Differences in Clinical Significance and Morphologic Features of *Blastocystis* sp Subtype 3

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**Abstract**

*Blastocystis* is a polymorphic intestinal parasite that is common in humans. A total of 51 asymptomatic and symptomatic patients positive for *Blastocystis* only were included in the study. Symptoms were mainly nonspecific gastrointestinal symptoms. *Blastocystis* isolates were xenically cultured and subtyped. *Blastocystis* species subtype 3 was the predominant subtype. Intrasubtype differences (vacuolar/amoeboid presence) in subtype 3 morphotypes were observed in 32 asymptomatic and symptomatic subtype 3 cases and could possibly be related to *Blastocystis* pathogenic potential. Diverse morphologic features (vacuolar transiting to amoeboid), probably reflecting the progression from an asymptomatic to a symptomatic state, were observed in an asymptomatic subtype 3 carrier who later had symptoms. Searching for amoeboid forms might be helpful to presumptively screen symptomatic patients with subtype 3 or to follow up an asymptomatic subtype 3 carrier in case symptoms become evident before antiprotozoal treatment was attempted. Further studies on the roles of morphologic features and variation within *Blastocystis* species subtypes as predictors of symptoms are encouraged.

*Blastocystis* is a cosmopolitan enteric parasite that is more commonly found in developing countries.1 It has been encountered in asymptomatic people and in patients with gastrointestinal (GI) and other symptoms.1,2 Diverse forms of *Blastocystis* have been reported by various researchers: vacuolar forms are observed in feces and in cultures, granular forms are considered degenerative products, and amoeboid stages of *Blastocystis* are detected whenever symptoms are reported.2-4 Ten subtypes of *Blastocystis* isolates have been recognized.5,6 A number of studies have attempted to correlate *Blastocystis* subtypes with clinical symptoms and found conflicting results.1

In the early 1990s, a dispute arose among prominent American researchers about whether *Blastocystis* is a mere commensal organism or a potential pathogen, an issue that remains unclear.7,8 More recently, it has again been suggested that the presence of subtype 3 correlates with symptoms.9,10 It has been hypothesized that a possible pathogenic variant of *Blastocystis*, originating from the Middle East, might be responsible for the increase in symptomatic cases in the United States, while subtype 3 has been predominantly detected.11,12 Greece is a country neighboring closely to the endemic Middle Eastern region11,12; however, it still remains a country of low endemicity despite a slight increase in *Blastocystis* infection seen during the past decade: a report from Greece, using data collected in the late 1990s, showed that *Blastocystis* was present in the stools of 9 (0.5%) of 1,879 people,13 while our recent results indicate a *Blastocystis* rate of 3.5%.14 Furthermore, in a previous study,15 our group found that subtype 3 was the most common *Blastocystis* species subtype identified in stool samples from Greece. In this study, we are reporting our observations on the differences of
the morphotypes within Blastocystis species subtype 3, and we suggest the possibility that there may be intrasubtype variability in the pathogenic potential of Blastocystis.

Materials and Methods

A total of 51 people older than 15 years, found positive for Blastocystis only during a 20-month period, were included in this study. All subjects were from the eastern metropolitan area of Athens, Greece, undergoing regular health checks and routinely submitted fresh, unreserved stool specimens (3 different specimens per person). We excluded subjects with a range of confounding factors, whether infectious or noninfectious. Recent travel (within the last 6 months), if any, to or from an endemic area was recorded. The results of clinical examination, laboratory testing, and, when indicated, endoscopy were also recorded. If case, sampling was repeated approximately 1 month after the initial visit because of the later-onset symptoms. Out of the 51 people, 36 were asymptomatic and 15 were symptomatic. All 15 symptomatic cases were resampled approximately 3 weeks after the end of treatment so that the therapeutic outcome could be evaluated.

Stool samples were examined immediately after specimen collection, and the following techniques were used to rule out any existing ova and parasites, including Dientamoeba fragilis, sporozoa, and microsporidia: saline and iodine wet mounts of fresh feces and then formalin-ether concentrations were performed, while trichrome-stained smears were prepared after immediate preservation and the modified acid-fast, modified-trichrome, and quick-hot-chromotrope stains were used on fixed feces. Blastocystis numbers per field were recorded using ×400 magnifications: 5 or more organisms per high-power field were reported as abundant1 Table 1. Immunoassay kits for Giardia (Ridascreen Giardia), Cryptosporidium (Ridascreen Cryptosporidium), and enteric viruses (Ridascreen Rotavirus, Ridascreen Adenovirus, and Ridascreen Norovirus) were used according to the manufacturer’s recommendations (R-Biopharm, Darmstadt, Germany). Bacterial enteropathogens, established or potential, and toxins were determined by standard diagnostic procedures.

Stool samples from each person were immediately processed for xenic culture in Robinson medium16,17 at 37°C. The sediment was checked by light microscopy to ensure that Blastocystis was the only enteric parasite and to record growth, motility, and morphologic features on days 2 and 4, as described previously.3,18 Using digital video recording, observations were made off line by frame-to-frame computer-assisted image analysis (PCTV, Pinnacle Systems, Mountain View, CA). Immobile irregular amoeboid forms of Blastocystis (with extension or retraction of pseudopods, which show no movement but appear to engulf, and with possible inclusions) were observed live, recorded, stained, and differentiated from round- or irregular-shaped forms of this parasite containing a vacuole (vacuolated) or granules (granular).3,19

The remainder of each sample was stored frozen after collection and used for polymerase chain reaction (PCR)-based detection of Blastocystis, and subtype assignment was performed using single-strand conformation polymorphism (SSCP) analysis and sequencing, as described previously.15 This method is routinely applied by our laboratory for detection and subtyping of Blastocystis. The same method was used for cultured parasites, harvested on day 4 and stored frozen until the PCR-SSCP analysis was performed. The PCR–restriction fragment length polymorphism method described previously20 was used to preliminarily analyze 6 subtype 3 strains isolated from asymptomatic and symptomatic patients.

Metronidazole (750 mg 3 times a day for 10 days) was the drug of choice for symptomatic patients.1 About 3 weeks after the completion of treatment, patients were reexamined. The evaluation of treatment outcome was based on clinical status. Posttreatment analysis of stool specimens was performed by microscopy and culture for the presence of Blastocystis, while direct and culture-based PCR-SSCP and sequencing15 were also used to determine Blastocystis species subtypes.

All analyses were made using the SPSS 13 software package for Windows (SPSS, Chicago, IL). Statistical significance was considered a P value of less than .05 and calculated by using the χ² test.

Results

Of the 51 people positive for Blastocystis, 27 were female and 24 male (mean age, 43.8 years). No one was immunocompromised or had traveled recently abroad. The 36 asymptomatic people reported no episode of diarrhea or other enteric symptoms within a 30-day period before being tested. The 15 symptomatic patients had nonspecific GI symptoms Table 2. Abdominal pain or discomfort (11/15) and diarrhea (9/15) were the main manifestations. One patient also had acute urticaria, as described in a previous report.21 An initially
asymptomatic patient became symptomatic after approximately 4 weeks, although recent exposure to Blastocystis could not be confirmed. The number of Blastocystis organisms in stool samples did not correlate with symptoms ($\chi^2 = 0.04; P = .85$; Table 1). Direct and culture-based PCR-SSCP and sequence analysis yielded identical single subtypes; no prominent evidence of mixed subtype infection or contamination was observed.

The Blastocystis species subtypes identified were distributed among the asymptomatic and symptomatic study subjects (Figure 1). The 31 people (18 asymptomatic and 13 symptomatic) with subtype 3 (61%) had an identical SSCP profile, suggesting a common nucleotide sequence, while 1 subtype 3 variant, which differed by 1 base, was detected in an asymptomatic person. Subtype 4 was identified in 6 (19%), 5 (14%), 1 (3%), and 5 (14%) asymptomatic people, respectively. Subtype 4 could not be confirmed. The number of Blastocystis species subtype distribution among asymptomatic (gray bars) and symptomatic (black bars) study subjects (Image 1).

In this study, direct-mount examination and permanent staining of feces disclosed vacuolar and granular forms but not amoeboïd forms (Image 1). Amoeboïd forms, found only in the cultures of the symptomatic isolates, were morphologically distinguishable from vacuolar, granular, and amoeboïd-like (irregular) forms seen in the cultures of the strains isolated from asymptomatic and symptomatic carriers of Blastocystis species (Image 1). Granular forms mostly appeared in older cultures of our isolates. None of the subtypes, seen only in our asymptomatic subjects, showed amoeboïd forms in culture. We observed amoeboïd forms in addition to vacuolar and granular forms during the cultivation of the 14 subtype 3 strains (Image 1) from symptomatic patients. No amoeboïd forms were shown in the cultures from the asymptomatic subtype 3 isolates. The amoeboïd forms of subtype 3 correlated significantly with the presence of symptoms, whereas vacuolar forms did not ($\chi^2 = 32; P < .001$) (Figure 2). Amoeboïd forms were also shown in the culture of 1 symptomatic subtype 4 strain (Image 2). Binary fission was seen in all cultures of the study strains (Image 1). Also, amoeboïd forms seemed to release individual vacuolar-like organisms (Image 2).
Morphologic features of *Blastocystis* isolates. *Blastocystis* forms measure 5 to 60 μm in diameter. **A**, Vacuolar forms of various sizes. Granular forms in older cultures (×400). **B**, Vacuolar forms and binary fission (arrow) (×400). **C, D, and E**, Unstained wet mounts of *Blastocystis* forms in culture (×400). **D**, A pseudopod retracting amoeboid form (arrowhead) and 2 amoeboid forms with extended pseudopods (arrows), one with a possible vacuolar-like structure connected with a thin band to cytoplasm (long arrow). Note the possible inclusions in the latter amoeboid form. **E**, A vacuolar form (arrow) and a large amoeboid-like *Blastocystis* that appears to be vacuolated (arrowhead) are observed. Note: No inclusions are seen in the amoeboid-like *Blastocystis*. **F** and **G**, Trichrome-stained *Blastocystis* in culture (×1,000). **F**, A vacuolar form (arrow) and a large amoeboid-like *Blastocystis* (arrowhead) are seen. Note: Granules are visible in the amoeboid-like *Blastocystis*. **G**, An amoeboid form with extended pseudopod (arrow, right side of image), a pseudopod retracting amoeboid form (arrow, top of image), one of the vacuolar forms (horizontal arrowhead), and barbell-like binary fission (vertical arrowhead). **H** and **I**, Immediate trichrome-stained preparations of stool samples (×1,000). **H**, Vacuolar forms of *Blastocystis* (arrows). **I**, Granular forms of *Blastocystis* (arrows).
Of note, of the 31 patients with subtype 3 with a common sequence, 14 (including the 1 asymptomatic subject with late-onset symptoms) had amoeboid forms that appeared during symptom manifestation. Furthermore, before and after symptom manifestation, PCR-SSCP showed identical patterns, implying identical variations within subtype 3.

Of the 15 symptomatic patients with subtype 3, 5 did not return for any follow-up appointment. Blastocystis remained detectable in 3 patients with subtype 3 experiencing improving symptoms after metronidazole discontinuation and in 2 patients with subtype 3 reporting poor compliance with therapy, with subtype 3 being the only subtype identified before and after treatment. In 1 patient with subtype 4 and 3 patients with subtype 3 who received metronidazole and recovered with no relapses at follow-up, posttreatment results of stool examination and PCR-SSCP and subtype analysis were negative for Blastocystis species.

Discussion

In this study, Blastocystis species subtype 3 was predominant, followed by subtype 1, a finding in agreement with previous reports. In our cases, we failed to confirm history of recent travel or exposure as risk factors. Long-standing infection also could not be excluded. We are unable to explain the relatively high prevalence of avian subtype 7 reported herein, but similar results have been reported in Japan; we are currently collecting samples from birds in the same study area to investigate Blastocystis species subtype distribution in the avian population and potential exposure sources. There was no difference in subtype distribution as assessed by culture-based analysis or direct analysis of the fecal samples, which is in accordance with the findings of a recent study. There is a controversy about the association of the number of Blastocystis organisms and the symptoms. We found no relationship between the presence of large numbers of Blastocystis and patient symptoms.

The results of our study support previous findings that the amoeboid forms of Blastocystis are present in fecal cultures from symptomatic patients. The findings from direct microscopy and permanent staining of feces from our symptomatic patients corroborate that amoeboid forms are rarely, if at all, seen in feces outside the context of acute diarrheal illness. Our results concur with some recent findings in which Blastocystis strains producing amoeboid forms in cultures were related to minor GI and other nonspecific symptoms.

By using arbitrarily primed PCR and subtype-specific sequenced-tagged site primers, it has recently been shown that the symptomatic subtype 3 isolates producing amoeboid forms formed a clade with more than 70% similarity among the isolates. In contrast, asymptomatic isolates that belonged to subtype 1 and subtype 2 and that did not show amoeboid forms in cultures were clearly separated from symptomatic isolates. Our results further support these findings in that the presence of amoeboid forms correlates well with the presence of subtype 3. We found 14 pathogenic subtype 3 strains with an identical SSCP profile that showed amoeboid forms in culture, whereas there were no amoeboid forms present in the nonpathogenic strains with different (highly
discriminating) profiles that belonged to various subtypes. However, our results suggest that subtype 3 can be detected in asymptomatic and symptomatic people, and similar findings have been reported by other investigators.29 Herein we report for the first time, to our knowledge, intrasubtype differences in morphotypes, specifically of subtype 3, which may be related to the pathogenic potential of *Blastocystis* species. Thus, the specific search for the presence or absence of amoeboid forms may be useful in the screening of patients with symptoms possibly due to *Blastocystis* species subtype 3.

This study is also the first to identify and describe an asymptomatic subtype 3 carrier in whom symptoms then developed within a short period after the initial testing for *Blastocystis* species. Our results indicate that the progression from an asymptomatic to a symptomatic state coincides with a transition from vacuolar and granular forms (normally found in an asymptomatic condition) to the amoeboid form (found in a symptomatic condition). A possible explanation for the observed diverse morphologic features is not reinfection with the same subtype, suggested by some researchers,11 but in line with the assumption already reported,26,27,30 that vacuolar forms can transform into amoeboid forms under specific conditions.

Nevertheless, it is of interest that in our study, amoeboid forms often released vacuolar forms, a phenomenon that, as previously suggested,22 may occur in certain situations. Taken together, these observations are consistent with an ongoing carriage of *Blastocystis*, as speculated previously.19

Whether *Blastocystis* species infections are asymptomatic or symptomatic might be affected by an interplay of host and parasitic factors, as in other enteric parasitic infections.12,19 The presence of the amoeboid forms may possibly represent situations in which physicochemical properties in the sample favor the development of amoeboid...
stages. This may be indicative of a specific situation in the gut.\textsuperscript{19} However, there may also be intrasubtype variability. A recent study using in vivo rat models has shown pathogenic and nonpathogenic variants within subtypes 3 and 4.\textsuperscript{31} In the in vitro cultures, larger cells and slower growth of cells were likely to facilitate phenotypic characterization of symptomatic isolates in comparison with asymptomatic isolates.\textsuperscript{10} Intrasubtype variability may result in the production of effectors contributing to \textit{Blastocystis} pathogenicity.

Similar to \textit{Entamoeba} isolates (which can be more or less virulent), symptomatic \textit{Blastocystis} isolates presenting amoeboid morphologic features have shown stronger fluorescent lectin binding (and possibly agglutination to \textit{Helix} lectin) compared with asymptomatic isolates. This implies that surface properties may influence the pathogenic potential of subtype 3.\textsuperscript{10} Still, cytopathic subtype 3 effects on host cell lines and cysteine protease activity in or within subtype 3 have not been explored, in contrast with recent in vitro studies on isolates of subtype 4 and subtype 7.\textsuperscript{7,1,32} Nevertheless, the existence of only 1 asymptomatic subtype 3 strain with a different variant cannot be indicative of either of the aforementioned models. Larger studies may reveal whether putative pathogenic amoeboid stages of \textit{Blastocystis} correlate with variations within subtype.

Still, as far as we know, today’s genotyping methods explore the ribosomal RNA gene, thus limiting the resolution.\textsuperscript{6,10} In the future, analysis of the \textit{Blastocystis} mitochondrion-like organelle, which is currently under investigation,\textsuperscript{33} might contribute to the study of the parasite’s pathogenicity. Genome analysis of \textit{Blastocystis} species and the application of modern techniques, such as microsatellites, microarrays, and differential display, coupled with proteomics and bioinformatics analyses are more likely to elucidate the matter of differences in morphologic features and pathogenicity of \textit{Blastocystis} species subtype 3 and unável virulence genes and their regulation.

Another finding of interest is the appearance of amoeboid forms, followed by the release of vacuolar-like cells, in the culture of the subtype 4 strain, which was isolated from a symptomatic patient. Our finding cannot provide a basis for making valid generalizations beyond that particular case, but it may begin to provide clinical and laboratory corroboration of epidemiologic and experimental findings in which subtype 4 may be regarded as pathogenic.\textsuperscript{32,34,35}

In the literature, cases have been reported in which \textit{Blastocystis} eradication has resulted in symptom resolution. However, resistance to metronidazole has been reported.\textsuperscript{1} In this study, posttreatment stool analysis showed that possible nonresponding isolates were identical to isolates identified before treatment, that is, no mixed subtype infection was evident. Reinfection is probably not responsible because recent exposure to \textit{Blastocystis} species subtype 3 was not confirmed.

Poor compliance to metronidazole treatment should be also taken into account. In our study, metronidazole antiprotozoal effect was rather modest. However, it seems appropriate that an asymptomatic subtype 3 carrier should be followed up and screened for amoeboid forms before any drug treatment is initiated, in case symptoms become evident.

Although our sample was small and the findings were limited by the retrospective nature of the study, our results may stimulate prospective studies on the roles of morphologic features and their variation within \textit{Blastocystis} species subtype as predictors of symptoms.

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