

The relationship between blastocystis hominis infection and Irritable Bowel Syndrome (IBS) and comparing direct wet mount, stool culture, Formalin-Ether and trichrome staining procedures for identifying organisms

Nashmin Moheemmi¹ Mohammad Moradi¹ Alireza Khalilian² Amir Hossein Maghsood³ Mohammad Fallah⁴

MSc of Parasitology¹, Assistant Professor Department of Internal Medicine², Associate Professor Department of Parasitology³, Professor Department of Parasitology⁴, Hamadan University of Medical Sciences, Hamadan, Iran.

(Received 17 Jun, 2013

Accepted 1 Feb, 2014)

Original Article

Abstract

Introduction: Blastocystis hominis is a parasite found in the stools of human as well as in the stools of other animals and recently its pathogenic potential has been increasingly recognized. Irritable Bowel Syndrome (IBS) is a digestive system's disorder which is featured by change in bowel habits, abdominal pains and other symptoms similar to Blastocystis hominis infection. There is little information about this syndrome and its etiology is still unknown. This study aims at analyzing the relationship between Blastocystis hominis and IBS.

Methods: In a case-control study, a total of 81 patients with IBS and 81 patients with other digestive disorders, but without IBS, who had referred to Shahid Beheshti Hospital in Hamedan City, were selected. The disease was diagnosed by a gastroenterologist in terms of the relevant criteria. Blastocystis hominis infection in this study was diagnosed using stool test through four techniques: direct wet mount, stool culture, formalin-ether concentration and trichrome staining.

Results: Totally, 43 women (53%) and 38 men (47%), in the age range of 13 to 64 years old, participated in this study. Using the stool culture technique, as the golden standard, infection in 37 members (45.67%) of the case group and 18 members (22.22%) of the control group was reported, which this difference was significant, statistically ($P < 0.001$). There was difference between case and control groups in terms of Blastocystis infection with all four techniques.

Conclusion: The results of this study indicated that since the infection rate in the case group was higher than that in the control group, a relationship between Blastocystis hominis infection and IBS is possible. Speaking about infection diagnosis, culture technique was more sensitive than other techniques used to diagnose Blastocystis hominis infection.

Key words: Blastocystis Hominis - Irritable Bowel Syndrome – Parasites

Correspondence:
Mohammad Fallah, MD.
Department of Parasitology
and Mycology, Medical
School Hamadan University
of Medical Sciences.
Hamadan, Iran
Tel: +98 9181113650
Email:
fallah@umsha.ac.ir

Citation: Moheemmi N, Moradi M, Khalilian A, Maghsood AH, Fallah M. The relationship between blastocystis hominis infection and Irritable Bowel Syndrome (IBS) and comparing direct wet mount, stool culture, Formalin-Ether and trichrome staining procedures for identifying organisms. Hormozgan Medical Journal 2015;19(2):67-73.

Introduction:

Blastocystis hominis is an intestinal protozoan which is abnormal for human being and a wide range of animals (1-4). It has a global distribution and is amongst the most prevalent parasites, which are separated through parasitological experiments. As a polymorphic organism, Blastocystis hominis is described as the organism with "a thousand faces". Its size varies from 5 to 30 micron. Its most important forms are vacuolar, granular, amoeboid, and cystic (5,6). Also there are other forms of this parasite including avacuolar and multivacuolar which are found rarely (7-11).

Human beings usually become infected through digesting the cysts excreted by another human. The digested cysts are opened near the stomach acid and intestinal enzymes and after converting into various forms, finally the new cysts produced in the bowel, are excreted by feces and move to another hosts and their life cycle repeats in this way (12).

A broad collection of epidemiological information, lab and experimental studies imply the pathogenic potential of Blastocystis hominis in certain condition. In the case of finding Blastocystis in feces where are free from other parasites, bacteria and viruses, it can be considered as the cause of diarrhea, cramps, nausea, fever, vomiting and abdominal pains which need to be treated. In a recent study on patients with IBS, presence of a considerable percent of Blastocystis was not considered casual and was related to occurrence of the syndrome (13). Researchers believe that there are many genotypes of Blastocystis in the nature and recent studies made it clear that human being is the host of many of its zoonotic genotypes. Infection to this parasite can be without symptom or with nonspecific clinical symptoms such as nausea, vomiting, abdominal pain, bloat, acute and chronic diarrhea and skin rashes (14-16).

IBS is a digestive system disorder which is featured with the change in bowel habits, abdominal pains and without structural anomalies. There is no clear diagnostic symptom for IBS; hence, all definitions proposed for the syndrome are based on its clinical manifestations. IBS is one of the most prevalent diseases which is found in clinical works; however there is very little information and knowledge about it. Patients with IBS always suffer from abdominal distension, more

burps and bloat. IBS pathogenesis is unknown yet (17). However, abnormal sensory and motor activities of bowel, central nervous function disorder, psychological disorders and bowel causes have been suggested (18,19).

Because of inflammation in the digestive tract, some studies on confirming this fact that IBS occurs by some undiscovered active infections have been conducted and some authors emphasize undiagnosed protozoan infections, as the IBS cause. Blastocystis is a protozoan organism which its infection can be without symptoms, acute or chronic symptoms. The parasite has sometimes suggested as the cause of certain symptoms such as abdominal pain contestation, diarrhea and change in bowel habits, which are similar to IBS; however, this report has been criticized by some physicians (20). The symptoms may continue be found as ongoing, alternative, individual for years, thus, it is necessary that parasitic infections are examined very precisely in such patients and their presence is confirmed or denied.

Although several studies about the relationship between this protozoan and even Dientamoeba fragilis and IBS have been carried out, their results in inconsistent (21). Unlike other parts of the world, no special study about this issue has been conducted in Iran. Since causes of this syndrome and the potential of involvement of racial factors or other unknown factors in its development, similar studies in various geographical regions and racial and ethnic groups can be justified. Therefore, the objective of this study is analyzing the relationship between infection with Blastocystis hominis and IBS in the Iranian patients and also comparing various diagnosis techniques including direct wet mount, stool culture, trichrome staining and formalin-ether concentration technique. Thus, in this study, only patients diagnosed with IBS based on scientific criteria and the results were compared with other digestive disorders, in terms of presence of Blastocystis hominis, in order to analyze the potential role of Blastocystis hominis in developing IBS.

Methods:

In a case-control study and under a 1:1 matching program in terms of age and gender, a

total of 81 patients with IBS and 81 patients with other digestive disorders, but without IBS, who had referred to Shahid Beheshti Hospital in Hamedan City, were selected. Patients with IBS, who have been diagnosed by a gastroenterologist and the project's clinical partner, were classified as case group and patients with other digestive disorders but without IBS were classified as control group. After signing the consent form and filling the questionnaire containing information about symptoms and personal data of patients, their fecal samples were collected. Samples were tested using for techniques including direct microscopic method, formalin-ether concentration, culture in the lab environment, and trichrome staining in the research lab of Parasitology Department for diagnosing Blastocystis. The definitive diagnosis of Blastocystis hominis using four mentioned techniques in order to compare diagnosis methods in both groups and making sure of lack of infection to other pathogenic parasites of bowel in the case model was used. In both groups, patients with any other parasitic disease than Blastocystis were excluded. Finally, the results were analyzed using chi-square test with SPSS version 16.

Results:

Totally, 43 women (53%) and 38 men (47%), in the age range of 13 to 64, participated in this study. In both control and case groups, there were 7 patients (8.6%) were younger than 20 years old, 27 patients (33.33%) were 20-30 years old, 19 patients (23.5% were 30-40 years old, 16 patients (19.8%)

were 40-50 years old, 10 patients (12.3%) were 50-60 years old and two patients were older than 60 years old. The age range of 20-30 years old (33.33%) had the most members.

The cumulative results of four mentioned techniques showed that Blastocystis infection rate in patients with IBS and in control group was 45.67% and 22.22%, respectively. In all four techniques, the infection with Blastocystis in the case group was more than that in control group and it was significant difference ($P < 0.0001$, $OR = 2.1$). Table 1 summarizes the rate of Blastocystis infection in both case and control groups measured with four diagnosis techniques.

In this study, using direct slide technique 28 patients (34.56%) in case group and 16 patients (19.75%) in control group; using formalin-ether concentration technique 31 patients (38.27%) in case group and 16 patients (19.75%) in control group; using trichrome staining technique 21 patients (25.92%) in case group and 14 patients (17.28%) in control group were found infected with Blastocystis. In all four methods, there was a significant different between case and control groups in terms of infection with Blastocystis. Culture technique was more sensitive than other technique, with 37 patients (45.67%) in case group and 18 patients (22.22%) in control group were diagnosed with Blastocystis infection. Trichrome staining technique showed the least sensitivity for diagnosing Blastocystis. Table 2 summarizes the frequency of various clinical symptoms in both case and control groups.

Table 1. Comparing sensitivity of diagnostic techniques in diagnosing Blastocystis in feces

Diagnosis technique	Case		Control	
	No.	Percentage	No.	Percentage
Culture	37	45.67%	37	45.67%
Direct slide	28	34.56%	28	34.56%
Formalin-ether	21	38.27%	21	38.27%
Tri-chrome staining	21	25.92%	21	25.92%

Table 2. Frequency of various clinical symptoms in both case and control groups

Symptoms	Case		Control	
	No.	Percentage	No.	Percentage
Abdominal pain	65	80.24	44	54.3
Nausea	12	14.8	20	24.7
Diarrhea	20	24.7	5	6.2
Constipation	60	74.1	18	22.2
Cramps	29	35.8	10	12.2
Vomiting	2	2.5	4	4.9
Bloat	70	84.4	17	21
Abdominal distension	53	65.4	5	6.2
Burps	37	45.7	8	9.9

Table 3. Infection severity: number of parasites in each microscopic field with 40 x magnifications

Number of Parasites	Case		Control	
	No.	Percentage	No.	Percentage
1-2	28	75.68	10	55.55
3-4	4	10.81	4	22.22
>5	5	13.51	4	22.22

Some researchers have argued that finding at least five Blastocystis parasites in any microscopic field at 40X magnification, provided that no other cause is found for the bowel disease, the parasite's pathogenesis must be considered. Since, in culture technique, 37 patients in case group and 18 patients in control group were diagnosed with Blastocystis infection, in this study 1-2 parasites in any microscopic field at 40x magnification, 28 out of 37 patients (75.68%) in case group in contrast to 10 out of 18 patients (55.55%) in control group were seen. In this magnification, 3-4 parasites in any microscopic field, in case group 4 out of 37 patients (10.81%) and in control group, 4 out of 18 patients (22.22%) were found. More than five parasites in each microscopic field with 40 x magnifications, in case group 5 out of 37 patients (13.51%) and in control group 4 out of 18 patients (22.22%) were found. The most number of seen parasites was 1-2 parasites in each microscopic field with 40 x magnifications. Table 3 shows infection severity with regard to number of parasites in each microscopic field with 40 x magnifications.

Regarding education level, there were 9 illiterates (11.1%), 16 persons with primary school education (19.8%), 9 persons with guidance school education (11.1%), 26 persons with diploma (32.15), and 21 persons with academic degrees (25.95%) in the case group; whereas, there were 11 illiterates (13.6%), 15 persons with primary school education (18.5%), 18 persons with

guidance school education (22.2%), 26 persons with diploma (32.1), and 11 persons with academic degrees (13.6%) in the control group. Diploma owners were the most population in both groups.

Conclusion:

This study showed that Blastocystis infection in patients with IBS is more than that in patients with other digestive problems. Likewise, this study indicated that the culture technique is the most sensitive technique in seeking Blastocystis in feces. Since this infection is prevalent in patients with IBS, it may play a key role in preparing these people to develop this syndrome. On the other hand, since some diseases are the result of several factors, it can be supposed that the parasite along with other unknown factors develop IBS. Blastocystis was already considered as a non-pathogenic organism. Since about thirty years ago, examining some patients with digestive disorder with stool test showed no other organism by this parasite and treating Blastocystis resulted in removing all digestive symptoms, hence it was concluded that Blastocystis would be the main cause of such digestive symptoms (18).

In many studies, the high prevalence rate of Blastocystis in people was without symptoms. In recent years it has been suggested that Blastocystis may have various pathogenic and non-pathogenic sub-species and many studies have been conducted

about this issue. Therefore, it is believed that pathogenic sub-species are effective in development of cases with symptoms (19-21). Because of these new observations and findings, *Blastocystis hominis* name was changed to *Blastocystis* spp. (22).

Regarding table 1, patients with *Blastocystis hominis* in case group were more than that in control group and it was a significant difference which was consistent with Yakoob et al. In his study, *Blastocystis hominis* was found in 53% of patients with IBS (90.171) in case group in contrast to the 16% of people (25.159) in control group ($P < 0.001$) (23). However, our study was not consistent with Ramirez-Miranda ($P = 0.43$) and Tuntrongchitr ($P = 0.87$) (24,25); with these researchers have not reported a significant relationship in *Blastocystis* infection in control and case groups. Surangsrirat compared 66 patients in IBS group with 60 subjects in control group in terms of *Blastocystis* infection. In the mentioned study, in both case and control groups, there was not a significant difference in terms of parasite prevalence ($P = 0.203$). Surangsrirat argues that in his study *Blastocystis* infection was a prevalent parasitological infection which was dominant in both IBS and control groups; however, *Blastocystis* in stool did not imply gastrointestinal symptoms in patients with IBS. This study suggests more studies on properties of *Blastocystis* sub-species in larger samples which may indicate a potential relationship between *Blastocystis* and patients with IBS (26). Ramirez-Miranda did not show a significant difference in the two groups, except in terms of bloating. This study indicated that although the relationship between *Blastocystis* and diarrhea in patients with IBS is not definitive, it was one of interesting findings of this study (24). Yakoob introduced Type 1 as the predominant genotype of *Blastocystis hominis* in IBS; whereas the predominant genotype in control group was type 3; thus it is supposed that there is a relationship between some genotypes of this parasite and symptoms of IBS (27,28). According to this study, the culture technique was more sensitive than other techniques and it was consistent with Yakoob, Tuntrongchitr, and Suresh et al. (29). Trichrome staining was introduced as the less sensitive method in contrast to other methods which was compatible

with relevant studies made in Turkey (30), Thailand (31), Britain (32), and Denmark (33).

Studies about different subtypes and genotypes in various geographical regions have focused on this sub-species and its relationship with clinical forms and its potential relationship with development of IBS (34-38). Accordingly, more than 8 sub-species in different African and European populations have been identified. In some cases, these sub-species have been verified as a key role in occurrence of recurrent digestive disorders symptoms (39,40). In a study made in Pakistan, genotype 1 was suggested as the predominant genotype in patients with IBS; whereas in the control group, genotype 3 was predominant (27); at the same time, *Dientamoeba* play a key role in this regard (23,41).

Sometimes Organism diagnosis faces limitations. One of targets of this study was determining sensitivity of various diagnostic techniques used to find parasites. Stool culture was one of techniques which was a valuable one, because in this technique even very few parasites can proliferate and their population will increase over time. However, regarding its results, there is a relationship between severity of natural *Blastocystis* infection and prevalence of clinical symptoms; hence, judging these results in terms of finding a relation between *Blastocystis* infection and IBS needs more studies with more samples and including severity of infection. The inability to make a cause and effect relationship between *Blastocystis* infection and IBS was a constraint in this study. For finding such relation we need an experimental study at least in animal model and conducting a genotyping experiment provides a more precise judgment. Likewise, it is suggested that in a clinical trial on *Blastocystis* pathogenesis patients are treated with a proper drug (e.g. metronidazole), if the symptoms are removed, the cause and effect relationship on its pathogenic potential in patients without IBS.

In this study, there was a significant relationship between severity of *Blastocystis hominis* infection and IBS and since this result was compatible with the results of studies made in Pakistan (Iran's eastern neighbor) and studies made in Turkey (Iran's northwestern neighbor) and is not consistent with results of studies made in Thailand

(southeastern Asia) and in Mexico (Latin America) and since, according to the new findings, *Blastocystis hominis* has different genotypes, it is possible that there is a certain genotype of *Blastocystis* in western Asian countries which has a relationship with IBS. Therefore, more studies on this issue with larger sample and more severe infection are needed.

Acknowledgement:

This article was prepared from Ms. Mohemi's M.S. thesis, registered with the reg. No. of 1542 by the Research Council of the university. The Research and Technology Deputy of Hamedan University of Medical Sciences, who financed a part of costs of the study, is appreciated. Likewise, Ms. Taheri, who assisted in collecting samples, and Dr. Muhammad Matini and Ms. Karimkhani, who helped us in conducting tests, are appreciated.

References:

1. Stenzel DJ, Boreham PF. *Blastocystis hominis* revisited. *Clin Microbiol Rev.* 1996;9:563-584.
2. Clark CG, van der Giezen M, Alfellani MA, Stensvold CR. Recent developments in *Blastocystis* research. *Adv Parasitol.* 2013;82:1-32.
3. Scanlan PD. *Blastocystis*: past pitfalls and future perspectives. *Trends Parasitol.* 2012;28:327-334.
4. Tan KS. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clin Microbiol Rev.* 2008;21:639-665.
5. John DT, Petri W. Markell and Voge's Medical Parasitology. 9th ed. Philadelphia: Saunders Press; 2006: 68-69.
6. Stenzel DJ, Boreham RE. *Blastocystis*, In: Gillespie HS, Pearson RD (eds.) *Principle and Practice of Clinical Parasitology*. New York: John Wiley & Sons Press; 2001: 359-372.
7. Zierdt CH, Rude WS, Bull BS. Protozoan characteristics of *Blastocystis hominis*. *Am J Clin Pathol.* 1967;48:495-501.
8. Suresh K, Smith H. Comparison of methods for detecting *Blastocystis hominis*. *Eur J Clin Microbiol Infect Dis.* 2004;23:509-511.
9. Tan KSW, Singh M, Yap EH. Recent advances in *Blastocystis hominis* research: hot spots in terra incognita. *Int J Parasit.* 2002;32:789-804.
10. Nascimento SA, Mda L. *Blastocystis hominis* and other intestinal parasites in a community of Pitanga city, Parana State, Brazil. *Rev Inst Med Trop Sao Paulo.* 2005;47:213-217.
11. Tan KS. New insights on classification, identification, and clinical relevance of *Blastocystis* spp. *Clin Microbiol Rev.* 2008;21:639-665.
12. Zierdt CH. *Blastocystis hominis*-past and future. *Clin Microbiol Rev.* 1991;4: 61-79.
13. Giacometti A, Cirioni O, Fiorentini A, Fortuna M, Scalise G. Irritable bowel syndrome in patients with *Blastocystis hominis* infection. *Eur J Clin Microbiol Infect Dis.* 1999;18:436-439.
14. Doyle PW, Helgason MM, Mathias RG, Proctor EM. Epidemiology and pathogenicity of *Blastocystis hominis*. *J Clin Microbiol.* 1990;28:116-121.
15. Clark CG. Extensive genetic diversity in *Blastocystis hominis*. *Mol Biochem Parasitol.* 1997;87:79-83.
16. Boorom KF, Smith H, Nimri L, Viscogliosi E, Spanakos G, Parkar U, et al. Oh my aching gut: irritable bowel syndrome, *Blastocystis*, and asymptomatic infection. *Parasit Vectors.* 2008;1:40.
17. Stensvold CR, Lewis HC, Hammerum AM, Porsbo LJ, Nielsen SS, Olsen KE, et al. *Blastocystis*: unravelling potential risk factors and clinical significance of a common but neglected parasite. *Epidemiol Infect.* 2009;137:1655-1663.
18. Longo D, Fauci A, Kasper D, Hauser S, Larry Jameson J, Loscalzo J, et al. *Harrison's Principles of Internal Medicine*. 18th ed. New York: McGraw-Hill Press; 2012;2496-2502.
19. Whipps CM, Boorom K, Bermudez LE, Kent ML. Molecular characterization of *Blastocystis* species in Oregon identifies multiple subtypes. *Parasitol Res.* 2010;106:827-832.
20. Stensvold CR, Suresh GK, Tan KS, Thompson RC, Traub RJ, Viscogliosi E, et al. Terminology for *Blastocystis* subtypes—a consensus. *Trends Parasitol.* 2007;23:93-96.
21. Wong KH, Ng GC, Lin RT, Yoshikawa H, Taylor MB, Tan KS. Predominance of subtype 3 among *Blastocystis* isolates from a major hospital in Singapore. *Parasitol Res.* 2008;102:663-667.
22. Santín M, Gómez-Muñoz MT, Solano-Aguilar G, Fayer R. Development of a new PCR protocol to

- detect and subtype Blastocystis spp. from humans and animals. *Parasitol Res.* 2011;109:205-212.
23. Yakoob J, Jafri W, Beg MA, Abbas Z, Naz S, Islam M, et al. Blastocystis hominis and Dientamoeba fragilis in patients fulfilling irritable bowel syndrome criteria. *Parasitol Res.* 2010;107:679-684.
 24. Ramirez-Miranda ME, /Hernandez-Castellanas R, Lopez-Escamilla E, Moncada D, Rodrigo Magallan A, Pogazamero C, et al. Parasites in Mexican patients with irritable bowel syndrome: a case-control study. *Parasites & Vectors.* 2010;3:96.
 25. Tungtrongchitr A, Manatsathit S, Kositchaiwat C, Ongrotchanakun J, Munkong N, Chinabutr P, et al. Blastocystis hominis infection in irritable bowel syndrome patients. *Southeast Asian J Trop Med Public Health.* 2004;35:705-710.
 26. Surangsrirat S, Thamrongwittawatpong L, Piyaniran W, Naaglor T, Khoprasert C, Taamasri P, et al. Assessment of the association between Blastocystis infection and irritable bowel syndrome. *J Med Assoc Thai.* 2010;93:119-124.
 27. Yakoob J, Jafri W, Beg MA, Abbas Z, Naz S, Islam M, et al. Irritable bowel syndrome: is it associated with genotypes of Blastocystis hominis. *Parasitol Res.* 2010;106:1033-1038.
 28. Yakoob J, Jafri W, Jafri N, Khan R, Islam M, Beg MA, et al. Irritable bowel syndrome: search of an etiology, role of Blastocystis hominis. *Am J Trop Med Hyg.* 2004;70:383-385.
 29. Suresh K, Smith H. Comparison of methods for detecting Blastocystis hominis. *Eur J Clin Microbiol Infect Dis.* 2004;23:509-511.
 30. Dogruman-Al F, Kustimur S, Yoshikawa H, Tuncer C, Simsek Z, Tanyuksel M, et al. Blastocystis subtypes in irritable bowel syndrome and inflammatory bowel disease in Ankara, Turkey. *Mem Inst Oswaldo Cruz.* 2009;104:724-727.
 31. Leelayoova S, Taamasri P, Rangsin R, Naaglor T, Thathaisong U, Mungthin M. In-vitro cultivation: a sensitive method for detecting Blastocystis hominis. *Ann Trop Med Parasitol.* 2002;96:803-807.
 32. Stensvold CR, Arendrup MC, Jespersgaard C, Molbak K, Nielsen HV. Detecting Blastocystis using parasitologic and DNA-based methods: a comparative study. *Diagn Microbiol Infect Dis.* 2007;59:303-307.
 33. Clark CG, van der Giezen M, Alfellani MA, Stensvold CR. Recent developments in Blastocystis research. *Adv Parasitol.* 2013;82:1-32.
 34. Meloni D, Sanciu G, Poirier P, El Alaoui H, Chabé M, Delhaes L. Molecular subtyping of Blastocystis sp. isolates from symptomatic patients in Italy. *Parasitol Res.* 2011;109:613-619.
 35. El Safadi D, Meloni D, Poirier P, Osman M, Cian A, Gaayeb L. Molecular epidemiology of Blastocystis in Lebanon and correlation between subtype 1 and gastrointestinal symptoms. *Am J Trop Med Hyg.* 2013;88:1203-1206.
 36. Forsell J, Granlund M, Stensvold CR, Clark GC, Evengård B. Subtype analysis of Blastocystis isolates in Swedish patients. *Eur J Clin Microbiol Infect Dis.* 2012;31:1689-1696.
 37. Alfellani MA, Stensvold CR, Vidal-Lapiedra A, Onuoha ES, Fagbenro-Beyioku AF, Clark CG. Variable geographic distribution of Blastocystis subtypes and its potential implications. *Acta Trop.* 2013;126:11-18.
 38. Soupart L, Sanciu G, Cian A, Wawrzyniak I, Delbac F, Capron M, et al. Molecular epidemiology of human Blastocystis isolates in France. *Parasitol Res.* 2009;105:413-421.
 39. Vogelberg C, Stensvold CR, Monecke S, Ditzén A, Stopsack K, Heinrich-Gräfe U. Subtype 2 detection during recurrence of gastrointestinal and urticarial symptoms. *Parasitol Int.* 2010;59:469-471.
 40. Stensvold CR, Nielsen HV, Mølbak K, Smith HV. Pursuing the clinical significance of Blastocystis--diagnostic limitations. *Trends Parasitol.* 2009;25:23-29.
 41. Stark D, van Hal S, Marriott D, Ellis J, Harkness J. Irritable bowel syndrome: a review on the role of intestinal protozoa and the importance of their detection and diagnosis. *Int J Parasitol.* 2007;37:11-20.