June 2014

Cytokine changes in colonic mucosa associated with Blastocystis spp. subtypes 1 and 3 in diarrhoea-predominant irritable bowel syndrome

Javed Yakoob
Aga Khan University

Zaigham Abbas
Aga Khan University

Muhammad Waqas Usman
Aga Khan University

Aisha Sultana
Aga Khan Unviersity

Muhammad Islam
Aga Khan University

See next page for additional authors

Authors
Javed Yakoob, Zaigham Abbas, Muhammad Waqas Usman, Aisha Sultana, Muhammad Islam, Safia Awan, Zubair Ahmad, Saeed Hamid, and Wasim Jafri

Cytokine changes in colonic mucosa associated with *Blastocystis* spp. subtypes 1 and 3 in diarrhoea-predominant irritable bowel syndrome

JAVED YAKOOB[1]*, ZAIGHAM ABBAS[1], MUHAMMAD WAQAS USMAN[1], AISHA SULTANA[2], MUHAMMAD ISLAM[3], SAFIA AWAN[1], ZUBAIR AHMAD[2], SAEED HAMID[1] *and* WASIM JAFRI[1]

[1] *Department of Medicine, Aga Khan University, Karachi, Pakistan*
[2] *Department of Pathology and Microbiology, Aga Khan University, Karachi, Pakistan*
[3] *Department of Community Health Sciences, Aga Khan University, Karachi, Pakistan*

(*Received 15 July 2013; revised 26 July and 20 August 2013; accepted 20 August 2013; first published online 5 March 2014*)

SUMMARY

We determined cytokines (e.g. interleukin-8, 10, 12 and TNF-α) expression by peripheral blood mononuclear cells (PBMCs) and in rectal mucosa in diarrhoea-predominant irritable bowel syndrome (D-IBS) with *Blastocystis* spp. Eighty patients with D-IBS and *Blastocystis* spp. infection were classified as 'cases' and 80 with D-IBS without *Blastocystis* spp. infection were classified as 'control'. Cases were subdivided into D-IBS and *Blastocystis* sp. defined type 1 (subtype-specific primer SB83) and type 3 (SB227). Stool microscopy and culture were performed. Rectal biopsies were obtained for histology and cytokines by real-time PCR for mRNA expression of cytokines. PBMCs IL-8 was similar in different groups but in type 1, IL-8mRNA was increased compared with type 3 ($P = 0{\cdot}001$) and control ($P = 0{\cdot}001$). In type 1, IL-10 by PBMCs had a low mean value ($14{\cdot}5 \pm 1{\cdot}6$) compared with ($16{\cdot}7 \pm 1{\cdot}5$) type 3 and ($16 \pm 2{\cdot}3$) in controls ($P < 0{\cdot}001$ and $P < 0{\cdot}001$, respectively). In *Blastocystis* sp. type 1, low IL-10 was associated with lymphocyte and plasma cell infiltration ($P = 0{\cdot}015$ and $P = 0{\cdot}002$, respectively). In *Blastocystis* sp. type 1 and type 3, IL-12 was associated with goblet cell depletion 23 (85%) ($P < 0{\cdot}001$) and 8 (29%) ($P = 0{\cdot}037$), respectively. In *Blastocystis* sp. type 1, low IL-10 was associated with a proinflammatory response characterized by IL-8.

Key words: D-IBS, *Blastocystis* spp., IL-12, IL-10, TNF-α, IL-8.

INTRODUCTION

Blastocystis spp. is a protozoan parasite commonly found in the human gastrointestinal tract. The symptoms attributed to infection with *Blastocystis* spp. include diarrhoea, abdominal pain or discomfort, etc. *Blastocystis* spp. from humans and animals exhibit similar morphological features. Extensive genetic variability has been reported in *Blastocystis* spp. of both human and animal origin. A number of molecular techniques used to study the genetic diversity of *Blastocystis* spp. include PCR-restriction fragment length polymorphism (RFLP) (Abe *et al.* 2003*a*, *b*, *c*), PCR followed by dideoxy sequencing (Abe, 2004; Yoshikawa *et al.* 2004; Stensvold *et al.* 2006), and PCR with subtype-specific (sequence-tagged site [STS]) primers (Abe *et al.* 2003*a*, *b*, *c*; Khan and Alkhalife, 2005; Yakoob *et al.* 2010).

Oral inoculation with 1×10^5 cysts of *Blastocystis* sp. strain RN94-9 in rats resulted in chronic infection in the caecum for 4 weeks after infection (Iguchi *et al.* 2009). Histological examination revealed only a slight increase in goblet cells in the caecal mucosa 1–3 weeks postinfection but no inflammatory cell infiltration nor mucosal sloughing. Significant upregulation of the expression of interferon-γ, IL-12 and TNF-α, but not IL-6 or granulocyte–macrophage colony stimulating factor (GM-CSF) was demonstrated in the caecal mucosa at 2 weeks post-infection. The induction of local host responses, including mild goblet cell hyperplasia, and significant upregulation of type-1 and proinflammatory cytokines, suggests that *Blastocystis* sp. strain RN94-9 is a weakly pathogenic organism that could elicit proinflammatory as well as protective responses in local tissues (Iguchi *et al.* 2009). The cysteine proteases of *Blastocystis ratti* WR1, a zoonotic isolate, can activate IL-8 gene expression in human colonic epithelial cells. The molecular mechanism by which *Blastocystis* activates IL-8 gene expression in human colonic epithelial T84 cells and the production of IL-8 is dependent on NF-κB activation (Puthia *et al.* 2008). Previously, an experimental *in vitro* study demonstrated *Blastocystis* spp. is able to trigger inflammatory cytokine response in colonic epithelial cells (Long *et al.* 2001). After 24 h incubation of *Blastocystis* spp. with the cell lines HT-29 and T-84, *B. hominis* cells were not able to

* Corresponding author: Department of Medicine, Aga Khan University, PO Box 3500, Stadium Road, Karachi-74800, Pakistan. E-mail: yakoobjaved@hotmail.com

doi:10.1017/S003118201300173X

cause cytopathic effects, but significantly increased the release of IL-8 and GM-CSF. However, after the first 6 h of co-incubation, the production of IL-8 was not increased in HT-29 cells and even reduced when *Escherichia coli* (bacteria or lipopolysaccharide) were present during co-incubation. Similar effects were observed using supernatants of *Blastocystis* spp. culture. These data indicate that *Blastocystis* spp. induces as well as modulates the immune response in intestinal epithelial cells (Long *et al*. 2001).

Irritable bowel syndrome (IBS) is common and has a significant medical and socioeconomic impact. It is considered to be a biopsychosocial disorder resulting from an interaction among many factors that included diet, gut microflora, visceral hyperalgesia, genetic and environmental factors, infection, inflammation, gut motility and psychological factors (Drossman *et al*. 2002). IBS is associated with abdominal pain or discomfort and an alteration in bowel habits (Drossman *et al*. 2002). There has been a suggestion that some patients with IBS may harbour bacterial overgrowth leading to low-grade inflammation (O'Leary and Quigley, 2003) and immune activation (O'Sullivan *et al*. 2000; Chadwick *et al*. 2002), and their symptoms may be ameliorated by its eradication (Pimentel *et al*. 2000). Imbalances in pro- and anti-inflammatory cytokine production may promote ongoing low-grade inflammation after an acute gastroenteritis, and subsequently lead to IBS (van der Veek *et al*. 2005). Thus abnormal neuroimmune interactions may contribute to the altered gastrointestinal physiology and hypersensitivity that underlies IBS (Barbara *et al*. 2002). Previously, we demonstrated that stool culture for *Blastocystis* spp. was positive in 46% of the patients with D-IBS (Yakoob *et al*. 2004). In the present study, we determined the cytokine profile in patients with D-IBS and in those with concomitant *Blastocystis* sp. type 1 and 3 infection. We evaluated cytokine production *in vitro* from PBMC and determined tissue cytokine levels of IL-8, IL-10, IL-12 and TNF-α. IL-12 is a marker of Th1 response and IL-10 of T regulatory response. The outcome of an immune response depends on the balance between pro- and anti-inflammatory responses. We aimed for a comprehensive overview of cytokine profiles including the anti-inflammatory cytokine IL-10.

MATERIALS AND METHODS

Study population

This prospective study was conducted at the Aga Khan University in Karachi, Pakistan. One hundred and seventy patients with symptoms suggestive of D-IBS, according to the Rome III criteria, who attended the gastroenterology clinic from December 2009 to December 2011, were enrolled. Rome III criteria define IBS as recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months that started at least 6 months before diagnosis, cannot be explained by a structural or biochemical abnormality, and is associated with at least two of the following: improvement with defecation, onset associated with a change in frequency of stool, and onset associated with a change in form (appearance) of stool (Drossman and Dumitrascu, 2006). Other symptoms that support the diagnosis but are not part of the criteria include abnormal stool frequency ($\leqslant$ 3 bowel movements per week or >3 bowel movements per day), abnormal stool form (lumpy/hard or loose/watery), defecation straining, urgency, or feeling of incomplete bowel movement, passing mucus and bloating. Four possible IBS subtypes include IBS with constipation (C-IBS), IBS with diarrhoea (D-IBS), mixed IBS (M-IBS) and un-subtyped IBS depending on the predominant stool pattern (Drossman and Dumitrascu, 2006). Of these ten patients were excluded; three had coinfection of *Giardia lamblia* and one *Entameba coli* with *Blastocystis* spp. while there were 2 each with *Blastocystis* sp. types 2 and 4 and 1 each with *Blastocystis* sp. types 5 and 6, respectively. Forty (25%) were infected with *Blastocystis* sp. type 1 and 40 (25%) with *Blastocystis* sp. type 3, respectively (Table 2). Eighty patients with D-IBS and *Blastocystis* spp. infection were defined as 'cases' and 80 D-IBS patients without *Blastocystis* spp. infection were 'control' (Table 1). Cases were subdivided into patients with D-IBS and *Blastocystis* spp. infection type 1 or type 3. Standardized subtype-specific (STS) primers for *Blastocystis* sp. subtype 1 (SB83) and subtype 3 (SB227) defined *Blastocystis* sp. type 1 and 3. The study was approved by the Ethics Committee of the Aga Khan University and written informed consent was obtained from all patients. After enrolment of eligible patients in the study, a detailed history and physical examination was carried out. Colonoscopy of the patient was performed to rule out any organic disease and four rectal biopsies were taken, two each for histopathology and cytokine levels. Blood samples were obtained for cytokines analysis. Three stool samples collected from each patient on alternate days were examined by microscopy and cultured for *Blastocystis* spp. Coeliac disease, small bowel bacterial overgrowth and lactose intolerance were excluded by measuring tissue transglutaminase antibodies (IgA and IgG) and lactose intolerance by hydrogen breath test, respectively. The inclusion criteria were adults from 18 to 61 years of age with D-IBS, satisfying ROME III criteria (Drossman and Dumitrascu, 2006). Exclusion criteria were pregnant and lactating females, inflammatory bowel disease and other systemic disease, patients on laxatives or anti-diarrhoeal drugs that could influence the motility of gut, patients on antibiotics that could alter the

Table 1. Comparison of characteristics of controls with and without *B. hominis* infection

	Blastocystis hominis positive, $n = 80$	*Blastocystis hominis* negative, $n = 80$	P value
Age (years)			
$\leqslant 35$ years	33 (41)	32 (40)	0·872
$\geqslant 36$ years	47 (59)	48 (60)	
Sex			
Male	60 (75)	58 (72)	0·719
Female	20 (25)	22 (28)	
Symptoms			
Abdominal pain			
Yes	68 (85)	65 (81)	0·527
No	12 (15)	15 (19)	
Bloating			
Yes	55 (69)	53 (66)	0·736
No	25 (31)	27 (34)	
Stool frequency			
$\leqslant 4$ per day	4 (5)	8 (10)	0·369
$\geqslant 5$ or more	76 (95)	72 (90)	
Stool microscopy			
Positive	55 (69)	0 (0)	$<0·001$
Negative	25 (31)	80 (100)	
Stool culture			
Positive	80 (100)	0 (0)	$<0·001$
Negative	0 (0)	80 (100)	
Histology			
Nonspecific colitis	58 (72)	53 (66)	0·391
Normal	22 (28)	27 (34)	
Neutrophil			
Absent	50 (63)	38 (47)	0·057
Mild	30 (37)	42 (53)	
Eosinophil			
Absent	37 (46)	47 (59)	0·113
Mild	43 (54)	33 (41)	
Lymphocyte			
Mild	62 (77)	64 (80)	0·699
Moderate	18 (23)	16 (20)	
Plasma cells			
Mild	62 (77)	58 (72)	0·465
Moderate	18 (23)	22 (28)	
Goblet cell depletion			
Absent	43 (54)	38 (47)	0·429
Mild	37 (46)	42 (53)	
Intraepithelial lymphocyte			
Absent	55 (69)	60 (75)	0·379
Mild	25 (31)	20 (25)	
PBMC cytokines			
IL-8			
$<12·4$ pg mL^{-1}	45 (56)	31 (39)	0·027
$\geqslant 12·4$ pg mL^{-1}	35 (44)	49 (61)	
IL-10			
$<15·8$ pg mL^{-1}	48 (60)	37 (46)	0·081
$\geqslant 15·8$ pg mL^{-1}	32 (40)	43 (54)	
IL-12			
$<5·8$ pg mL^{-1}	55 (69)	62 (77)	0·212
$\geqslant 5·8$ pg mL^{-1}	25 (31)	18 (23)	
TNF-α			
$<9·3$ pg mL^{-1}	28 (35)	32 (40)	0·514
$\geqslant 9·3$ pg mL^{-1}	52 (65)	48 (60)	

Table 2A. The sequence-tagged site (STS) primer used

Subtypes	STS primer sets	Product size (bp)	Sequences of forward (F) and reverse (R) primers (5′ to 3′)		GenBank accession no.
1	SB83	351	F	GAAGGACTCTCTGACGATGA	AF166086
			R	GTCCAAATGAAAGGCAGC	
2	SB155	650	F	ATCAGCCTACAATCTCCTC	AF166087
			R	ATCGCCACTTCTCCAAT	
3	SB227	526	F	TAGGATTTGGTGTTTGGAGA	AF166088
			R	TTAGAAGTGAAGGAGATGGAAG	
4	SB332	338	F	GCATCCAGACTACTATCAACATT	AF166091
			R	CCATTTTCAGACAACCACTTA	
5	SB340	704	F	TGTTCTTGTGTCTTCTCAGCTC	AY048752
			R	TTCTTTCACACTCCCGTCAT	
6	SB336	317	F	GTGGGTAGAGGAAGGAAAACA	AY048751
			R	AGAACAAGTCGATGAAGTGAGAT	
7	SB337	487	F	GTCTTTCCCTGTCTATTCTGCA	AY048750
			R	AATTCGGTCTGCTTCTTCTG	

Table 2B. Cytokines primer sequence

Serial no.	Gene	Sequence	
1.	IL-12	5′-CACTCCAGACCCAGGAATGTTC-3′	F
		5′-TTGTCTGGCCTTCTGGAGCAT-3′	R
2.	IL 10	5′- ACGGCGCTGTCATCGATT-3′	F
		5′ GGCATTCTTCACCTGCTCCA-3′	R
3.	IL-8	5′-ACTGCGCCAACACAGAAATT-3′	F
		5′-TTCTCCACAACCCTCTGCAC-3′	R
4.	TNF-α	5′-CCCTGGTATGAGCCCATCTATC-3′	F
		5′-AAAGTAGACCTGCCCAGACTCG-3′	R
5.	GAPDH	5′-GAAGGTGAAGGTCGGAGTC-3′	F
		5′-GAAGATGGTGATGGGATTTC-3′	R

F = Forward primer; R = Reverse primer.
Tsukada *et al*. (2002).

enteric flora, patients with any ongoing infection, not willing to participate and those with *Blastocystis* spp. infection of more than one genotype. We chose to study only D-IBS patients as they were more frequently documented to be positive for *Blastocystis* spp. in our patients previously as compared with other IBS subtypes (Yakoob *et al*. 2004). The stool samples from D-IBS patients were screened with the seven kinds of STS primers 1, 2, 3, 4, 5, 6 and 7. However, we studied only genotypes of *Blastocystis* sp. type 1 and 3 which are the predominant types of *Blastocystis* spp. identified among our D-IBS patients (Yakoob *et al*. 2010).

Histology

Colonic biopsy specimens were obtained from rectum at the recto-sigmoid junction and were fixed immediately in 10% formalin in sodium phosphate buffer and sent to the Department of Pathology for processing. Biopsies were embedded in paraffin, and histological sections were stained with haematoxylin and eosin for evaluation. Histological inflammation was graded as 0–3 (0 – nil or without evidence of histopathology, 1 – mild, 2 – moderate, 3 – severe) according to the infiltration by neutrophils, eosinophils, lymphocytes, plasma cells and epithelial cell damage e.g. goblet cell depletion, intraepithelial cell lymphocytes (IEL). The pathologist was blinded to cytokine data.

Microscopy of fecal smear

Fecal sample microscopy was done as described before (Zaman and Khan, 1994). Briefly, approximately 2 mg of feces was thoroughly emulsified on a glass slide in one drop of physiological 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. Three fecal samples were examined on alternate days before giving a negative diagnosis of infection with an intestinal protozoan.

Culture of feces

Blastocystis spp. culture was done by inoculating approximately 50 mg of feces into Jones' medium. *Blastocystis* spp. culture was performed using Jones medium without starch (Zaman and Khan, 1994). The cultures were incubated at 37 °C and examined after 2–3 days for one week to exclude slow-growing parasites. The sediment was examined under both the low power (×10) and high dry (×40) objectives. The positive samples were subcultured for another 3 days using fresh media.

Extraction of genomic DNA

Genomic DNA of *Blastocystis* spp. was extracted by using a Stool DNA Extraction kit (Qiagen) according to the manufacturer's protocol. Extracted DNA was stored at −20 °C until PCR was carried out for *Blastocystis* spp. genotyping.

Blastocystis *spp. genotyping by PCR with STS primers*

Seven kinds of STS primers developed for typing the *Blastocystis* spp. isolates were used as described previously (Table 2A) (Yoshikawa *et al*. 1998, 2000, 2003; Abe *et al*. 2003*a*, *b*; Yan *et al*. 2006; Li *et al*. 2007*a*, *b*). Seven standardized subtype-specific STS primers were used, namely SB83 (351 bp) for subtype 1, SB340 (704 bp) for subtype 2, SB227 (526 bp) for subtype 3, SB337 (487 bp) for subtype 4, SB336 (317 bp) for subtype 5, SB332 (338 bp) for subtype 6, and SB155 (650 bp) for subtype 7 according to a recent classification terminology (Stensvold *et al*. 2007). Typing of the *Blastocystis* spp. isolates was conducted through PCR amplification on the basis of the presence or absence of the products with parallel control. The PCR conditions consisted of one cycle denaturing at 94 °C for 3 min, 30 cycles including annealing at 59 °C for 30 s, extending at 72 °C for 60 s, denaturing at 94 °C for 30 s, and additional cycle with a 5-min chain elongation at 72 °C (PCR System 9700, Perkin Elmer, USA). 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.

Isolation of peripheral blood mononuclear cells

Blood samples from patients with D-IBS (approximately 4 mL) were collected in sterile ethylene diaminetetra-acetate (EDTA) containing tubes. Peripheral blood mononuclear cells were isolated using Histopaque (Sigma-Aldrich, USA) according to the density gradient centrifugation method (Chomczynski and Sacchi, 1987). The collected PBMCs were washed twice with Roswell Park Memorial Institute (RPM 1640) culture medium and resuspended in growth medium of RPMI supplemented with 10% foetal bovine serum (FBS), 100 U mL^{-1} penicillin-streptomycin and 2·5 μg mL^{-1} of fungizone at 37 °C in a CO_2 incubator containing 5% CO_2, 95% air and 100% humidity. The supernatant was removed after 3 days and stored at −70 °C until further testing.

Cytokine assays

Cell culture supernatants were harvested and analysed for cytokines by ELISA techniques with commercially available kits. Human IL-8, 10, 12 and TNF-α kits were obtained from BD OptEIA. All cytokine assays were calibrated against the World Health Organization international standards by the kit manufacturer. The lower limits of detection for the individual assays are as follows: IL-8, 0·8 pg mL^{-1}; IL-10, 2 pg mL^{-1}; IL-12, 4 pg mL^{-1} and TNF-α, 2 pg mL^{-1}.

Biopsy cytokines using real-time quantitative PCR with SYBR Green

TRIzol. Intestinal biopsy specimens obtained for RNA extraction were collected in an Eppendorf vial containing TRIzols Reagent (Invitrogen Corporation, USA) and stored in a liquid nitrogen container for transport to the laboratory and stored at −70 °C until further use. Total RNA was extracted from endoscopic biopsy samples of colonic mucosa with TRIzol method described previously. Reverse transcription of the extracted RNA was performed using RNase H-deficient reverse transcriptase (Superscript II, Life Technologies) and oligo (dT) primers (Life Technologies). Aliquots (2 μL) of reverse transcription reaction mixture (20 μL) were used for quantitation of IL-12, IL 10, IL-8 TNF-α and GAPDH gene expression by real-time PCR assays (Table 2B) (Tsukada *et al*. 2002). The SYBR Green QRT PCR was used to quantify IL-12, IL-10, IL-8, TNF-α and GAPDH gene expression (PE Applied Biosystems, Foster City, CA). The PCR reactions were performed using the SYBR Green QRT PCR kit (PE Applied Biosystems) as described previously (Heid *et al*. 1996). After activation for 10 min at 95 °C, 40 cycles of 15 s at 95 °C and 1 min at 62 °C was carried out in model icycler (Biorad). Real-time fluorescence measurements was recorded and the threshold cycle (Ct) value for each sample calculated by the above sequence detector (Heid *et al*. 1996). For IL-12, TNF-α and GAPDH standard curves of Ct values were obtained from real-time

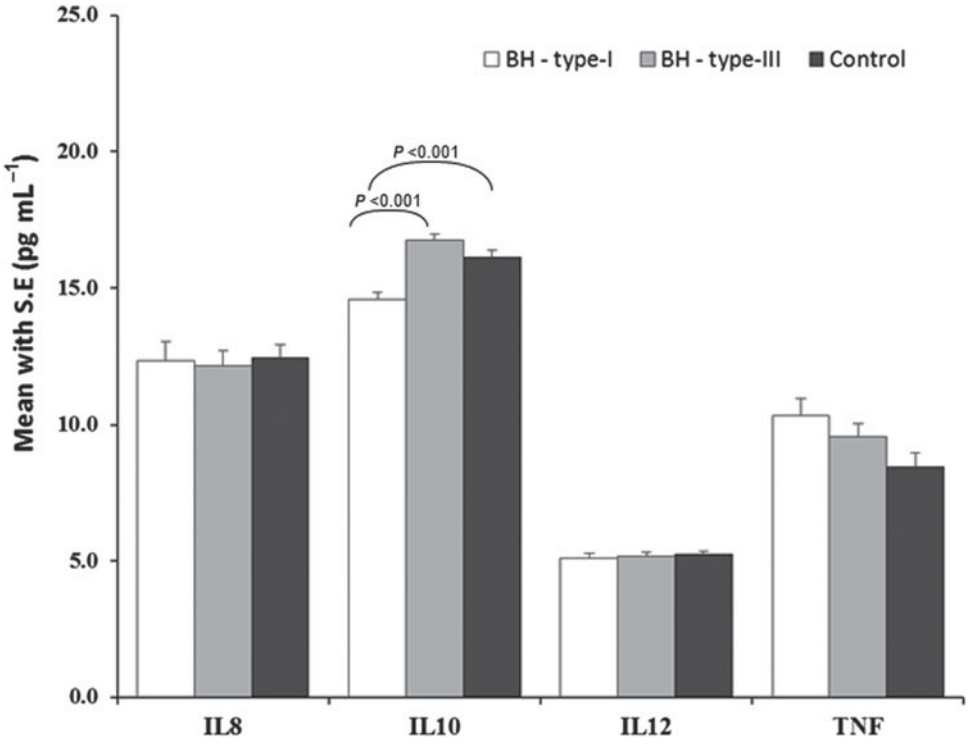

Fig. 1. Comparison of PBMC cytokines with *Blastocystis* sp. type 1 and 3 and control.

PCR of pMFGhTNF, pBSKIhIL-12 and PCRII GAPDH (Reference plasmids). The relative fold change in mRNA expression of the target gene was calculated with $2^{-\Delta\Delta CT}$ method using the software GENEX. The $2^{-\Delta\Delta CT}$ method presents the data as fold change in mRNA expression of the target gene, normalized to the mRNA expression of the housekeeping gene. Glyceraldehyde-3-phosphate dehydrogenase was used as the housekeeping gene in all the QRT-PCR experiment. Each biopsy sample obtained from the same patient was tested in duplicate, and the average of two Ct values was used in this study.

Statistical methods

Results are expressed as mean ± S.D. for continuous normally distributed variables whereas median with IQR (interquartile range) for non-normal variables and number (percentage) for categorical data. Differences in mean comparison of characteristics of IBS patients with and without *Blastocystis* sp. infection type-I and 3 with control group for normally distributed variables were performed by using the analysis of variance (ANOVA) and Tukey's HSD test was used for multiple comparison, however for non-normal variables a non-parametric Kruskal–Wallis test and Mann–Whitney test were used for within-group comparison. Differences in proportion were compared by Pearson Chi-square or Fisher exact 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 19.

RESULTS

Ninety-five (59%) of the patients were over 35 years of age. Abdominal pain, bloating and frequency of stool were equally common in both groups (Table 1). Fecal smear microscopy for *Blastocystis* spp. was positive in 55 (69%) and *Blastocystis* spp. culture was positive in 80 (50%) (Table 1).

Blastocystis *spp. subtypes and histology*

Non-specific colitis was present in 58 (72%) with *Blastocystis* spp. infection and in 53 (66%) without (P = 0·391) (Table 1). Of the 58 *Blastocystis* positive D-IBS with non-specific colitis, *Blastocystis* sp. subtype 1 was 30 (75%) compared with control of 53 (66%) (P = 0·328) and *Blastocystis* sp. subtype 3 was 28 (70%) compared with control of 53 (66%) (P = 0·679). There was no significant difference in the distribution of neutrophils, eosinophils, mononuclear cells or evidence of epithelial cell damage in the colonic mucosal biopsies in the two groups (Table 1).

Blastocystis *spp. types and PBMC cytokines*

Patients with D-IBS infected with *Blastocystis* spp. demonstrated low PBMC production of IL-8 in

Table 3. Comparison of characteristics of controls with *B. hominis* type genotype

	B. hominis Type 1, $n = 40$	Control, $n = 80$	*P* value	*B. hominis* Type 3, $n = 40$	Control, $n = 80$	*P* value
Age (years)						
⩽ 35 years	18 (45)	32 (40)	0·600	15 (37)	32 (40)	0·791
⩾ 36 years	22 (55)	48 (60)		25 (63)	48 (60)	
Gender						
Male	28 (70)	58 (73)	0·774	32 (80)	58 (72)	0·371
Female	12 (30)	22 (27)		8 (20)	22 (28)	
Abdominal pain						
Yes	28 (70)	65 (81)	0·164	35 (87)	65 (81)	0·386
No	12 (30)	15 (19)		5 (13)	15 (19)	
Bloating						
Yes	23 (57)	53 (66)	0·348	32 (80)	53 (66)	0·118
No	17 (43)	27 (34)		8 (20)	27 (34)	
Stool frequency						
⩽ 4 per day	4 (10)	8 (9)	0·999	6 (15)	8 (10)	0·421
⩾ 5 per day	36 (90)	72 (91)		34 (85)	72 (90)	
Stool microscopy						
Positive	30 (75)	0 (0)	< 0·001	25 (62)	0 (0)	< 0·001
Negative	10 (25)	80 (100)		15 (38)	80 (100)	
Stool culture						
Positive	40 (100)	0 (0)	< 0·001	40 (100)	0 (0)	< 0·001
Negative	0 (0)	80 (100)		0 (0)	80 (100)	
Histology						
Nonspecific colitis	30 (75)	53 (66)	0·328	28 (70)	53 (66)	0·679
Normal	10 (25)	27 (34)		12 (30)	27 (34)	
Neutrophil						
Absent	26 (65)	38 (47)	0·070	24 (60)	38 (47)	0·196
Mild	14 (35)	42 (53)		16 (40)	42 (53)	
Eosinophil						
Absent	17 (42)	47 (59)	0·093	20 (50)	47 (59)	0·363
Mild	23 (58)	33 (41)		20 (50)	33 (41)	
Lymphocyte						
Mild	25 (63)	64 (80)	0·039	28 (70)	64 (80)	0·222
Moderate	15 (37)	16 (20)		12 (30)	16 (20)	
Plasma cells						
Mild	30 (75)	58 (73)	0·770	32 (80)	58 (73)	0·371
Moderate	10 (25)	22 (27)		8 (20)	22 (27)	
Goblet cell depletion						
Absent	27 (67)	38 (47)	0·038	16 (40)	38 (48)	0·436
Mild	13 (33)	42 (53)		24 (60)	42 (52)	
Intraepithelial lymphocyte						
Absent	23 (57)	60 (75)	0·050	32 (80)	60 (75)	0·542
Mild	17 (43)	20 (25)		8 (20)	20 (25)	

Table 4. Comparison of peripheral cytokines with *B. hominis* types and control

			P value			
		Mean ± S.D.	Over all	*B hominis* type 1 *vs* 3	*B hominis* type 1 *vs* control	*B hominis* type 3 *vs* control
Blood IL-8	BH type1 ($n=40$) BH type 3 ($n=40$) Control ($n=80$)	12·3 ± 4·5 12·2 ± 3·5 12·5 ± 4	0·929	NS	NS	NS
Blood IL-10	BH type1 ($n=40$) BH type 3 ($n=40$) Control ($n=80$)	14·5 ± 1·6 16·7 ± 1·5 16·0 ± 2·3	<0·001*	<0·001*	<0·001*	0·367
Blood IL-12	BH type1 ($n=40$) BH type 3 ($n=40$) Control ($n=42$)	5·0 ± 1 5·2 ± 1 5·2 ± 1	0·786	NS	NS	NS
Blood TNF-α	BH type1 ($n=40$) BH type 3 ($n=40$) Control ($n=82$)	10·4 ± 4·0 9·5 ± 3·2 8·5 ± 4·5	0·051	NS	0·054	NS

* *P* value <0·05 was considered as statistically significant; NS = not significant; S.D. = Std. deviation.

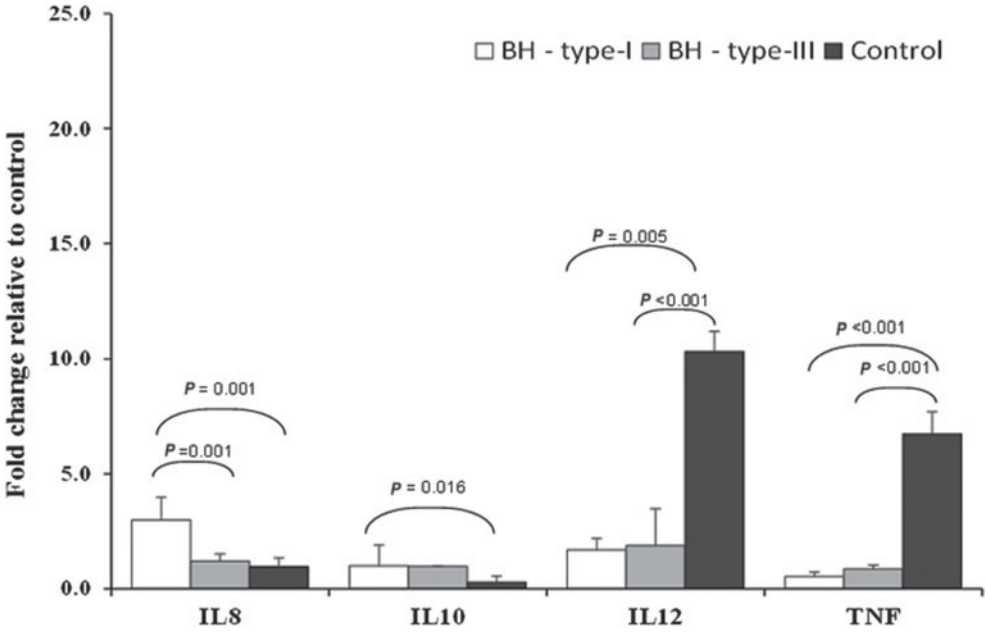

Fig. 2. Comparison of cytokine mRNA expression in colonic mucosa between *B. hominis* subtypes and control group.

45 (56%) ($P=0·027$) compared with control 31 (39%) (Table 1). There was no difference in the PBMC production of IL-10, IL-12 and TNF-α by PBMCs *in vitro* in the *Blastocystis* spp. infected and control groups (Table 1). Patients with D-IBS infected with *Blastocystis* sp. type 1 demonstrated low PBMC production of IL-10 in 36 (90%) compared with control 37 (46%) ($P<0·001$) (Fig. 1). The mean concentration of IL-10 ($14·5 \pm 1·6$ pg mL^{-1}, $P<0·001$) was low in *Blastocystis* sp. type 1 compared with those with *Blastocystis* sp. type 3 ($16·7 \pm 1·5$ pg mL^{-1}) and controls ($16 \pm 2·3$ pg mL^{-1}) (Table 4), and these differences remained significant after adjustment for multiple comparisons, $P<0·001$ and $P<0·001$, respectively (Table 4).

Comparison between Blastocystis *spp. types with PBMC expressed cytokines and histology*

In *Blastocystis* sp. type 1, mucosal lymphocyte and IEL infiltration were 15 (37%) ($P=0·039$) and 17 (43) ($P=0·050$), respectively compared with control of 16 (20%) and 20 (25%) (Table 3). In *Blastocystis* sp. type 1, IL-8 was associated with mild eosinophil infiltration in 18 (72%) ($P=0·024$); IL-10 with lymphocyte in 25 (69%) ($P=0·015$), plasma cell

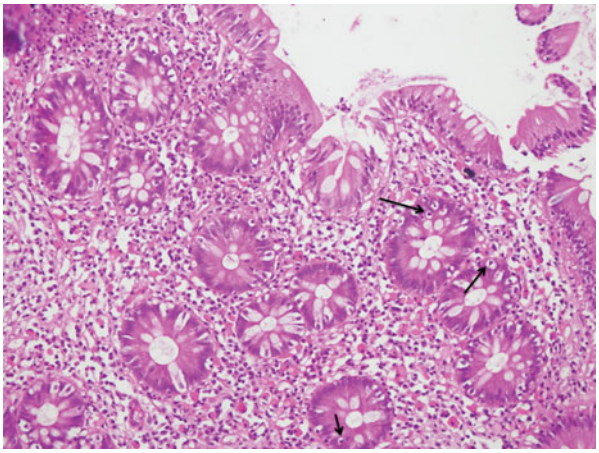

Fig. 3. Colonic glands showing intraepithelial lymphocytes in the glandular lining epithelium. H&E ×10.

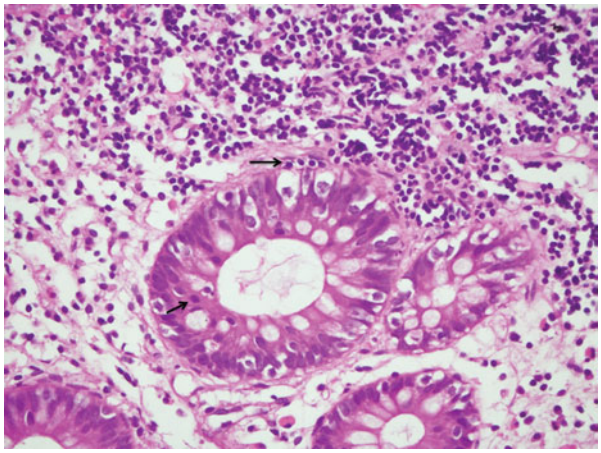

Fig. 4. Colonic glands showing intraepithelial lymphocytes in the glandular lining epithelium H&E ×20.

infiltration in 30 (83%) ($P = 0{\cdot}002$) and IEL in 23 (64%) ($P = 0{\cdot}026$) (Figs 3–5); IL-12 was associated with goblet cell depletion in 13 (100%) ($P < 0{\cdot}001$) and IEL in 9 (69%) ($P = 0{\cdot}038$) and TNF-α also with plasma cell infiltration in 12 (100%) ($P = 0{\cdot}019$) (Table 5). In *Blastocystis* sp. type 3, IL-8 was associated with goblet cell depletion in 16 (80%) ($P = 0{\cdot}010$) and IEL in 8 (40%) ($P = 0{\cdot}003$); IL-10 in above mean values with mild lymphocyte in 24 (86%) ($P = 0{\cdot}002$) and plasma cell infiltration in 28 (100%) ($P < 0{\cdot}001$); IL-12 was associated with goblet cell depletion in 20 (71%) ($P = 0{\cdot}037$) and TNF-α was associated with lymphocyte in 12 (50%) ($P = 0{\cdot}001$) and plasma cell infiltration in 8 (33%) ($P = 0{\cdot}013$) (Table 5).

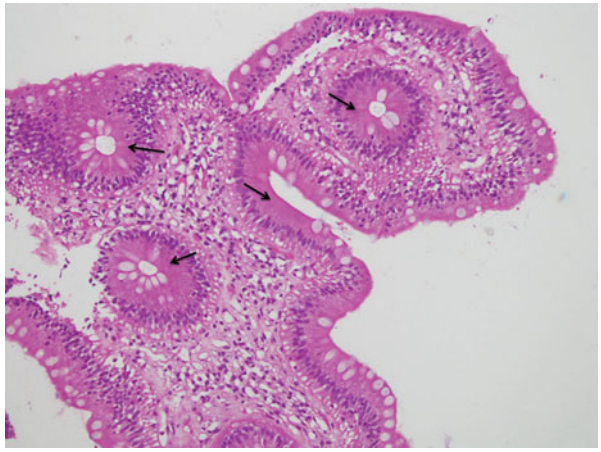

Fig. 5. Colonic glands showing mucin (goblet cell) depletion. H&E ×20.

Comparison between Blastocystis *spp. types and cytokine mRNA expression in colonic mucosa*

The expression of IL-8 mRNA (pg mL^{-1}) in colonic mucosa in *Blastocystis* sp. type 1 (median with interquartile range) was 3·4 (1·04–7·30), type 3 was 1·2 (0·21–2·14) and for control was 1 (0·27–9·8) ($P = 0{\cdot}016$). The expression of IL-10 mRNA (pg mL^{-1}) in colonic mucosa in *Blastocystis* sp. type 1was 0·78 (0·34–5·7), type 3 was 1 (0·97–0·99) and for control was 0·27 (0·04–3·6) ($P = 0{\cdot}153$). The expression of IL-12 mRNA (pg mL^{-1}) in colonic mucosa in *Blastocystis* sp. type 1 was 1·5 (1·2–5·15), type 3 was 2 (0·22–7·06) and for control was 1 (2·1–13·8) ($P = 0{\cdot}005$). The expression of TNF-α mRNA (pg mL^{-1}) in colonic mucosa in *Blastocystis* sp. type 1 was 0·54 (0·05–1·5), type 3 was 0·87 (0·53–1·8) and for control was 6·7 (2·03–14·7) ($P < 0{\cdot}001$). The IL-8mRNA expression was increased in *Blastocystis* sp. type 1 compared with type 3 ($P = 0{\cdot}001$) and control ($P = 0{\cdot}001$) (Fig. 2). The expression of mRNA of IL-10 was low in the control group and *Blastocystis* sp. type 3 and type 1 (Fig. 2). The IL-10 mRNA expression was increased in *Blastocystis* sp. type 1 compared with control ($P = 0{\cdot}016$) (Fig. 2). The expression of mRNA of IL-12 was higher in the control group compared with *Blastocystis* sp. type 3 and type 1 ($P < 0{\cdot}001$ and $0{\cdot}005$, respectively) (Fig. 2). The expression of mRNA of TNF-α was higher in the control group compared with *Blastocystis* sp. type 3 and type 1 ($P = < 0{\cdot}001$ and $0{\cdot}001$, respectively) (Fig. 2).

DISCUSSION

Blastocystis spp. is an extracellular luminal organism. It is able to evade the host immune response by suppressing iNoS production and cleaving immunoglobulin (Puthia *et al*. 2005; Mirza *et al*. 2011). This may also compromise these protective responses to other coinfecting pathogens allowing them to infect host epithelia and cause disease. There have been studies investigating the priming of human immune responses by intestinal protozoa (Djuardi *et al*. 2010). The presence of *Blastocystis* spp. as the predominant

Table 5. Comparison between *B. hominis* types, peripheral blood mononuclear cells cytokines and histological parameters

			IL8			IL-10			IL-12			TNF		
			$<12{\cdot}4$ pg mL^{-1}	$\geqslant 12{\cdot}4$ pg mL^{-1}	*P*	$<15{\cdot}8$ pg mL^{-1}	$<15{\cdot}8$ pg mL	*P*	$<5{\cdot}2$ pg mL	$<5{\cdot}2$ pg mL^{-1}	*P*	$<9{\cdot}3$ pg mL^{-1}	$\geqslant 9{\cdot}3$ pg mL	*P*
B. Hominis TYPE 1	Lymphocyte Grade	Mild	15 (60)	10 (67)	0·673	25 (69)	0	**0·015**	16 (59)	9 (69)	0·542	9 (75)	16 (57)	0·477
		Moderate	10 (40)	5 (33)		11 (31)	4 (100)		11 (41)	4 (31)		3 (25)	12 (43)	
	Plasma cell Grade	Mild	19 (76)	11 (73)	0·850	30 (83)	0	**0·002**	19 (70)	11 (85)	0·451	12 (100)	18 (64)	**0·019**
		Moderate	6 (24)	4 (27)		6 (17)	4 (100)		8 (30)	2 (15)		0 (0)	10 (36)	
	Neutrophil Grade	Absent	18 (72)	8 (53)	0·231	22 (61)	4 (100)	0·278	18 (67)	8 (61)	1	6 (50)	20 (71)	0·193
		Mild	7 (28)	7 (47)		14 (39)	0		9 (33)	5 (39)		6 (50)	8 (29)	
	Eosinophil Grade	Absent	7 (28)	10 (67)	**0·024**	17 (47)	0	0·123	10 (37)	7 (54)	0·314	7 (58)	10 (36)	0·185
		Mild	18 (72)	5 (33)		19 (53)	4 (100)		17 (63)	6 (46)		5 (42)	18 (64)	
	Goblet cell depletion Grade	Absent	12 (48)	11 (73)	0·117	19 (53)	4 (100)	0·123	23 (85)	0 (0)	**<0·001**	3 (25)	20 (71)	**0·013**
		Mild	13 (52)	4 (27)		17 (47)	0		4 (15)	13 (100)		9 (75)	8 (29)	
	Intra-epithelial cell Grade	Absent	12 (48)	11 (73)	0·187	23 (64)	0	**0·026**	19 (70)	4 (31)	**0·038**	7 (58)	16 (57)	0·944
		Mild	13 (52)	4 (27)		13 (36)	4 (100)		8 (30)	9 (69)		5 (42)	12 (43)	
B. Hominis TYPE 3	Lymphocyte Grade	Mild	16 (80)	12 (60)	0·168	4 (33)	24 (86)	0·002	16 (57)	12 (100)	0·007	16 (100)	12 (50)	0·001
		Moderate	4 (20)	8 (40)		8 (67)	4 (14)		12 (43)	0 (0)		0	12 (50)	
	Plasma cell Grade	Mild	16 (80)	16 (80)	0·999	4 (33)	28 (100)	**<0·001**	20 (71)	12 (100)	0·079	16 (100)	16 (67)	0·013
		Moderate	4 (20)	4 (20)		8 (67)	0		8 (29)	0 (0)		0	8 (33)	
	Neutrophil Grade	Absent	12 (60)	12 (60)	0·999	4 (33)	20 (71)	**0·037**	16 (57)	8 (67)	0·729	12 (75)	12 (50)	0·144
		Mild	8 (40)	8 (40)		8 (67)	8 (29)		12 (43)	4 (33)		4 (25)	12 (50)	
	Eosinophil Grade	Absent	12 (60)	8 (40)	0·206	8 (67)	12 (43)	0·301	16 (57)	4 (33)	0·301	8 (50)	12 (50)	0·999
		Mild	8 (40)	12 (60)		4 (33)	16 (57)		12 (33)	8 (67)		8 (50)	12 (50)	
	Goblet cell depletion Grade	Absent	12 (60)	4 (20)	**0·010**	4 (33)	12 (43)	0·729	8 (29)	8 (67)	**0·037**	8 (50)	8 (33)	0·292
		Mild	8 (40)	16 (80)		8 (67)	16 (57)		20 (71)	4 (33)		8 (50)	16 (67)	
	Intra Epithelial cell lymphocyte Grade	Absent	20 (100)	12 (60)	0·003	8 (67)	24 (86)	0·211	24 (86)	8 (67)	0·211	16 (100)	16 (67)	**0·013**
		Mild	0	8 (40)		4 (33)	4 (14)		4 (14)	4 (33)		0	8 (33)	

B. Hominis	Lymphocyte											
non	Grade	Mild	27 (87)	37 (76)	0·260	25 (68)	39 (91)	**0·010**	46 (74)	18 (100)	**0·017**	24 (75)	40 (83)	0·361
infective		Moderate	4 (13)	12 (24)		12 (32)	4 (9)		16 (26)	0 (0)		8 (25)	8 (17)	
	Plasma cell													
	Grade	Mild	27 (87)	31 (63)	**0·020**	23 (60)	35 (81)	0·055	42 (68)	16 (89)	0·132	24 (75)	34 (71)	0·683
		Moderate	4 (13)	18 (37)		14 (40)	8 (19)		20 (32)	2 (11)		8 (25)	14 (29)	
	Neutrophil													
	Grade	Mild	20 (64)	18(35)	**0·015**	12(32)	26 (60)	0·012	26 (42)	12 (67)	0·064	16 (50)	22 (46)	0·715
		Moderate	11 (35)	31 (65)		25 (68)	17 (40)		36 (58)	6 (33)		16 (50)	26 (54)	
	Eosinophil													
	Grade	Mild	27 (87)	20 (41)	**<0·001**	19 (51)	28 (65)	**0·212**	31 (50)	16 (89)	**0·003**	20 (62)	27 (56)	0·578
		Moderate	4 (13)	29 (59)		18 (49)	15 (35)		31 (50)	2 (11)		12 (38)	21 (44)	
	Goblet cell depletion													
	Grade	Mild	8 (26)	30 (61)	**0·002**	12 (32)	26 (61)	**0·012**	34 (55)	4 (22)	**0·015**	8 (25)	30 (62)	**0·001**
		Moderate	23 (74)	19 (39)		25 (68)	17 (39)		28 (45)	14 (78)		24 (75)	18 (38)	
	Intra epithelial cell lymphocyte													
	Grade	Mild	23 (74)	37 (75)	0·895	25 (68)	35 (81)	0·154	46 (74)	14 (78)	1	26 (81)	34 (71)	0·292
		Moderate	8 (26)	12 (25)		12 (32)	8 (19)		16 (26)	4 (22)		6 (19)	14 (29)	

Histological inflammation was graded as 0 = nil, 1 = mild, 2 = moderate, 3 = severe.

species in pregnant mothers was shown to dampen the innate and adaptive responses to purified protein derivative (PPD) of mycobacterium tuberculosis (Djuardi *et al*. 2010). Earlier studies have shown that *Blastocystis* spp. infection can be associated with impaired intestinal permeability (Mirza *et al*. 2012) as well as lower total leucocyte and neutrophil count (Reiman *et al*. 2006).

In this study, colonoscopic examination of D-IBS patients with *Blastocystis* spp. demonstrated no pathological changes or only non-specific inflammation without evidence of invasion with *Blastocystis* spp., in keeping with previous studies (Chen *et al*. 2005). Also, *Blastocystis* strain RN94-9 induced neither epithelial injury nor inflammatory cell infiltration in rat caecal mucosa (Iguchi *et al*. 2009). The non-invasive nature of strain RN94-9 was further confirmed by normal expression levels of epithelial tight junction proteins. In D-IBS with *Blastocystis* spp. infection, PBMCs IL-8 and IL-10 responses were less marked in *Blastocystis* sp. type 1 compared with control (Table 3). The mean IL-10 was significantly lower in *Blastocystis* sp. type 1 compared with *Blastocystis* sp. type 3 and control (Table 4). In *Blastocystis* sp. type 1, there was colonic mucosal eosinophil infiltration associated with IL-8 compared with *Blastocystis* sp. type 3 (Table 5) and lymphocyte infiltration and goblet cell depletion compared with control (Table 3). In contrast, in a mouse model of *Blastocystis* spp. infection (Iguchi *et al*. 2009) goblet cell hyperplasia was reported. Moreover, goblet cell hyperplasia has also been reported in *Giardia* infections as well (Ponce-Macotela *et al*. 2008). It is known that T helper type 2 cytokines (IL-13 and IL-4) regulate the development of goblet cell hyperplasia in the gut during infection and an increased number of goblet cells plays an important role in host protective immunity against infection (Khan and Alkhalife, 2005). Goblet cell hyperplasia, increased mucin and fluid secretion and enhanced intestinal propulsive activity results in the eviction of noxious agents from the gut lumen. However, a single administration of recombinant adenovirus vector expressing IL-12 (Ad5IL-12) in *Trichinella spiralis*-infected mice inhibited infection-induced muscle hypercontractility and goblet cell hyperplasia. This also correlated with upregulated interferon-γ (IFN-γ) expression and downregulated IL-13 expression in the muscularis externa layer (Khan *et al*. 2001). These results indicate that transfer and overexpression of the IL-12 gene during Th2-based infection shifts the immune response towards Th1 and abrogates the physiological responses to infection, attenuating both muscle hypercontractility and goblet cell hyperplasia (Khan *et al*. 2001). O'Malley *et al*. (2010) reported goblet cell hyperplasia in a rat model of anxiety and depression. The finding of goblet cell depletion in both case and control in this study is

surprising and may be explained on the basis that Th2 reaction when replaced by a proinflammatory reaction leads to goblet cell depletion in D-IBS in humans.

IL-12 plays a key role in induction of Th1 immune responses by stimulating the production of IFN-γ and TNF-α from T and natural killer (NK) cells. In this study, IL 12 was associated with goblet cell depletion in both cases and control (Table 5). Long *et al*. (2001) in an *in vitro* study reported *Blastocystis* sp. type 1 elicited a significant increase in proinflammatory IL-8 in HT-29 and T-84 IEC human colonic cell lines. This was observable after 24 h exposure to parasites (Long *et al*. 2001). However, the PBMCs culture supernatants harvested after 72 h of incubation did not show significant cytokine variation among *Blastocystis* spp. types and control (Tables 4 and 5). It has been previously reported that cytokine changes presented within 6 h (Long *et al*. 2001) and normalized after that period. IL-10, having a regulatory role, is produced by activated lymphocytes in later phases of the immune response (Schröder *et al*. 1987; De Waal Malefyt *et al*. 1991). The anti-inflammatory cytokine IL-10 inhibits monocytes production of pro-inflammatory cytokines including TNF-α which subsequently regulates both early neutrophilic infiltration and eosinophil recruitment (Asseman *et al*. 1999). Secretion of IL-10 is also induced by TNF-α linking its production to inflammation (Powrie *et al*. 1994). In *Blastocystis* spp. type 1 and type 3, IL-12 and TNF-α mRNAs expression were also low compared with in control (Fig. 2). The low IL-12 and TNF-α in coinfection with *Blastocystis* spp. may be attributed to the immune modifying effect of *Blastocystis* spp. Previously, the downregulation of IFN-γ and TNF-α together with the upregulation of IL-6, IL-8, as well as NF-κB gene expressions were seen in the PBMCs stimulated with $1\,\mu g\,mL^{-1}$ of *Blastocystis* antigen. This suggested that *Blastocystis* antigen stimulated the humoral immune responses in PBMCs, which may lead to inflammatory reactions and propagation of the cells to combat the infection (Chandramathi *et al*. 2010).

IL-10 is important in regulating inflammatory response as it reduces the production of chemotactic factors, such as IL-8 (Turner *et al*. 1997; Olivo-Diaz *et al*. 2012). Previously in animal models, gene transcription of type 1 and proinflammatory cytokines IFN-γ, IL-12 and TNF-α was significantly upregulated in the caecal mucosa. These results suggest that *Blastocystis* infection in rats induces local host responses to exposed antigens (Iguchi *et al*. 2009). In D-IBS with *Blastocystis* spp., the IL-8mRNA expression was increased in *Blastocystis* sp. type 1 compared with type 3 ($P = 0{\cdot}001$) and control ($P = 0{\cdot}001$) (Fig. 2). In keeping with the previous studies that showed *Blastocystis* strains N and II that belong to subtype 1 and WR1 belonging to subtype 4 stimulated the release of chemokine IL-8 from a human colonic epithelial cell line *in vitro*, possibly mediated by the organism-derived cysteine protease (Long *et al*. 2001; Puthia *et al*. 2005).

Blastocystosis may not be a highly inflammatory disease (at least in the context of these isolates) as evidenced by low levels of cytokine among *Blastocystis* spp. types and control. The severity of *Blastocystosis* spp. infection may be mediated by host factors. Secretion of high levels of enteric anti-*Blastocystis* IgA has been found to be a common factor in all symptomatic, but not asymptomatic, cases of *Blastocystis* (Mahmoud and Saleh, 2003). This would also explain why some individuals can be carriers of the organism without expressing symptoms (Markell and Udkow, 1986).

The limitation of this study is that the case-to-control patient ratio of 1:1 is too small to draw any firm conclusion. A case-to-control ratio of 2:1 or 3:1 would have been preferable. The size of the case group was limited by the number of eligible patients fulfilling the Rome III criteria for D-IBS infected with *Blastocystis* sp. subtype 1 or 3 and the resources required for experiments on this sample size. In conclusion, in patients with D-IBS and concomitant *Blastocystis* sp. type 1 infection, there is a low IL10 response compared with type 3 and D-IBS without *Blastocystis* spp. and this may be attributable to an immune modifying effect of *Blastocystis* sp. infection in D-IBS.

ACKNOWLEDGEMENT

We are grateful to the staff of the JUMA research building for their help during the completion of this work.

CONFLICT OF INTERESTS

All authors declare they have no conflict of interest.

REFERENCES

Abe, N. (2004). Molecular and phylogenetic analysis of *Blastocystis* isolates from various hosts. *Veterinary Parasitology* **120**, 235–242.

Abe, N., Wu, Z. and Yoshikawa, H. (2003*a*). Molecular characterization of *Blastocystis* isolates from birds by PCR with diagnostic primers and restriction fragment length polymorphism analysis of the small subunit ribosomal RNA gene. *Parasitology Research* **89**, 393–396.

Abe, N., Wu, Z. and Yoshikawa, H. (2003*b*). Molecular characterization of *Blastocystis* isolates from primates. *Veterinary Parasitology* **113**, 321–325.

Abe, N., Wu, Z. and Yoshikawa, H. (2003*c*). Zoonotic genotypes of *Blastocystis hominis* detected in cattle and pigs by PCR with diagnostic primers and restriction fragment length polymorphism analysis of the small subunit ribosomal RNA gene. *Parasitology Research* **90**, 124–128.

Asseman, C., Mauze, S., Leach, M. W., Coffman, R. L. and Powrie, F. (1999). An essential role for interleukin 10 in the function of regulatory T cells that inhibit intestinal inflammation. *Journal of Experimental Medicine* **190**, 995–1004.

Barbara, G., De Giorgio, R., Stanghellini, V., Cremon, C. and Corinaldesi, R. (2002). A role for inflammation in irritable bowel syndrome? *Gut* **5**, 41–44.

Chadwick, V., Paulus, B., Bethwaite, P., Tie, A. and Wilson, I. (2002). Activation of the mucosal immune system in irritable bowel syndrome. *Gastroenterology* **122**, 1778–1783.

Chandramathi, S., Suresh, K. and Kuppusamy, U. R. (2010). Solubilized antigen of *Blastocystis hominis* facilitates the growth of human colorectal cancer cells, HCT116. *Parasitology Research* **106**, 941–945.

Chen, C. C., Louie, S., McCormick, B., Walker, W. A. and Shi, H. N. (2005). Concurrent infection with an intestinal helminth parasite impairs host resistance to enteric *Citrobacter rodentium* and enhances *Citrobacter*-induced colitis in mice. *Infection and Immunity* **73**, 5468–5481.

Chomczynski, P. and Sacchi, N. (1987). Single-step method of RNA isolation by acid guanidinium-thiocyanate-phenol-chloroform extraction. *Analytical Biochemistry* **162**, 156–159.

De Waal Malefyt, R., Abrams, J., Bennett, B., Figdor, C. G. and de Vries, J. E. (1991). Interleukin-10 (IL-10) inhibits cytokine synthesis by human monocytes: an autoregulatory role of IL-10 produced by monocytes. *Journal of Experimental Medicine* **174**, 1209–1220.

Djuardi, Y., Sartono, E., Wibowo, H., Supali, T. and Yazdanbakhsh, M. (2010). A longitudinal study of BCG vaccination in early childhood: the development of innate and adaptive immune responses. *PLoS ONE* **5**, e14066. doi: 10.1371/journal.pone.0014066.

Drossman, D. A. and Dumitrascu, D. L. (2006). Rome III: new standard for functional gastrointestinal disorders. *Journal of Gastrointestinal and Liver Disease* **15**, 237–242.

Drossman, D. A., Camileri, M., Mayer, E. A. and Whitehead, W. E. (2002). AGA technical review on irritable bowel syndrome. *Gastroenterology* **123**, 2108–2131.

Heid, C. A., Stevens, J., Livak, K. J. and Williams, P. M. (1996). Real time quantitative PCR. *Genome Research* **6**, 986–994.

Iguchi, A., Yoshikawa, H., Yamada, M., Kimata, I. and Arizono, N. (2009). Expression of interferon gamma and proinflammatory cytokines in the cecal mucosa of rats experimentally infected with *Blastocystis* sp. strain RN94–9. *Parasitology Research* **105**, 135–140.

Khan, W. I., Blennerhasset, P., Ma, C., Matthaei, K. I. and Collins, S. M. (2001). Stat6 dependent goblet cell hyperplasia during intestinal nematode infection. *Parasite Immunology* **23**, 39–42.

Khan, Z. A. and Alkhalife, I. S. (2005). Prevalence of *Blastocystis hominis* among "healthy" food handlers in Dammam, Saudi Arabia. *Journal of Egyptian Society of Parasitology* **35**, 395–401.

Li, L. H., Zhou, X. N., Du, Z. W., Wang, X. Z., Wang, L. B., Jiang, J. Y., Yoshikawa, H., Steinmann, P., Utzinger, J., Wu, Z., Chen, J. X., Chen, S. H. and Zhang, L. (2007*a*). Molecular epidemiology of human *Blastocystis* in a village in Yunnan province, China. *Parasitology International* **56**, 281–286.

Li, L. H., Zhang, X. P., Lv, S., Zhang, L., Yoshikawa, H., Wu, Z., Steinmann, P., Utzinger, J., Tong, X. M., Chen, S. H. and Zhou, X. N. (2007*b*). Cross sectional surveys and subtype classification of human *Blastocystis* isolates from four epidemiological settings in China. *Parasitology Research* **102**, 83–90.

Long, H. Y., Handschack, A., König, W. and Ambrosch, A. (2001). *Blastocystis hominis* modulates immune responses and cytokine release in colonic epithelial cells. *Parasitology Research* **87**, 1029–1030.

Mahmoud, M. S. and Saleh, W. A. (2003). Secretory and humoral antibody responses to *Blastocystis hominis* in symptomatic and asymptomatic human infections. *Journal of the Egyptian Society of Parasitology* **33**, 13–30.

Markell, E. K. and Udkow, M. P. (1986). *Blastocystis hominis*: pathogen or fellow traveller? *American Journal of Tropical Medicine Hygiene* **35**, 1023–1026.

Mirza, H., Wu, Z., Kidwai, F. and Tan, K. S. (2011). A metronidazole-resistant isolate of *Blastocystis* spp. is susceptible to nitric oxide and downregulates intestinal epithelial inducible nitric oxide synthase by a novel parasite survival mechanism. *Infection Immunity* **79**, 5019–5026.

Mirza, H., Wu, Z., Teo, J. D. and Tan, K. S. (2012). Statin pleiotropy prevents rho kinase-mediated intestinal epithelial barrier compromise induced by *Blastocystis* cysteine proteases. *Cell Microbiology* **14**, 1474–1484.

O'Leary, C. and Quigley, E. M. (2003). Small bowel bacterial overgrowth, celiac disease and IBS: what are the associations? *American Journal of Gastroenterology* **98**, 720–722.

Olivo-Diaz, A., Romero-Valdovinos, M., Gudiño-Ramirez, A., Reyes-Gordillo, J., Jimenez-Gonzalez, D. E., Ramirez-Miranda, M. E., Martinez-Flores, W. A., Martinez-Hernandez, F., Flisser, A. and Maravilla, P. (2012). Findings related to IL-8 and IL-10 gene polymorphisms in a Mexican patient population with irritable bowel syndrome infected with *Blastocystis*. *Parasitology Research* **111**, 4874–4891.

O'Malley, D., Dinan, T. G. and Cryan, J. F. (2010). Alterations in colonic corticotropin-releasing factor receptors in the maternally separated rat model of irritable bowel syndrome: differential effects of acute psychological and physical stressors. *Peptides* **3**, 662–670.

O'Sullivan, M., Clayton, N., Breslin, N. P., Harman, I., Bountra, C., McLaren, A. and O'Morain, C. A. (2000). Increased mast cells in the irritable bowel syndrome. *Neurogastroenterology Motility* **12**, 449–457.

Pimentel, M., Chow, E. J. and Lin, H. C. (2000). Eradication of small bowel bacterial overgrowth reduces symptoms of irritable bowel syndrome. *American Journal of Gastroenterology* **95**, 3503–3506.

Ponce-Macotela, M., González-Maciel, A., Reynoso-Robles, R. and Martínez-Gordillo, M. N. (2008). Goblet cells: are they an unspecific barrier against *Giardia intestinalis* or a gate? *Parasitology Research* **102**, 509–513.

Powrie, F., Leach, M. W., Mauze, S., Menon, S., Caddle, L. B. and Coffman, R. L. (1994). Inhibition of Th1 responses prevents inflammatory bowel disease in SCID mice reconstituted with CD45RBhi CD4+ T cells. *Immunity* **1**, 553–562.

Puthia, M. K., Vaithilingam, A., Lu, J. and Tan, K. S. (2005). Degradation of human secretory immunoglobulin A by *Blastocystis*. *Parasitology Research* **97**, 386–389.

Puthia, M. K., Lu, J. and Tan, K. S. (2008). *Blastocystis ratti* contains cysteine proteases that mediate interleukin-8 response from human intestinal epithelial cells in an NF-kappaB-dependent manner *Eukaryotic Cell* **7**, 435–443.

Reiman, R. M., Thompson, R. W., Feng, C. G., Hari, D., Knight, R., Cheever, A. W., Rosenberg, H. F. and Wynn, T. A. (2006). Interleukin-5 (IL-5) augments the progression of liver fibrosis by regulating IL-13 activity. *Infection and Immunity* **74**, 1471–1479.

Schröder, J. M., Mrowietz, U., Morita, E. and Christophers, E. (1987). Purification and partial biochemical characterization of a human monocyte-derived, neutrophil-activating peptide that lacks interleukin-1 activity. *Journal of Immunology* **139**, 3474–3483.

Stensvold, R., Brillowska-Dabrowska, A., Nielsen, H. V. and Arendrup, M. C. (2006). Detection of *Blastocystis hominis* in unpreserved stool specimens by using polymerase chain reaction. *Journal of Parasitology* **92**, 1081–1087.

Stensvold, C. R., Suresh, G. K., Tan, K. S. W., Thompson, R. C., Traub, R. J., Viscogliosi, E., Yoshikawa, H. and Clark, C. G. (2007). Terminology for *Blastocystis* subtypes – a consensus. *Trends in Parasitology* **23**, 93–96.

Tsukada, Y., Nakamura, T., Iimura, M., Iizuka, B. E. and Hayashi, N. (2002). Cytokine profile in colonic mucosa of ulcerative colitis correlates with disease activity and response to granulocytapheresis. American Journal of Gastroenterology **97**, 2820–2828.

Turner, D. M., Williams, D. M., Sankaran, D., Lazarus, M., Sinnott, P. J. and Hutchinson, I. V. (1997). An investigation of polymorphism in the interleukin-10 gene promoter. *European Journal of Immunogenetics* **24**, 1–8.

van der Veek, P. P., van den Berg, M., de Kroon, Y. E., Verspaget, H. W. and Masclee, A. A. (2005). Role of tumor necrosis factor-alpha and interleukin-10 gene polymorphisms in irritable bowel syndrome. *American Journal of Gastroenterology* **100**, 2510–2516.

Yakoob, J., Jafri, W., Jafri, N., Khan, R., Islam, M., Beg, M. A. and Zaman, V. (2004). Irritable bowel syndrome: in search of an etiology: role of *Blastocystis hominis*. *American Journal of Tropical Medicine Hygiene* **70**, 383–385.

Yakoob, J., Jafri, W., Beg, M. A., Abbas, Z., Naz, S., Islam, M. and Khan, R. (2010). Irritable bowel syndrome: is it associated with genotypes of *Blastocystis hominis*. *Parasitology Research* **106**, 1033–1038.

Yan, Y., Su, S., Ye, J., Liao, H., Ye, J., Li, X., Luo, X. and Chen, G. (2006). Genetic variability of *Blastocystis hominis* isolates in China. *Parasitology Research* **99**, 597–601.

Yoshikawa, H., Nagano, I., Wu, Z., Yap, E. H., Singh, M. and Takahashi, Y. (1998). Genomic polymorphism among *Blastocystis hominis* strains and development of subtype-specific diagnostic primers. *Molecular Cellular Probes* **12**, 153–159.

Yoshikawa, H., Abe, N., Iwasawa, M., Kitano, S., Nagano, I. and Takahashi, Y. (2000). Genomic analysis of *Blastocystis hominis* strains isolated from two long-term health care facilities. *Journal of Clinical Microbiology* **38**, 1324–1330.

Yoshikawa, H., Abe, N. and Wu, Z. (2003). Genomic polymorphism among *Blastocystis* isolates and development of PCR-based identification of zoonotic isolates. *Journal of Eukaryote Microbiology* **50**, 710–717.

Yoshikawa, H., Wu, Z., Kimata, I., Iseki, M., Ali, I. K., Hossain, M. B., Zaman, V., Haque, R. and Takahashi, Y. (2004). Polymerase chain reaction based genotype classification among human *Blastocystis hominis* populations isolated from different countries. *Parasitology Research* **92**, 22–29.

Zaman, V. and Khan, K. (1994). A comparison of direct microscopy with culture for the diagnosis of *Blastocystis hominis*. *Southeast Asian Journal of Tropical Medicine Hygiene Public Health* **25**, 792–793.