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ORIGINAL PAPER

Diagnostic Features of *Blastocystis* Life Cycle Forms in the Small Intestine in an HIV-Infected Patient

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Abstract

Purpose *Blastocystis* spp. are parasites of the intestinal tract found in many hosts including humans. This pathogen is commonly found in immunocompetent in asymptomatic individuals and in patients with gastrointestinal and extra-intestinal symptoms. Recently, it has been implicated as an important cause of diarrheal illness in immunocompromised individuals, including HIV-infected patients. At least six life cycle stages have been described in faeces and cultures, namely vacuolar, granular, multi-vacuolar, avacuolar, ameboid and cyst forms. The aim of the present study was to describe the histological findings of *Blastocystis* infection in an adult HIV-infected patient with gastrointestinal symptoms.

Methods Parasitological techniques and PCR were applied to stool samples. Histological analysis was performed on duodenal biopsy specimens.

Results Standard parasitological methods revealed vacuolar, granular, cyst and multi-vacuolar forms of *Blastocystis* in faecal samples with the presence of *Blastocystis* DNA being confirmed by PCR. DNA sequencing revealed *Blastocystis* subtype ST1. Histological findings in duodenal samples showed an inflammatory infiltrate with plasma cells and lymphocytes. We identified cyst, granular, ameboid and multi-vacuolar forms in the lumen.

Conclusion To our knowledge, there are no previous peer review reports describing these four different forms of *Blastocystis* in histological sections from the lumen and the brush border of the enterocytes.

Keywords *Blastocystis* · Histology · HIV · Diarrhoea

Introduction

Blastocystis spp. are parasites of the intestinal tract found in many hosts including humans [32]. *Blastocystis* has been isolated from a wide range of hosts including primates, pigs, rodents, birds, reptiles, amphibians and insects [2]. This organism is a unicellular endosymbiont and is classified taxonomically within the complex and heterogenous evolutionary assemblage of the Stramenopiles according to the sequence of the *Blastocystis* ssrRNA gene [34].

This pathogen is commonly found in asymptomatic individuals and in patients with gastrointestinal and extra-intestinal symptoms, such as diarrhoea, nausea, abdominal pain, bloating, vomiting or anorexia, with an almost equal prevalence [3, 6, 19, 31, 33, 45]. *Blastocystis* has been described as an opportunistic pathogen and has recently been implicated as an important cause of diarrheal illness in immunocompromised individuals, including HIV-infected patients [1, 11, 13, 21, 29, 42, 54].

Blastocystis is detected using standard parasitological methods, which are also employed to detect other intestinal parasites [37]. Examination of multiple stool specimens is recommended to enhance the detection [40]. The use of concentration methods has been controversial [40]. Light microscopic examination of wet mounts, either unstained or stained with iodine, may be used [16, 40, 56].

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Trichrome staining of permanent smears can be employed for routine diagnosis of *Blastocystis* [12, 40].

At least six life cycle stages have been described in faeces and cultures, namely vacuolar, granular, multivacuolar, avacuolar, ameboid and cyst forms. They vary in size from 2 to > 200 µm and morphology [35, 37, 39, 40, 43, 44, 46, 50]. Although *Blastocystis* is usually identified microscopically by the presence of the vacuolar form, this variability of forms and occurrence in stools should be taken in account to an accurate diagnosis [37, 40].

Biopsies from those who are infected by the parasite mostly show normal appearances in the intestinal mucosa, as *Blastocystis* usually does not penetrate nor invade the tissues [20]. Tissue invasion of *Blastocystis* parasites in the appendix or in the colon mucosa, associated with acute or chronic inflammation, has been sporadically reported [9, 10, 14].

The genetic diversity of *Blastocystis* has led to its classification in multiple subtypes (STs) based on polymorphic regions of the SSU rDNA gene [38]. Currently, 29 subtypes are known, of which ST1 to ST9 and ST12 have been identified in humans [15, 22, 36].

The present study describes the histological findings of *Blastocystis* infection in an adult HIV-infected patient with gastrointestinal symptoms.

Materials and Methods

Case History

A 53-year-old man was admitted to the hospital with vomiting, and chronic intermittent diarrhoea. He had been diagnosed of HIV infection and was being treated with dolutegravir, lamivudine and abacavir. He was a cholecystectomised patient, with hypertension and type II diabetes. One month prior his hospitalisation, he had been diagnosed of renal lithiasis and hydronephrosis.

Laboratory evaluation showed a haemoglobin level of 12.0 g/dl and a leukocyte count of 6000/mm^3. Liver function tests were normal. The CD4 lymphocyte count was 190 cells/mm^3 and the viral load was 50 copies/ml.

Stool examination revealed *Blastocystis*. After 2 weeks, the patient continued with diarrhoea and vomiting. An upper endoscopy with small intestinal biopsy sampling was performed. The endoscopy showed granular duodenum and esophageal candidiasis. At the time the endoscopy was performed, the patient started a regimen with ethambutol, ciprofloxacin, and clarithromycin to treat a *Mycobacterium avium*-intracellulare complex infection. He continued to have intermittent diarrhoea and vomiting. One week later the patient died due to sepsis and multiorgan failure.

Stool Samples

Stool specimens were collected daily for seven consecutive days into 5% formalin saline solution and 70% ethanol. The formalin-fixed samples were then processed by ethyl ether concentration. The concentrated samples were used to prepare wet mounts and iodine-stained smears, in addition to smears that were stained with Kinyoun's modified acid-fast, Gram chromotrope [27] and modified Weber's modified trichrome [49]. Both stained and unstained preparations were examined by light microscopy.

Routine bacterial stool cultures were also performed. The stool samples were cultured on agar plates and in liquid medium, to detect and identify *Salmonella*, *Shigella* and *Escherichia coli*. Stool specimens were also assessed simultaneously for *Clostridium difficile* glutamate dehydrogenase antigen and toxins A and B using a commercial enzyme immunoassay (TECHLAB® C. DIFF QUIK CHEK COMPLETE®, USA).

Duodenal Tissues

Five biopsy specimens from the distal duodenum were obtained by an upper gastrointestinal endoscopy (UGE) with Pentax EPM 2000 equipment. They were fixed in 10% formalin, embedded in paraffin, and stained with hematoxylin and eosin (H&E) and Giemsa. Stained preparations were examined by light microscopy.

Molecular Analysis

Ten millilitres of gauze-filtered faeces, that had been collected in 70% ethanol, were concentrated by centrifugation and the pellet was washed twice with saline solution. Then, DNA purification was carried out by standard phenol–chloroform extraction and ethanol precipitation according to previous protocols [48]. The purified DNA was resuspended in 20 µl of bidistilled water and kept at − 20 °C until use.

To confirm the presence of *Blastocystis* DNA in the stool samples a PCR assay was employed. The target sequence was a fragment of the SSU rDNA gene, and the set of primers were Blast 505–532 (5′ GGA GGT AGT GAC AAT AAA TC 3′) and Blast 998–1017 (5′ TGC TTT CGC ACT TGT TCA TC 3′) previously described [30], based on nucleotide positions 445–464 and 905–924 of the reference nucleotide sequence U51151.

Each 50-µl PCR mixture contained 10 mM Tris–HCl (pH 8.8), 50 mM KCl, 0.08% (v/v) Nonidet P40, 200 µM (each) dNTP, 1.5 mM $MgCl_2$, 0.6 µM of each primer, 2.5 U of Taq DNA polymerase (Invitrogen.), and 0.048% BSA. The amplifications were carried out in a MyCycler thermocycler

(Biorad, USA). The PCR protocol included an initial step of 4 min at 95 °C, followed by 35 cycles of denaturation for 30 s at 95 °C, annealing for 30 s at 54 °C, and extension for 30 s at 72 °C, with a final step of 5 min at 72 °C [30]. The negative control corresponded to human DNA. Positive control was DNA from a confirmed stool sample with *Blastocystis* (kindly provided by Dr. Florencia Mongi, Universidad de Córdoba). For the blank reaction mixture, DNA template was replaced by distilled water. Twenty microliters of obtained amplicons were run on 2% agarose gels stained with GelRed® (Biotium, Inc., Fremont, CA) and then viewed under UV transillumination.

PCR product was sequenced in both directions by a commercial sequencing service (Macrogen, Korea). The resulting sequence was analysed using the Blast program of the National Center for Biotechnology Information and the *Blastocystis* 18S database available at http://pubmlst.org/blastocystis.

The research protocol was approved by the Ethical Committee for Research, Hospital Francisco J. Muñiz, protocol No. 274. Informed consent in writing was obtained from the patient.

Results

Stool Findings

Microscopic examination of concentrated faecal material with addition of Lugol's iodine solution disclosed vacuolar, cyst, granular and multi-vacuolar forms of *Blastocystis* (Fig. 1a–d).

The morphology of the vacuolar forms was mainly spherical, showing diameter from 6.33 to 7.18 µm. They presented a large central body or vacuole and a peripheral cytoplasmic rim usually containing two nuclei (Fig. 1a).

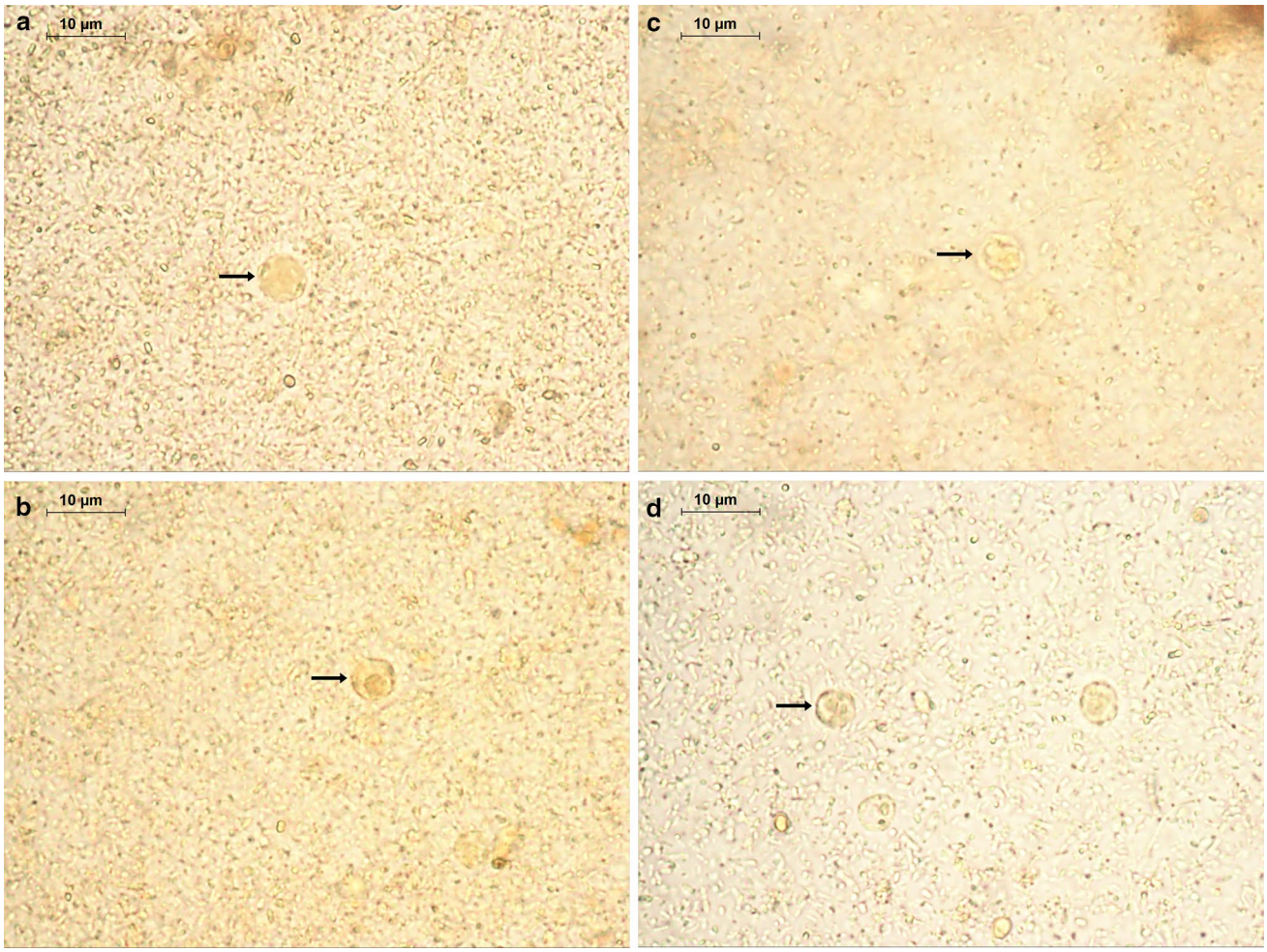

Fig. 1 Morphological heterogeneity of *Blastocystis* in concentrated patient's faeces stained with iodine. **a** Vacuolated form. **b** Cyst form. **c** Granular form. **d** Multi-vacuolated form. Magnification × 400

The cysts were of 4.82–5.64 µm in diameter, with a thick multi-layered wall surrounding the organism and a condensed cytoplasm containing many small vacuoles (Fig. 1b).

The granular forms were spherical in shape, with diameters of 6.61–6.79 µm. They showed a thin peripheral band of cytoplasm surrounding a large central vacuole containing granules (Fig. 1c).

The multi-vacuolar forms were approximately 5.57–5.84 µm in diameter, with multiple small vacuoles of different sizes, one or two nuclei and a thick surface coat (Fig. 1d).

Stool bacterial cultures were negative. *C. difficile* immunoassay was also negative.

Histological Findings

Mucosal architecture was abnormal showing villous atrophy and expansion of the lamina propria by an inflammatory infiltrate that was predominantly mononuclear (comprised by plasma cells and lymphocytes).

Different forms of *Blastocystis* were identified in the lumen, including the cyst, the granular, the ameboid and the multi-vacuolar forms. Some of the structures were placed on the microvilli of the enterocytes.

The cysts were spherical in shape, 7.01 µm in diameter (average measure). They had a thick refractile multi-layered cyst wall surrounded by loose irregular outer coat. The cytoplasm was condensed (Fig. 2a).

The granular forms displayed a thin peripheral band of cytoplasm and a large central vacuole, with the presence of granules in both. The average diameter of the granular forms was 6.56 µm (Fig. 2b).

The amoeboid forms were irregular in shape, with the presence of an extended pseudopodia, multiple small vacuoles in the cytoplasm and one nucleus were present, located at the centre of the cell. They measured in average 9.91 × 6.92 µm in size (Fig. 2c).

The multi-vacuolar forms measured in average 6.08 µm in diameter. They had multiple small, interconnected vacuoles and usually two nuclei (Fig. 2b).

Molecular Analysis

DNA obtained from ethanol-preserved faeces was employed in the PCR assay. After amplification, a fragment of the expected size corresponding to 480 base pairs was obtained (Fig. 3).

Sequence analysis of the 480-bp fragment of the SSU rDNA gene identified *Blastocystis* subtype ST1, with 99.77–98.65% homology with previously reported sequences (with 100% coverage) from different geographical localisations belonging to human hosts.

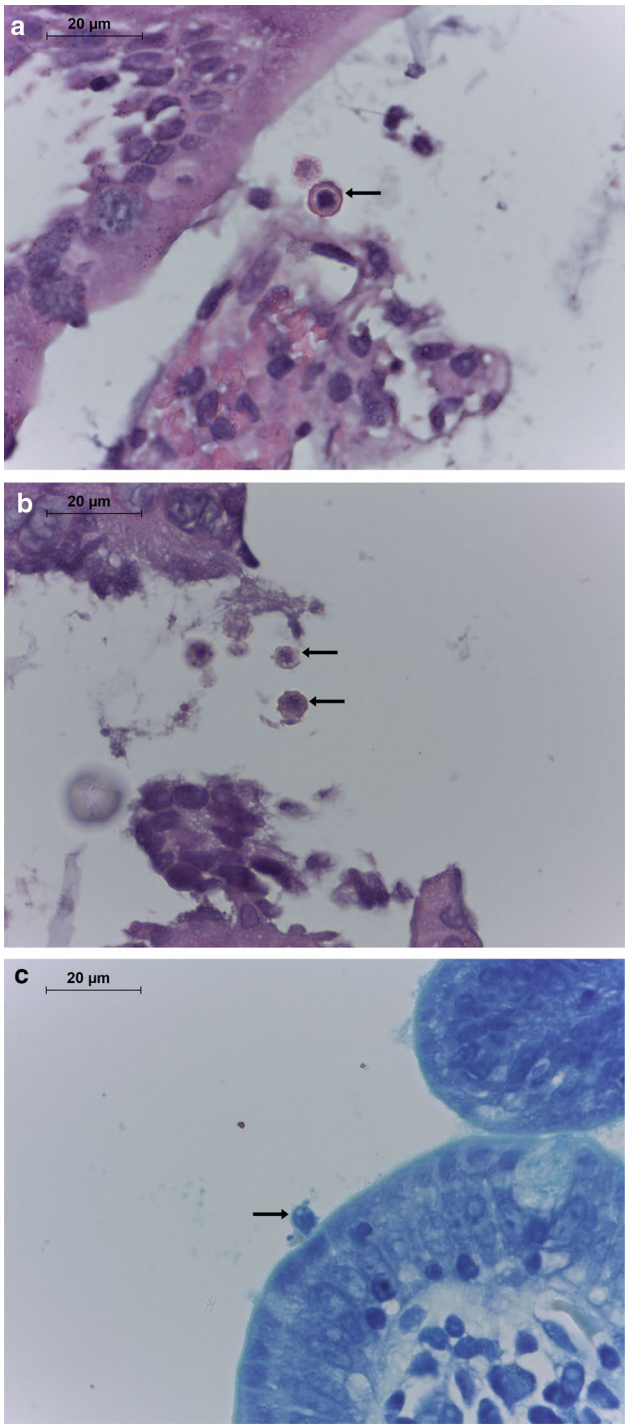

Fig. 2 Micrographs showing histopathologic examination of duodenal samples from the HIV-infected patient with *Blastocystis* infection (magnification × 1000). **a** Cyst form showing the thick multi-layered cyst wall and the condensed cytoplasm (hematoxylin–eosin stain). **b** Different forms of *Blastocystis* in the lumen. A granular form with accumulations of granules in the central vacuole can be identified. Multi-vacuolar forms can also be noted, with variable appearance of the small vacuoles (hematoxylin–eosin stain). **c** An ameboid form, irregular in shape, with an extended pseudopodium (Giemsa stain)

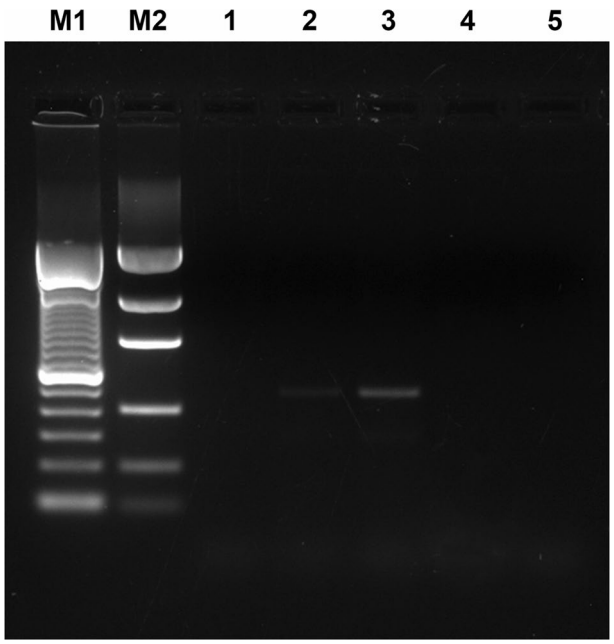

Fig. 3 Electrophoresis of amplification products after PCR of a fragment of the SSU rDNA gene of *Blastocystis* in 2% agarose gel stained with GelRed®. Lane M1, 100 bp ladder; lane M2, low DNA mass ladder (Thermo Fisher), lane 1, negative control; lane 2, positive control; lane 3, patient's stool sample; lane 4, opened reaction mixture; lane 5, closed reaction mixture

Discussion

Blastocystis is a polymorphic organism. The four forms commonly described in the literature are the vacuolar, granular, amoeboid and cyst forms. Other less frequently encountered forms such as avacuolar and multi-vacuolar have also been described [40].

The vacuolar form is predominant in axenised liquid cultures and is also commonly observed in faecal samples. This form varies greatly in size (2 to > 200 µm), with the average diameter of cells usually being between 4 and 15 µm [40].

The granular form is commonly seen in non-axenised and older cultures. Morphologically, these are identical to vacuolar forms except that granules are observed in the cytoplasm or, more commonly, in the central vacuole. There is considerable variation in the appearance of the granules seen within the central vacuole, and these have been described as myelin-like inclusions, small vesicles, crystalline granules and lipid droplets [7]. Granular form has also been described in faecal samples [8], with sizes varying from 6.5 to 80 µm [40].

The amoeboid form of *Blastocystis* has been reported infrequently and its morphological descriptions have yielded conflicting and confusing reports [7, 23, 43, 56]. Their sizes were about 2.6–7.8 µm. It has been observed in older cultures, cultures treated with antibiotics and occasionally in faecal samples [55].

The cystic form was last confirmed and described [24–26, 39, 51–53]. The delay in these findings was probably attributed to the smaller size (3–10 µm) and distinct appearance of the cyst, which could also be easily confused with faecal debris. Furthermore, these faecal cysts are seldom seen in axenised cultures.

The appearance of cells isolated from colonoscopy samples was distinctly different from the vacuolar and granular forms [39], but it was similar to what was described in an earlier study of *Blastocystis* obtained from a patient with severe diarrhea [57]. These were termed avacuolar forms for their lack of a central vacuole. In contrast to culture forms, these cells were smaller (approximately 5 µm in diameter) and lacked a surface coat. Differences in the morphology of the mitochondria were also noted.

Interestingly, these avacuolar cells [39] could not be established in culture and so it remains to be seen if these were indeed *Blastocystis* cells.

Multi-vacuolar forms have been reported in fresh human faecal material [40, 41]. These were relatively small cells (5–8 µm in diameter) containing multiple small vacuoles varying in size and content and possessed thick surface coats.

However, small vacuolar forms were also clearly discernable from the micrographs indicating that the multi-vacuolar form is not the sole form in faecal material. In a separate study [18] involving 81 isolates, vacuolar and, in some instances, amoeboid forms were the only *Blastocystis* cell types seen in faecal samples. The avacuolar and multi-vacuolar forms have been described as *in vivo* stages of the parasite while the larger vacuolar and granular forms were predominantly found in culture [2].

Our case presented four of the six described forms of *Blastocystis* in faeces. They included vacuolar, granular, cyst and multi-vacuolar forms. Morphology and sizes of the different forms were in concordance with the described in the literature.

Multiple vacuolated, amoeboid, vacuolated and avacuolar forms have been described in biopsy samples from intestine in cases of invasive *Blastocystis* infection [10, 14, 20, 41]. Pathologic examination in those cases showed infiltration by neutrophils, eosinophils, plasma cells, and lymphocytes.

Although we did not observe invasive forms of *Blastocystis*, the histological findings in our samples showed an inflammatory infiltrate with plasma cells and lymphocytes. We identified cyst, granular, ameboid and multi-vacuolar forms in the lumen. These forms and their locations are drawn in Fig. 4. To our knowledge, this is the first report describing each of four different forms of *Blastocystis* as histological findings at the lumen and the brush border of the enterocytes. This location should be taken in account

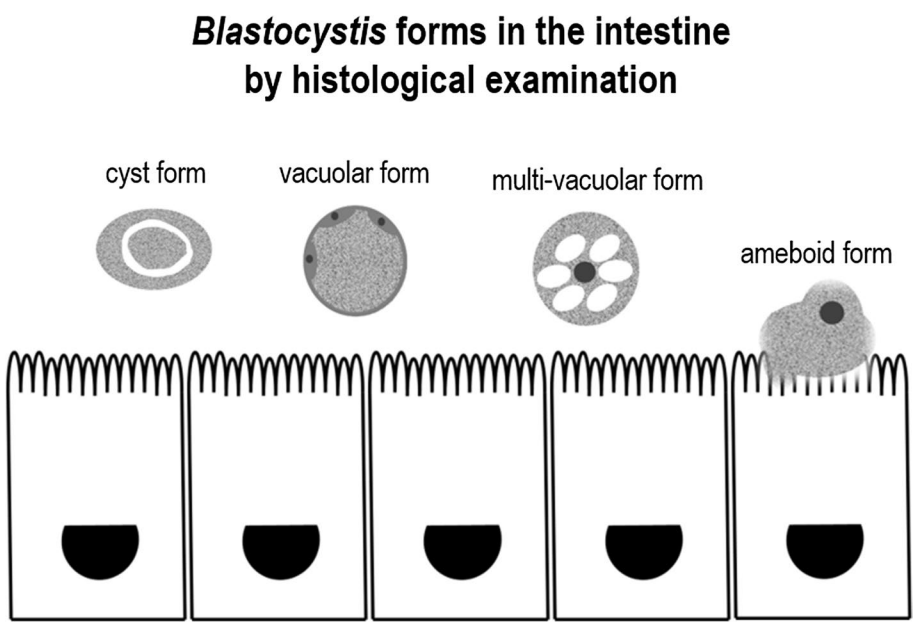

Fig. 4 Schematic representation of *Blastocystis* forms identified by histological examination of patient's duodenal samples

for routine histological diagnosis because it is shared with *Cryptosporidium* spp. [47]. Although *Blastocystis* varies widely in size, small organisms of about 5 µm of the different morphological forms can be detected, and they should be differentiated from different stages of *Cryptosporidium* spp.

Blastocystis can cause deterioration of health in immune-compromised patients, being harmful to HIV-positive patients [5, 17]. Garavelli *et al.* [11] reported for the first time two cases with persistent generalised lymphadenopathy who complained of diarrhoea, abdominal pain, and tenesmus, in which *Blastocystis* was found in the stools in significantly pathogenic amounts. They confirmed the pathogenic potential of *Blastocystis* and its possible role in causing diarrhoea in HIV-infected patients. After that one, there were several reports of *Blastocystis* infection associated with chronic diarrhoea in patients with AIDS including adults and children [4, 11, 21].

The case we described in this report was an HIV-infected patient that had been hospitalised with intermittent chronic diarrhoea that continued for 2 weeks after the admission, in whom the only diagnosis was *Blastocystis* by stool and histological examination. This is in concordance with the previous reported cases in which *Blastocystis* was the cause of diarrhoea.

The association of *Blastocystis* infection and low CD4 + T cell count is currently controversial. Our case has a CD4 lymphocyte count lower than 200 cells/mm^3 and this is in concordance with previous descriptions [54]. Although *Blastocystis* is not yet considered an opportunistic parasite, its pathogenic potential as cause of diarrhoea in HIV-infected patients with low CD4 cell count should be established.

Blastocystis ST1 is regarded as a pathogenic subtype and associated with irritable bowel syndrome diarrhoea (IBS-D). *Blastocystis* ST1 has been reported as a pathogenic subtype and associated with diarrhoea in HIV-infected patients [28]. Molecular analysis of the stool sample from our case showed the presence of *Blastocystis* ST1. This result is in concordance with previous reports, as the patient had chronic intermittent diarrhoea.

In summary *Blastocystis* is usually detected using standard parasitological methods but examination of biopsy specimens showing the presence of the different forms in the intestinal lumen and microvilli of cells is an important data to consider this parasite as another cause of diarrheal illness in HIV-infected patients.

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Declarations

Conflict of interest The authors declare that they have no conflict of interest.

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