

Association of *Blastocystis hominis* genetic subtypes with urticaria

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Abstract Although intestinal parasites are a possible cause of skin disorders, there are few case reports concerning the role of *Blastocystis hominis* in urticaria. To clarify this association, we determined the frequency of *B. hominis* genetic subtype in urticarial patients by stool culture and polymerase chain reaction (PCR) and evaluated the clinical and parasitological recovery of urticarial patients after treatment with metronidazole. Of 54 urticarial patients (group I), 18 (33.3%) were diagnosed as acute urticaria (group IA) and 36 (66.7%) were diagnosed as chronic (group IB). Thirty-three (61.1%) out of 54 urticarial (group I) patients were *Blastocystis* positive by stool culture and PCR. Out of these 33 patients, 21 were symptomatic and 12 were asymptomatic. The amoeboid form was found in 20 (95.2%) out of 21 symptomatic *Blastocystis* urticarial patients assuring their pathogenic potential. Of 50 normal control group (group II), four (8%) *Blastocystis* isolates were found with no amoeboid

form. *B. hominis* subtype 3 was the only detected genotype in both groups. Of 20 symptomatic *Blastocystis* urticarial patients, 12 (60%) patients recovered symptomatically and parasitologically after one course of metronidazole. Recovery reached 100% on repeating the treatment for a second course with disappearance of the amoeboid form. It was concluded that acute urticaria of unknown etiology and chronic idiopathic urticaria patients who are resistant to the ordinary regimen of urticaria treatment might be examined for infection with *B. hominis*, in order to prescribe the proper specific anti-protozoan treatment.

Introduction

Blastocystis hominis is a common parasite among humans with surveys revealing infection rates of up to 10% in developed countries and 50% in developing countries (Clark 1997 and Wong et al. 2008). Numerous epidemiological surveys carried out in different countries identify *Blastocystis* as the most common eukaryotic organism reported in human fecal samples (Souppart et al. 2009a). Prevalence reaches to 33.3% in Egypt (Rayan et al. 2007). It has been proposed that genetically different genotypes (subtypes) may be correlated with the pathogenic potential of *Blastocystis* (Domínguez-Márquez et al. 2009; Eroglu et al. 2009; Hussein et al. 2008; Tan et al. 2008; Yan et al. 2006). *Blastocystis* isolates from humans and other animals have been reported to be morphologically similar. However, extensive genetic variation among numerous isolates has been identified using molecular methods including, in most cases, polymerase chain reaction (PCR)–restriction fragment length polymorphism, random amplified polymorphic DNA, and PCR using sequence-tagged site primers (Stensvold et al. 2007; Tan 2008). *Blastocystis* has been

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found both in patients with gastrointestinal symptoms and in asymptomatic individuals (Tan et al. 2002). The concept of luminal protozoa as being causative agents of allergy-like cutaneous lesions is interesting and has been suggested to be linked to the activation, by parasite molecules, of certain specific immune cell subsets such as interleukin 3 (IL-3)-, IL-4-, IL-5-, or IL-13-secreting Th2 cells, which mediate IgE allergic responses (Pasqui et al. 2004).

Idiopathic urticaria is a relatively common debilitating disease. It is characterized by recurrent urticarial wheals (Kang and Shin 2009). Urticarial patients are usually treated with oral antihistamines and 50% respond well to this treatment; however, the other 50% do not respond to antihistamines and need a more aggressive approach, such as short or prolonged courses of oral corticosteroids or cyclosporine. Potential adverse effects, however, limit this regimen (Shahar et al. 2006). Only few cases were reported regarding the association of *Blastocystis* infection with urticaria. One of those reports pointed *Blastocystis* species subtype 2 to be the causative factor (Shahar et al. 2006) and another pointed subtype 3 (Katsarou-Katsari et al. 2008), a finding that may affect the response to therapy. In addition to a single Turkish study on small number of cases without identifying the isolated subtypes (Dogruman-Al et al. 2009). In this regard, the presence of *Blastocystis* infection in patients with urticaria may indicate a causal role of the parasite, and appropriate therapy should be considered (Tan 2008). This work aims to find out the association of *B. hominis* with urticaria with and without gastrointestinal symptomatology. In such condition, the responsible *Blastocystis* genetic subtype will be determined and the response to anti *Blastocystis* specific therapy will be studied.

Materials and methods

Patient selection

Patients were collected from the Dermatology outpatient clinic Ain Shams University Hospital, Cairo, Egypt. Patients stool samples were examined and cultured at the Parasitology Research and Diagnostic Laboratory Parasitology Department, Ain Shams University, Cairo, Egypt. *Blastocystis* genotyping was performed at the Molecular Department of Medical Research Center, Ain Shams University Hospital, Cairo, Egypt. The study was carried from June 2009 till June 2010 and included 104 patients. The study design is a case control prospective study including two phases. Phase I: a case control study, while phase II: was to follow up the response of *Blastocystis hominis*-positive urticarial patients to metronidazole for one or two courses. Patients in study phase I were classified into

two groups; group I (GI): 54 patients (3–59 years) with history of urticaria; this group was further classified into GIA: 18 patients with acute urticaria and wheals of less than 6 weeks duration, GIB: 36 patients with chronic urticaria and wheals of more than 6 weeks duration (Vogelberg et al. 2010). Group II (GII): 50 apparently healthy individuals (7–65 years). Patients in study phase II included 20 symptomatic *B. hominis* (stool culture and PCR) urticarial patients who were subjected to treatment with metronidazole 750 mg (10 mg/kg/dose in children) orally, three times a day for 5–10 days for one or two courses with follow up for 1 year. All patients were subjected to complete history taking. Patients with personal and family history for nasal allergies, asthma and/or allergy, and hypersensitivity to particular foods or drugs were excluded. Patients with a history of drug intake within 2 weeks before the appearance of lesion were also excluded. Fever, arthralgia, or insect bites prior to onset of symptoms were also exclusion criteria in our patients. Patients were selected having no pets and did not travel recently. Laboratory tests to identify any underlying cause were performed. A search for a possible underlying internal disorder was done (hepatic, renal diseases, autoimmune diseases, or endocrinological diseases). Other parasitic diseases were excluded by stool and urine examination. Skin sensitivity tests for common allergens were negative. *Blastocystis*-positive patients were considered as “symptomatic” (presenting variously with diarrhea, abdominal pain, vomiting, flatulence, some in association with fatigue, and fever) and “Asymptomatic” based on history and examination.

Stool culture

B. hominis isolates were established in culture from human fecal samples using a biphasic egg-slant medium overlaid with Locke's solution and inactivated horse serum. The cultures were screened for *Blastocystis* cells by standard light microscopy and subculture for an additional 2–3 days in fresh medium (Zierdt 1991) with identification of the various forms of *Blastocystis* morphologically (Figs. 1 and 2). Parasites were harvested from culture medium by centrifugation (500×g, 5 min) and washed twice with sterile phosphate-buffered saline (PBS; pH 7.4). Subsequently, parasites were purified using a density gradient centrifugation technique to reduce bacterial contamination and to concentrate *Blastocystis* organisms from culture supernatants (Upcroft et al. 1989). Briefly, organisms were pelleted by centrifugation at 400×g for 10 min, and the parasite pellet was resuspended in 3 ml PBS. Parasites were layered onto 10 ml of Ficoll-Hypaque separating media (Pharmacia Biotech Inc. Piscataway, NJ, USA) in a 12-ml centrifuge tube and were separated from bacteria by centrifugation (2,000×g

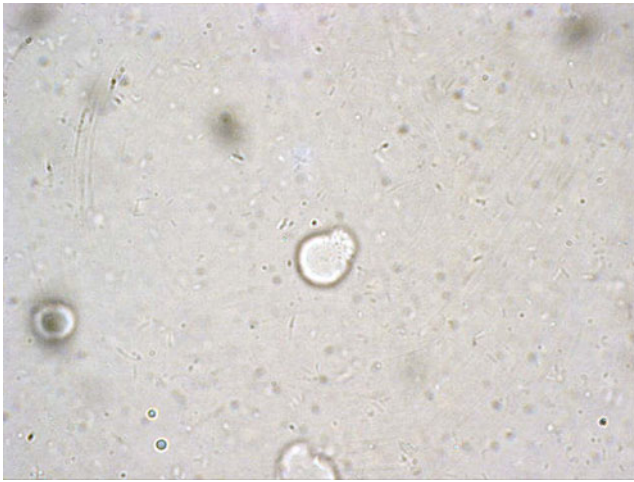


Fig. 1 Amoeboid and granular forms of *Blastocystis hominis* in stool culture (5×1,000)

for 20 min). *B. hominis* cells banded approximately 1 cm from the top of the Ficoll-Hypaque solution, while bacteria pelleted at the bottom of the tube. Purified parasites were recovered by aspirating the band, washed in sterile PBS and pelleted by centrifugation (500×*g* for 5 min). This was repeated for five times, then a portion of the final pellet was examined under bright field microscopy at the magnification of ×1,000 to ensure the purity of the sample and the pellet was stored at −70°C until processed for genomic DNA extraction.

DNA extraction from stool culture

Genomic DNA was purified from cultured *B. hominis* isolates using QIAamp® stool mini kit (QIAGEN Co., GmbH, D-40724 Hilden). Amplification of DNA was

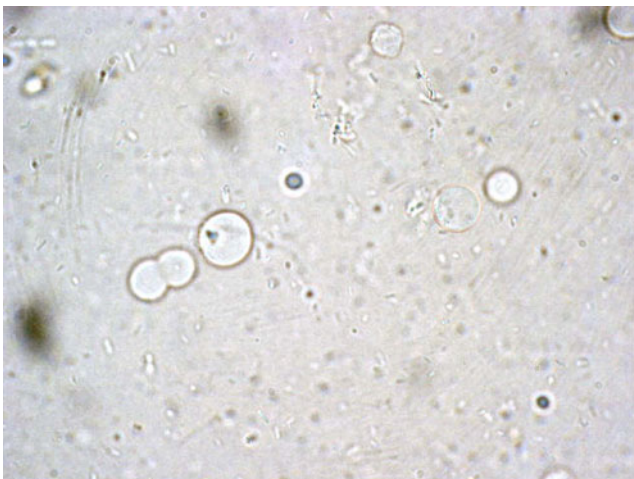


Fig. 2 Binary fission of the vacular form of *Blastocystis hominis* in stool culture (×1,000)

carried out with the primers R1 5'(GGAGGTAGTGACAA-TAAATC)3' and reverse, 5' (CGTTCATGATGAACAAT-TAC) 3'(Böhm-Glönig et al. 1997) PCR conditions (Perkin Elmer 9700, USA) consisted of one cycle denaturation at 94°C for 4 min, 35 cycles including denaturation at 94°C for 30 s, annealing 55°C for 30 s, extension at 72°C for 30 s and final extension 72°C for 5 min. The amplification products were digested with 3 restriction enzymes *AluI* *HinfI* and *RsaI* (Promega, USA) in a reaction mixture of 2 µl of 10× Multi-core buffer, 0.5 µl of acetylated bovine serum albumin (10 µg/µl), 1 µl of DNA (1 µg/µl), 0.5 µl (10U/µl) of restriction endonuclease, and 16 µl of sterile distilled water to a final volume of 20 µl, at 37°C for 4 h then at 65°C for 20 min. The digested products were separated by electrophoresis with a size marker (100 bp ladder marker, Promega, USA) in 2% agarose gel containing ethidium bromide. The specific band was visualized with Transilluminator under ultraviolet light (Cole-Parmer, Chicago, USA) and the restriction fragments of each isolate were analyzed.

Statistical analysis

All data are reported as the mean value including the standard deviation. *T* test was used to assess the statistical significance of the difference between two study group means. Chi-square test was used to examine the relationship between two qualitative variables. Fisher's exact test: was used to examine the relationship between two qualitative variables when the expected count is less than five in more than 20% of cells. Results with *p* values less than or equal to 0.05 are considered statistically significant.

Results

Out of the 54 urticarial patients (group I), 13 (24.1%) had diarrhea, eight (14.8%) had abdominal pain, two (3.7%) had constipation, and one (1.8%) patient only with irritable bowel syndrome.

There was no statistical difference between *Blastocystis* symptomatic and asymptomatic urticarial and patients regarding age, sex, and type of urticaria.

There was no statistical difference between *Blastocystis* acute and chronic urticaria patients as regards the relief of symptoms after first course of treatment. After the first course of treatment with metronidazole for the 20 symptomatic *Blastocystis* urticarial patients, 12 (60%) became clinically and parasitologically free. After a second course of treatment with metronidazole, for the remaining non-relieved eight patients, they became clinically and parasitologically free with disappearance of the amoeboid form.

All the positive *B. hominis* cases were of subtype 3 (Table 1).

Discussion

Urticaria could result from a variety of pathophysiological mechanisms (Hennino et al. 2006). Antigens might cause urticaria and concomitant exposure could precipitate this reaction by increasing antigen absorption or by modulating the release of mediators (Cassano et al. 2005, Gupta and Parsi 2006). Mast cell activation might involve membrane receptors for the complement molecules (Hennino et al. 2006); this mechanism might explain toxic-allergic reactions possibly caused by *Blastocystis* (Valsecchi et al. 2004 and Stenzel and Boreham 1996). *B. hominis* is a common parasite among humans with surveys revealing infection rates of up to 10% in developed countries and 50% in developing countries (Clark 1997 and Wong et al. 2008). Interestingly, the present study showed a prevalence of 61.1% of *Blastocystis* infection among patients with urticaria (group I; Table 2). This is supported by a recent survey showed a high prevalence of *Blastocystis* isolation in patients with allergic skin disease (Özcakir et al. 2007). On the other hand, 8% of the normal control group (group II) had *Blastocystis* (Table 2) in their stool culture. This is different from a prevalence of 33.3% reported in Egypt by Rayan et al. (2007) as the author carried out his study on patients presumed to be infected with intestinal parasites. The presence of the parasite was seen concurrently in patients with acute or chronic urticaria (Armentia et al. 1993; Biedermann et al. 2002; Katsarou-Katsari et al. 2008 and Valsecchi et al. 2004). In the present study, out of the 18 acute urticarial patients, nine (50%) were *Blastocystis*-positive and out of 36 chronic urticarial patients, 24 (66.7%) were *Blastocystis* positive (Table 1). Actually, it cannot be managed that there is a significant statistical difference comparing acute with chronic cases of urticaria. This is on the basis that out of the 54 randomly selected patients with urticaria (group I), chronic patients were more than the acute ones (Table 1).

Subtype 3 is the most frequently isolated genotype in epidemiological surveys and is probably the only genotype of human origin (Böhm-Glönig et al. 1997, Kaneda et al. 2001, Yoshikawa et al. 2004 and Wong et al. 2008). In the present study, all the *B. hominis* isolates were of subtype 3 (Fig. 3). This is supported by other studies indicated no association between disease and parasite genotype (Böhm-Glönig et al. 1997 and Yoshikawa et al. 2004). In contrast, Hussein et al. (2008) concluded that subtype 1 was the most virulent, while subtypes 3 and 6 consisted of pathogenic and nonpathogenic strains. This explains the presence of subtype 3 in asymptomatic patients included in GI&GII. Recent clinical studies have reported the association of skin rash with *Blastocystis* infection (Ozyurt et al. 2008 and Amin 2006). A Greek case report of one patient with *Blastocystis*-associated rash identified the genotype infecting the patient as *Blastocystis* sp subtype 3 (Katsarou-Katsari et al. 2008). Moreover, dermatologists have found *Blastocystis* infection at a statistically significant rate in patients with allergic skin conditions (Giacometti et al. 2003). Indeed, there is a strong association between *Blastocystis* infection and skin rash as reported by the previous studies. However, accusation of other factors in addition to this genotype like genetic predisposition and proteolytic activity are suggested. This is evident by the present study where subtype 3 was reported in urticarial patients as well as normal control group included in phase I (group I, group II). Subtypes 4, 6, and 7 appear to show some degree of host specificity (Noel et al. 2005). The host specificity may be responsible for the absence of these subtypes in the present study as well as for the low prevalence of these subtypes studies of the human population (Clark 1997). Souppart et al. (2009b) reported that subtype 3 was the most common subtype (61.90%) in the present Egyptian population. Moreover, current data suggest that subtype 3 is the only subtype of human origin; therefore, it is considered as the true *B. hominis* (Tan 2008). It was observed that drinking unboiled water was associated with subtype 3 infections (Li et al. 2007). This is another evidence for the detection of subtype 3 only in the Egyptian

Table 1 Comparison between acute and chronic urticarial patients regarding sex, *Blastocystis* in stool culture, amoeboid form and gastrointestinal (GIT) symptoms

		Urticaria		χ^2	<i>p</i>	Sig
		Acute, n.18 (%)	Chronic, n.36 (%)			
Sex	Male	9 (50)	14 (38.9)	0.606	0.436	NS
	Female	9 (50)	22 (61.1)			
<i>Blastocystis</i> in stool	Negative	9 (50)	12 (33.3)	1.403	0.236	NS
	positive	9 (50)	24 (66.7)			
Amoeboid form	Negative	10 (55.6)	24 (66.7)	0.635	0.425	NS
	Positive	8 (44.4)	12 (33.3)			
GIT symptoms	Asymptomatic	10 (55.6)	23 (63.9)	0.351	0.554	NS
	Symptomatic	8 (44.4)	13 (36.1)			

Table 2 Comparison between patients with urticaria (group I) and healthy controls (group II) regarding sex, *Blastocystis hominis* and amoeboid form

		Patients, n.54 (%)	Controls, n.50 (%)	χ^2	<i>p</i>	Sig
Sex	Male	23 (42.6)	21 (42)	0.004	.951	NS
	Female	31 (57.4)	29 (58)			
Blastocystis in stool	Negative	21 (38.9)	46 (92.0)	10.93	0.001	HS
	Positive	33 (61.1)	4 (8)			
Amoeboid form	Negative	34 (63)	50 (100)	22.92	0.001	HS
	Positive	20 (37)	0 (0.0)			

patients included in the present study, since consumption of contaminated water is a common health problem in Egypt and could be the main source of *Blastocystis* infection. Considerably, the large-scale survey of Dogruman-Al et al. (2008) and Dogruman-Al et al. (2009) did not show any correlation between subtypes and symptomatology. The present study shows no statistical significant difference between acute and chronic urticarial patients as regard sex, *B. hominis* in stool culture, amoeboid form, and gastrointestinal symptoms. Out of 33 *B. hominis* patients with urticaria included in this study, 21 (63.6%) were symptomatic and 12 (36.4%) were asymptomatic (Table 3). Generally, this is in agreement with a number of recent surveys where *B. hominis* was reported to be found with higher incidences in immunocompetent individuals suffering from intestinal disorders than in the asymptomatic group (Graczyk et al. 2005 and Kaya et al. 2007). On the other hand, the symptomatic *Blastocystis* urticarial patients (63.6%) included in the present study were less than that of a recent study by Kaya et al. (2007), who reported intestinal symptoms in 46 of 52 (88.4%) individuals infected with *Blastocystis* spp. This could be explained by the

selective group of urticaria included in the present study. As regards the pathogenic potential of *Blastocystis*, it was widely debated in the literature during the two last decades because the organism can be found in both symptomatic and asymptomatic patients. However, numerous recent in vivo and in vitro studies strongly suggest that this organism is a pathogen (Stark et al. 2007; Boorom et al. 2008 and Tan 2008). This is proved in the present study where *B. hominis* was detected in stool culture in both symptomatic and asymptomatic patients. Major symptoms associated with *Blastocystis* infection in the present study included diarrhea (24.1%), abdominal pain (14.8%), constipation (3.7%), and irritable bowel syndrome (1.8%). This is in agreement with that reported by Yakoob et al. (2004); Boorom et al. (2008), and Jones et al. (2009). The present study showed no statistical significance difference between acute and chronic patients with urticaria regarding detection of *B. hominis* in stool culture. Concerning the association of the amoeboid form of *Blastocystis* with the type of urticaria, it was of no statistical significance difference. A recent study revealed that acute urticaria was associated with amoeboid forms belonging to subtype 3 (Micheloud et al. 2007). Moreover,

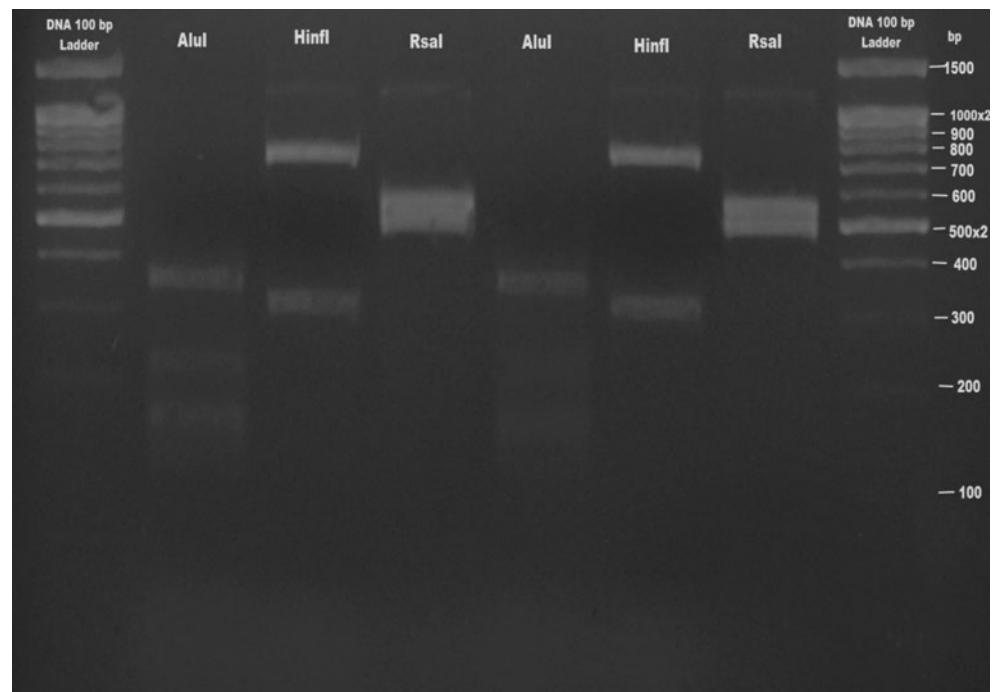
Fig. 3 Restriction profiles of part of the 16 S-like rDNA of different *B. hominis* isolates on 2% agarose gel. 1 and 8 molecular weight marker 100 bp ladder. DNA in 2 and 5 was digested with *AluI*, DNA in 3 and 6 was digested with *HinfI*, and DNA in 4 and 7 was digested with *RsaI*. Restriction digests in 2–7 stand for subgroup III

Table 3 Comparison between *Blastocystis* symptomatic and asymptomatic urticarial patients regarding amoeboid form in stool culture

Amoeboid form	urticarial patients (no.33)		P	Sig
	Asymptomatic, n.12 (%)	Symptomatic, n.21 (%)		
Positive	0 (0.0)	20 (95.2)	0.001	HS
Negative	12 (100.0)	1 (4.8)		

*Fisher exact test

the amoeboid form was found in 20 (95.2%) out of 21 symptomatic urticarial patients (Table 3). This finding assures the pathogenic potential of the amoeboid form of the *Blastocystis* subtype 3. This agrees with Yan et al. (2006) and Katsarou-Katsari et al. (2008) who speculated that subtype 3 might contribute to *Blastocystis* sp. pathogenic potential only when the amoeboid form is present. In symptomatic patients, amoeboid forms of *Blastocystis* sp. might adhere to the epithelial cells lining the gut (Tan et al. 2002 and Tan and Suresh 2006b). The irregular amoeboid form has been suggested as the pathogenic form (Tan and Suresh 2006a, b). Contrarily, Souppart et al. (2009a) suggested that the presence of the amoeboid form may be poorly correlated with patient symptoms. In phase II of the present study, 20 symptomatic *B. hominis* urticarial patients with amoeboid form were submitted to treatment for one or two courses of metronidazole. Stool culture was done with subculture for three times, 1 day apart. Patients were considered free when all the recognized forms of the parasite were completely eradicated. After the first course of treatment, 12 (60%) patients out of the 20 *Blastocystis* patients with urticaria became clinically and parasitologically free. All the 12 (100%) relieved patients did not show any forms of *B. hominis* in their stool culture while all of the eight (100%) non-relieved patients showed different forms of *Blastocystis* in their stool culture (Table 4). On repeating the treatment after 10 days as a second course, the remaining eight patients turned negative clinically and parasitologically. So by the end of the second course of treatment, 100% of the patients recovered from both urticarial symptoms and *B. hominis* in stool culture. Clinical and parasitological relieve was reported in 100% of subgroup A (acute urticaria) and in 50% of subgroup B (chronic urticaria) after the first course of treatment. The remaining 50% of subgroup B recovered only after the second course of treatment. This is evidence

that infection of *B. hominis* may be one of the underlying causes of resistance to urticaria treatment. Another explanation could be the metronidazole itself as Vogelberg et al. (2010) reported a clinical case which possibly links *Blastocystis* sp. ST2 infection with the simultaneous occurrence of gastrointestinal illness and generalized chronic urticaria. The authors found that despite repeated chemotherapy with different antimicrobial drugs, both the gastrointestinal and cutaneous disorders reoccurred after short symptom-free intervals. In the present study, the follow up of those patients included in study phase II continued for 1 year by clinical and parasitological examination and no reoccurrence of symptoms was reported. This is in agreement with that reported by Tan (2008) where out of 41 individuals who submitted a second stool specimen after metronidazole therapy, with clinical cure in 36 of the 39 (92.3%) patients. Moreover, Valsecchi et al. (2004); Biedermann et al. (2002); Gupta and Parsi (2006); Zuberbier and Maurer (2007), and Katsarou-Katsari et al. (2008), reported that the successful eradication of *Blastocystis* producing amoeboid forms during the cultivation period resulted in complete resolution of urticarial lesions. In the present study, there was no statistical difference between the stage of urticaria (acute/chronic) and the early response to treatment. Since 50% of the relieved patients were acute and 50% were chronic after the first course of treatment. Therefore, treatment should be administered for both acute and chronic cases of urticaria with *Blastocystis* and repeated for a second course till complete elimination of the parasite and complete recovery of the patients. It was concluded that *Blastocystis* is a strong suggestive pathogen for urticaria reported in 61% of urticarial patients. Moreover, the amoeboid form was detected in symptomatic patients with urticaria assuring the pathogenic potential of this form. Metronidazole for two repeated courses resulted in 100% elimination of *Blastocystis*

Table 4 Comparison between the relief of urticarial symptoms after the first course of metronidazole treatment as regards sex and *Blastocystis* in stool culture

		Urticarial symptoms				<i>P</i> ^a	Sig
		Relieved		Not relieved			
		<i>N</i>	%	<i>N</i>	%		
Sex	Male	5	41.7	2	25.0	0.642	NS
	Female	7	58.3	6	75.0		
<i>Blastocystis</i> amoeboid form in stool culture	Negative	12	100.0	0	0.0	0.001	HS
	Positive	0	0.0	8	100.0		

^a Fisher exact test

and cure of urticaria. Further studies are needed in different countries and populations on urticarial patients trying to detect the pathogenic subtype in each population, the factors influencing the pathogenicity and the source of the organism in an attempt to avoid infectivity and pathogenicity of such parasite.

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