Case report

*Blastocystis hominis* transmission by non-potable water: a case report in Italy

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Running title: *Blastocystis* in unpotable water: a case report

**SUMMARY**

In the reported case, a 41-year-old Italian man came to the clinician’s observation reporting cramps, bloating and watery diarrhoea a few days after drinking water indicated as unpotable from a fountain in a farm area. The medical suspicion was directed at both gluten intolerance and enteric infection, eventually of waterborne origin. Gluten intolerance was investigated by intestinal biopsy and excluded, while stool analyses ruled out infective bacteriological or viral agents and parasites. Subsequently, a persistent eosinophilia was revealed and a parasitological analysis was again suggested, planning for a more sensitive molecular method. Therefore, a multiplex-PCR of enteric protozoa species DNA was performed on an intestinal biopsy and faecal samples revealing only *Blastocystis hominis* protozoa, subsequently typed as subtype 1 by RFLP-PCR method. *B. hominis* is an anaerobic protozoa found in the human and animal intestinal tract, recently associated with a pathogenic role characterized by chronic development. Since blastocystosis has been demonstrated as a waterborne infection, a sample of water matrix was analysed, revealing the *B. hominis* subtype 1 DNA inside. A probable water transmission of *Blastocystis* infection has been demonstrated in this case report. Only a probiotic treatment based on *Saccharomyces boulardii* was administered to the patient and this apparently resolved the infection. In summary, the case described here is a chronic blastocystosis of possible waterborne origin, controlled by assuming a yeast treatment.

**Key words:** Blastocystosis, Drinking water, Chronic infection, Waterborne parasite.

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INTRODUCTION

*B. hominis* is a eukaryotic microbe frequently found in the human and animal intestinal tract. It can be part of the gut microbiota generally resulting harmless to the subject (Scanlan *et al.*, 2014; Audebert *et al.*, 2016). In some cases, it is a pathogen causing gastrointestinal symptoms such as diarrhoea, nausea, abdominal pain, vomiting and bloating (Wawrzyniak *et al.*, 2013). *B. hominis* has a global distribution and a faecal-oral transmission route has been suggested. From the taxonomic point of view it was previously considered a yeast, or fungus or ameoboid protozoa since in 1991 it was classified it as a protozoon by morphology (Zierdt, 1991). Afterwards small-subunit ribosomal DNA sequencing definitively assigned this protist to the *Phylum Heterokonta* (*Stramenopiles*), including also water molds, diatoms, brown algae and other photosynthetic protozoa (Cavalier-Smith, 1999; Arisue *et al.*, 2002). Four major cellular forms of this species have been described in stools samples and *in vitro* cultures: vacuolar, granular, amoeboid and cystic. Upon ingestion of cysts by the host, this protozoon undergoes excystation in the large intestine and develops into vacuolar forms. These vacuolar forms divide developing amoeboid and granular forms. Then, encystation may occur along the colon, followed by cysts excretion in the faeces (Suresh *et al.*, 2009). *B. hominis* is the only *Stramenopile* known to colonize humans and animals. From the epidemiological point of view it is considered the most common protist inhabitant of the human gut and has been also associated with inflammatory bowel disease and irritable bowel syndrome (Nagler *et al.*, 1993; Poirier *et al.*, 2012; Azizian *et al.*, 2016). The different behaviour shown by *B. hominis* could be associated with the high genetic diversity found in this species (Arisue *et al.*, 2003). In fact, using isoenzymes and karyotyping techniques, these species have been divided into subgroups showing different pathogenic skills (Stenzel and Boreham, 1996; Clark, 1997; Bohn-Gloning *et al.*, 1997). In particular, the mammalian and avian *Blastocystis* spp. groups were subdivided into seventeen subtypes (STs), nine of which, referred to as ST1 to ST9, have also been found in humans (Stensvold and Clark, 2016). It is a rising idea that some subtypes of *Blastocystis* may be more likely associated with symptoms and it has been demonstrated that several of them show different degrees of pathogenicity in experimentally inoculated rats, subtype 1 being the most virulent (Hussein *et al.*, 2008). A very exhaustive study, published in the current year, described the very high biodiversity of *Blastocystis* genotypes, differing especially in the coding sequences for proteins implicated in the pathogenic molecular machine (Gentekaki *et al.*, 2017). According to different studies, *B. hominis* is also associated with cutaneous manifestations and chronic urticaria (Zuel-Fakkar *et al.*, 2011; Verma and Delfagnan, 2013; Haamed *et al.*, 2011) also in Italy (Pasqui *et al.*, 2004). A recent study showed a strong association between an allele of ST3 *B. hominis* and urticaria recurrence in patients (Casero *et al.*, 2015) and ST4 has been highly associated with acute gastroenteric symptoms (Stensvold *et al.*, 2011). Additionally, it was
observed that *B. hominis* can frequently be found in mixed infections together with *Dientamoeba fragilis* (Stensvold *et al.*, 2009), another intestinal protozoan able to cause urticaria (Windsor and Johnson, 1999) and intestinal disorders (Stensvold *et al.*, 2007). Moreover, substantial molecular evidence for zoonotic transmission of *Blastocystis* was provided (Yoshikawa *et al.*, 2000) and its transmission route being oro-faecal, environmental faecal contamination plays a key role. In effect, *Blastocystis* spp. was detected in sewage in Malaysia and Scotland, and other studies provided evidence for waterborne transmission of the parasite (Lee *et al.*, 2012; Leelayoova *et al.*, 2002; Baldursson and Karanis, 2011). Thick-walled cysts have been correlated with environmental transmission, while thin-walled cysts might be responsible for internal reinfection within the host gut (Stenzel *et al.*, 1991; Singh *et al.*, 1995). Finally, *Blastocystis* has been correlated with outbreaks due to the use of unfiltered drinking water (Tan, 2008). In conclusion, the asserted pathogenicity of most *Blastocystis* spp. strains, a possible association with inflammatory gut diseases, environmental transmission and zoonotic potential, contribute to currently considering this protist an emergent parasite.

**CASE REPORT**

A 41-year-old Italian man came to a doctor’s observation reporting symptoms of acute gastroenteritis, considered a self-limiting viral affection. After several weeks, cramps, bloating and watery diarrhoea were persistent and associated with weight loss, general malaise and anorexia, so clinicians suggested an investigation for celiac disease. Therefore, the patient underwent a gastro-duodenoscopy with an intestinal biopsy, which showed atrophy of the intestinal villi, a mild-chronic nonspecific inflammation and a duodenal lymphocytic infiltrate (intraepithelial lymphocytes/enterocytes 23/100). However, he resulted negative for celiac disease after serological tests and gluten-free diet. Further clinical investigations revealed deficiency of D vitamin assimilation and increased duodenal lymphocytic infiltrate CD3+ (intraepithelial lymphocytes/enterocytes 30/100), but gastric ulcer, colon cancer and inflammatory bowel disease were excluded. Therefore, the patient's history highlighted an intestinal malabsorption and chronic intestinal inflammation of possible infective origin.

Since intestinal symptoms persisted and the patient reported drinking unpotable water from a rural fountain a few days before the acute symptomatology, clinicians performed a microbiological stool analysis. Stool analysis by microscope for bacteria and parasites and by serological diagnosis for virus excluded enteric bacterial / parasitological as well as viral infections (Table 1). In particular, infections by *Giardia* spp. and *Cryptosporidium* spp. waterborne parasites were ruled out. In the following months, the patient complained of persistent muscle-tendon pain, tremors, and intermittent gastro-enteric disturbance; blood-analyses were again performed and a peripheral persistent
eosinophilia was highlighted. Therefore, a parasitic infection was again strongly suspected and he arrived at our laboratory for a molecular parasitological analysis more sensitive than the microbiological analysis. Firstly, to investigate the patient’s infective initial stage and to increase the diagnostic sensitivity/specificity degree by molecular technique, it was decided to analyse a paraffin-fixed slide specimen of the gut biopsy, already performed several months earlier. A slide scraping was then carried out to extract tissue material and total DNA production was performed according to the Promega Wizard Genomic DNA Purification Kit. A multiplex PCR amplification, previously standardized, was applied to search for different parasitic species DNA (Giardia, Cryptosporidium, Cyclospora, Dientamoeba, Blastocystis) using a standardized primer panel shown in Table 2. Among the target sequences, only B. hominis SSU rRNA gene target (Scicluna et al., 2006) was amplified, showing the specific DNA in the sample. The molecular diagnosis on the biotic material confirmed the previously demonstrated negativity for other enteric parasites (Table 1), showing only a high gut colonization by Blastocysts protozoa, not searched for before. Since the patient reported drinking unpotable water from a rural fountain, and Blastocystis has been isolated from surface water also in Italy (Angelici et al., 2013; Ade et al., 2011), this event was assumed to be the putative cause of infection. That fountain is normally intended for watering livestock and provides canalized water from the nearby river contaminated by animal faeces. The fountain was designated as NON-POTABLE water. Due to the faecal contamination of river water by livestock, a risk of zoonotic waterborne infections appeared consistent. Therefore, the fountain water was monitored a few months after the patient’s infection occurred. The same environmental conditions and livestock presence in the fountain area were, indeed, confirmed. Five litres of water were collected and concentrated by an initial filtration and then serial centrifugations to obtain a final pellet. Cell fraction in the pellet was lysed by Promega Genomic DNA Purification Kit while PCR and DNA amplification was performed by the same primer panel shown in Table 2. The presence of Giardia duodenalis and B. hominis was revealed. A genotyping protocol of the Blastocystis isolates by restriction enzymes analysis of SSU rRNA, 1800bp amplicon (RFLP-PCR analysis) from both human and environmental specimens was then performed, as suggested by Clark (Clark, 1997). Concerning the amplified gene target of B. hominis, and the Rsa1-RFLP electrophoretic pattern, results are reported in Fig.1 and Fig.2, respectively. As the electrophoretic Rsa1 restriction pattern corresponds to B. hominis Subtype 1, in agreement with Clark (Clark, 1997) in both the specimens, we can assert they belong to the same genotype 1. This datum supports the hypothesis that the patient was infected by drinking the contaminated water from the fountain some days before the onset of enteric symptomatology.

The patient was recommended for metronidazole treatment, but he declined, reporting his intolerance of nitroimidazole derivatives, previously tested (genetic polymorphism of the coding gene for
CYP2C19 substrate). Consequently, he was recommended to assume a probiotic treatment, containing Lactobacillus and Bifidobacterium, to control the intestinal dysbiosis and a month after he returned to our observation he had no intestinal symptoms, but claimed chronic fatigue, muscle cramps, pallor, chronic urticaria and psychological malaise. A faecal analysis by PCR on three serial specimens was then performed, with multiplex PCR (Table 2), again detecting B. hominis and suggesting that the parasite had chronically colonized the intestinal tract of this subject. Furthermore, faecal analysis revealed the presence of Dientamoeba fragilis, showing a co-infection between these two parasites, often described during the chronic stage of a blastocystosis (Stensvold et al., 2007; Windsor and Johnson, 1999). He was also diagnosed with a high level of faecal lactoferrin, a sensitive and specific marker identifying intestinal inflammation (Kane et al., 2003), suggesting that, almost certainly, the situation had not yet resolved. He continues to be affected by general malaise, muscle-tendon pain, pinprick sensation in his legs, chronic itching especially at calf level, and persistent peripheral eosinophilia. Clinicians suggested the patient take a different kind of probiotic, containing Saccharomyces boulardii. Recently, 18 months after the last positivity for blastocystosis, the patient twice repeated the molecular search for B. hominis in faecal samples and resulted negative showing parasite eradication. He resulted negative for B. hominis and D. fragilis parasites also by microscope assay and, now, specific symptoms like eosinophilia, enteric disturbance and urticaria are no longer claimed.

DISCUSSION
B. hominis has been definitively demonstrated as a human gut pathogen, mostly characterized by a high genetic variability with subtypes showing different degrees of pathogenicity (Stensvold and Clark, 2016). It has also been implicated as cause of long-term chronic inflammation and difficult to eradicate (Vitetta et al., 2016; Roberts et al., 2014). The case reported here showed a symptomatic Blastocystis infection with a chronic course because of the lack of pharmacological treatment and a possible waterborne origin of this infection was demonstrated. Indeed, the patient’s symptoms appeared immediately after drinking from a contaminated fountain. A molecular analysis of both the patient’s biological samples and the watery matrix of the fountain specimens indicated the subtype 1 B. hominis DNA, suggesting possible infection by the contaminated water. Subtype 1 has been very frequently isolated from human cases also in Italy (Mattiucci et al., 2016) and often isolated in animals, showing a certain zoonotic potential (Wang et al., 2013; Wang et al., 2014). The high prevalence of Blastocystis as a parasite in the Italian population has been previously demonstrated by clinical studies (Calderaro et al., 2010), but it is not yet correlated to specific routes of transmission and risk factors. Usually, an enteric microorganism transmitted by the faecal-oral route with a zoonotic cycle should be easily transmitted by contact with contaminated surface water. Indeed, B.
hominis has been isolated in surface water (Elshazly et al., 2007) and in different animal species (Stensvold and Clark, 2016), proving both a waterborne and zoonotic parasite. Therefore, a possible waterborne origin of the infection was proposed. This is the first case in Italy in which the diagnostic approach indicates a water-related transmission risk factor. Few data are available on the waterborne origin of B. hominis infections in Italy, neglecting a very important aspect in its transmission to humans and animals (Angelici et al., 2013; Ade et al., 2011).

The case presented here shows the chronic appearance of intestinal symptoms, eosinophilia, diffuse pain at muscle level and positivity for faecal lactoferrin, strongly suggesting a chronic infection by Blastocystis as the only etiological agent isolated by repeated diagnosis in the patient’s intestinal tract. Specific symptoms like chronic fatigue (Tan, 2008), persisting urticaria (Windsor and Johnson, 1999) and general discomfort support this diagnosis. In addition, D. fragilis co-infection was also revealed confirming data from other authors that these infections can occasionally occur simultaneously (Stensvold et al., 2007; Windsor and Johnson, 1999). The long-term chronic symptoms associated with the infection claimed by the subject could be caused by the lack of therapy giving rise to the chronic pathology. In any case, a specific treatment by only long-term probiotic intake finally resolved the infection especially when Saccharomyces was supplied. This yeast is a eukaryotic microorganism like the protozoon Blastocystis and a possible trophic competition between them could be the reason inhibiting parasite survival. Likely, in this clinical case S. boulardii administration was sufficient to control the Blastocystis infection, as reported by several authors (Sekar and Shanthi, 2013; Dinleyici et al., 2011). In summary, this case report highlights for the first time a possible transmission of B. hominis through contaminated drinking water in Italy, and the relevance of considering Blastocystis a pathogen able to support a chronic infection. This enteric protozoon is very common, but highly neglected in the clinical diagnostic panel and its chronicity is often not revealed. The role of B. hominis as a potential pathogenic agent responsible for intestinal and extra-intestinal disorders is still underestimated at clinical level, although scientific knowledge exists at a basic research level (Wawrzyniak et al., 2012; Basak et al., 2014). For this reason, this parasite is often not investigated and can occasionally produce an alteration of the intestinal flora (Vitetta et al., 2016), exposing the subject to other co-infections. Since B. hominis infection is associated with chronic intestinal diseases or other gut disturbances, we strongly recommend considering the risk of transmission through surface water contaminated by faeces.

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REFERENCES


Table 1. Enteric microorganisms researched in the stool sample. Diagnoses by and serological microscopy analyses. All the diagnosis results were negative.

<table>
<thead>
<tr>
<th>Bacteria</th>
<th>E. coli O157, Salmonella spp., Brucella spp., Helicobacter pilori, Borrelia spp., Clostridium difficile and C. difficile toxin A and B, Yersinia spp., Campylobacter jejuni, Proteus vulgaris, Mycobacterium spp., Rickettsia prowazekii.</th>
</tr>
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<tbody>
<tr>
<td>Viruses</td>
<td>HIV, HCV, HBV, HHV-6, Adenovirus.</td>
</tr>
<tr>
<td>Parasites</td>
<td>Giardia intestinalis, Cryptosporidium spp., Entamoeba histolytica, Toxoplasma gondii, Leishmania donovani, Balantidium coli, Cyclospora cayetanensis, Cystoisospora belli, Microsporidia spp., Taenia saginata, Enterobius vermicularis, Echinococcus granulosus, Strongyloides stercoralis, Trichinella spiralis, Filariaidae.</td>
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</tbody>
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Table 2. Target genes used for diagnosis of enteric protozoa in the intestinal biopsies.

<table>
<thead>
<tr>
<th>Protozoan Species</th>
<th>Target Gene / Amplicon bp</th>
<th>Reference</th>
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<tbody>
<tr>
<td>Cyclospora cayetanensis</td>
<td>ITS 2 / 116 bp</td>
<td>(Lalonde and Gajadhar, 2008)</td>
</tr>
<tr>
<td>Cryptosporidium parvum</td>
<td>COWP nested / 311 bp</td>
<td>(Yu et al., 2009)</td>
</tr>
<tr>
<td>Giardia duodenalis</td>
<td>16S rRNA nested / 292 bp</td>
<td>(Appelbee et al., 2003)</td>
</tr>
<tr>
<td>Dientamoeba fragilis</td>
<td>5.8S rRNA / 98bp</td>
<td>(Verweij et al., 2007)</td>
</tr>
<tr>
<td>Blastocystis hominis</td>
<td>SSU rRNA / 600 bp</td>
<td>(Scicluna et al., 2006)</td>
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**Fig. 1.** PCR amplification of 2 fecal samples (lines 1 and 2) and 2 water samples (lines 3 and 4), marker standard (1kb) (line 5) and negative control (line 6).

**Fig. 2.** Enzymatic digestion of two samples: a fecal sample of the patient (line 2), a water sample from the fountain (line 3), lines 1 and 4 are standard markers (1kb).