrome stain under ×100 magnification

The implications of this study are that *B. hominis* are found with a higher frequency in our IBS patients compared to *D. fragilis*. The coinfection of *B. hominis* and *D. fragilis* was seen in only IBS group and none in the fecal samples from control group comprising of healthy individuals without gastrointestinal symptoms. This study has shown that *D. fragilis* was associated with IBS-like symptoms in our patients, though it has a low prevalence. It is still possible that we were not able to document all the *D. fragilis* infections in spite of using three different modalities, i.e., microscopy with modified trichrome staining of fixed stool specimen, culture of stool specimens, and PCR examination of unfixed stool specimens. It is possible that if we examined three fixed and three unfixed stool specimens from each of these patients, the yield might have increased. Also, increasing number of symptomatic patients would have helped. Windsor et al. found *D. fragilis* culture has the best yield compared to microscopy with modified iron-hematoxylin staining of stool specimen (Windsor et al. 2003). An Australian study previously described an association between *D. fragilis* and IBS with 21 IBS patients diagnosed with concurrent *D. fragilis* infection (Brody et al. 2002). In another prospective study, 6,750 fecal samples were examined for *D. fragilis*. Trophozoites of *D. fragilis* were detected in 60 (0.9%) patients by permanent staining, using a modified iron-hematoxylin stain that was confirmed by PCR (Stark et al. 2005).

Chronic symptoms were present in 32% with diarrhea and abdominal pain present in all of these patients (Stark et

al. 2005). In the control group comprising of 900 stool samples from patients without gastrointestinal symptoms, no *D. fragilis* was detected by permanent staining (Stark et al. 2005). Our results concur with that of Stark et al. (2005). This is the first report of documentation of *D. fragilis* in our population. It was found infrequently in our patients with IBS. *D. fragilis* is a pathogenic organism rather than a commensal, as diarrhea and abdominal pain were the most common symptoms in both acute and chronic infections (Johnson et al. 2004; Stark et al. 2005; Dobell 1940; Windsor and Johnson 1999; Yang and Scholten 1977). We also did not find any correlation between *D. fragilis* and *E. vermicularis*, a proposed vector of transmission for *D. fragilis* in keeping with previous study (Stark et al. 2005).

The results of this study concur with a previous study that culture of *B. hominis* has a better yield than that of microscopy (Zaman and Khan 1994). However, *B. hominis* culture yield was not significantly different from *B. hominis* PCR. In recent years, studies have suggested that there are genotypes of *B. hominis* associated with symptomatic and asymptomatic states (Hussein et al. 2008; Tan 2008; Yan et al. 2006). Different degrees of pathological changes were present among infected rats by symptomatic subtypes 1, 3, and 4 compared with asymptomatic subtypes, e.g., 2, 3, and 4 (Hussein et al. 2008). The moderate and severe degree of pathological changes was found only in symptomatic subtypes infected rats, while mild degree was found only in asymptomatic subtypes infected rats (Hussein et al. 2008). The intestinal cell permeability was increased in symptomatic subtype 1 compared to symptomatic subtypes, e.g., 3 and 4, infected rats (Hussein et al. 2008). Minimal effects were found in the asymptomatic and control groups. These results proved that subtype 1 was clinically and statistically highly relevant to the pathogenicity of *B. hominis*, while subtype 2 was irrelevant (Hussein et al. 2008). Also, the results suggested the presence of pathogenic and nonpathogenic strains among subtypes 3 and 4 (Hussein et al. 2008). A study from China also described a possible relationship between subtype 1 and a pathogenic potential of *B. hominis* (Yan et al. 2006). Similarly, *Blastocystis* subtype 3 was described as the most dominant genotype in asymptomatic individual, and subtype 1 determined all of symptomatic patients in studies from Turkey and Pakistan (Eroglu et al. 2009; Yakoob et al. 2010). *D. fragilis* prevalence appears to be low in our IBS population.

In conclusion, we demonstrated presentation of *B. hominis* and *D. fragilis* infections with symptoms of IBS; however, only a prospective study with a larger sample size is required to confirm this association of *D. fragilis* with IBS-like symptoms in our population.

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Conflict of interest The authors declare they have no conflict of interests.

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