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# Multimethodological Approach to Gastrointestinal Microsporidiosis in HIV-Infected Patients

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## Abstract

**Purpose** Microsporidiosis is an opportunistic infection that produces chronic diarrhoea and cholangiopathy in patients with AIDS, mainly caused by two species of microsporidia, *Enterocytozoon bieneusi* and *Encephalitozon intestinalis*. The aim of this work was to develop an integral system for the diagnosis of microsporidiosis of the intestine and biliary tract in HIV-infected patients, comprising microscopic and molecular techniques.

**Methods** The study population comprised 143 adult patients of both sexes with diagnosis of HIV infection, with chronic diarrhoea, and with or without HIV-associated cholangiopathy. Stool studies for microsporidia identification of spores were performed on each patient. A video esofagogastroduodenoscopy with biopsy collection was also carried out for routine histology and semi-thin sections stained with Azure II. Species identification was carried out by transmission electron microscopy and/or polymerase chain reaction for the species *E. bieneusi* and *E. intestinalis*.

**Results** Out of the 143 patients a total of 12.6% ($n=18$) were infected with microsporidia. Microsporidia species identified in most cases was *E. bieneusi* (16/18 cases), followed by *E. intestinalis* (4/18), all of these last ones in coinfection with *E. bieneusi*.

**Conclusions** Clinical, imaging, microscopic and molecular analyses, when applied in a systematic and integrated approach, allow diagnosis and identification of microsporidia at species level in AIDS patients with chronic diarrhoea, and with or without HIV-associated cholangiopathy.

**Keywords** Microsporidiosis · *Enterocytozoon bieneusi* · *Encephalitozoon intestinalis* · HIV

## Introduction

Microsporidia are obligate intracellular eukaryotic organisms which lack mitochondria, with non-typical Golgi apparatus and peroxisomes and ribosomes resembling those of bacteria [18, 19, 30, 53, 88]. This phylum consists of more than 1200 species that infect a wide range of vertebrates and invertebrates [16, 109]. To date there are ten genera of microsporidia that have been identified as causing human infections including *Encephalitozoon*, *Enterocytozoon*, *Trachipleistophora*, *Pleistophora*, *Anncalia*, *Nosema*, *Vittaforma*, *Tubulinosema*, *Microsporidium* and *Endoreticulatus* [26, 43, 78, 102].

Microsporidia have been recognized as infectious agents worldwide, with reported cases in developed and developing countries [6, 14, 15, 37, 39, 48, 50, 51, 70, 95]. In Argentina the characteristics of the disease are unknown in terms of prevalence, morbidity and mortality.

Standard diagnostic methods depend on the detection of the organisms in fluids, stool and biopsy specimens by light or electron microscopy [104, 105]. Light microscopy presents a challenge due to the small size of the organisms (about 3 um), with a detection limit of 103–104 spores/

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gram of stool [83]. Meanwhile, electron microscopy is the definitive procedure for the identification of species, but requires expensive and specialized equipment and has low sensitivity. The identification of species of microsporidia is clinically useful because of the existence of differences in the response to the therapy and depends on the ultrastructural and/or molecular analysis. Molecular techniques that have been developed for the diagnosis of human microsporidiosis correspond to PCR, restriction fragment length polymorphism (RFLP), Southern blot analysis, single-strand conformational polymorphism (SSCP), in situ hybridization, DNA sequencing, real-time PCR, Luminex, loop-mediated isothermal amplification (LAMP) and multiplex commercial kit [20, 21, 29, 32–34, 42, 44, 45, 61, 62, 67, 69, 71–73, 91, 92, 96, 97, 99, 110].

*Enterocytozoon bieneusi* and *E. intestinalis* are the most common species infecting the gastrointestinal tract. In AIDS patients *E. bieneusi* has been identified in different tissues such as intestinal epithelium, lamina propria, bile duct, pancreatic, bronchial and nasal epithelium. *E. intestinalis* has been identified in the intestinal epithelium and lamina propria, endothelial, renal tubule cells, biliary tract, nasal and bronchial epithelium and other tissues [43, 104]. Co-infection of more than one species of microsporidia can be found in this group of patients [9]. Patients with AIDS and microsporidiosis have a variety of clinical manifestations ranging from asymptomatic to severe medical conditions that depend on the location of the infection and CD4 cell count [56–58]. The clinical presentations in this group of patients include asymptomatic, symptomatic intestinal and symptomatic extraintestinal. Asymptomatic clinical forms have a prevalence of about 2–11% [2, 86] and are associated with high CD4 cell count. In the intestinal symptomatic forms, patients with a CD4 count greater than 200 cells/mm$^3$ present acute or persistent diarrhoea [81]. With CD4 counts higher than 200 cells/mm$^3$, acute and chronic diarrhoea may evolve towards healing [81]. Patients with CD4 counts between 100 and 200 cells/mm$^3$ present chronic diarrhoea. The CD4 counts lower than 100 cells/mm$^3$ are associated with symptoms of diarrhoea that can persist for years and lead to extraintestinal sites producing cholangiopathy and pancreatitis [43, 105]. Watery diarrhoea, dehydration, hypovolemia and death are associated with a CD4 count lower than 100 cells/mm$^3$ [13, 37, 52]. Intestinal perforation has been described in a patient infected with *E. intestinalis*. The infection of the biliary tract is one of the most frequent extraintestinal clinical manifestations and has been described in 10% of patients with AIDS and intestinal microsporidiosis without antiretroviral therapy [59]. The species *E. bieneusi* causes alithiasic cholecystitis, pancreatitis and sclerosing cholangitis [80]. The species *E. intestinalis* causes acalculous cholecystitis, sclerosing cholangitis, pancreatitis and disseminated infection [17, 108]. The level of CD4 cells in this group of patients is generally lower than 100 cells/mm$^3$ [80].

Before the use of antiretroviral therapies, microsporidiosis had been reported in at least 15% (and up to 85%) of HIV/AIDS patients [89]. Since the advent of antiretroviral therapy (ART) and its progressive introduction starting in 1996, opportunistic infections such as microsporidiosis have substantially decreased in developed countries [40] due to the reconstitution of the immune system of the patients. This contrasts with the situation in developing countries, with limited or no access to ART, where opportunistic infections, including those due to microsporidia, remain problematic [38, 87, 94]. A recent review and meta-analysis [101] showed that the global prevalence of microsporidia infection in HIV-infected people ranged between 0.7 and 81.3%. The estimated pooled prevalence was 11.8% overall, significantly higher in low-income countries than in middle-income countries. The infection rates of the species *E. bieneusi* in HIV-infected patients are usually in the range of 1.3–11.6%, with higher rates reported in Nigeria, Iran, and Australia [60].

The aim of this work was to develop an integral system for the diagnosis of microsporidiosis of the intestine and biliary tract in HIV-infected patients, comprising microscopic and molecular techniques in fluid and biopsy specimens.

## Materials and Methods

### Studied Population and Sample Collection

The studied population comprised 143 adult patients, with ages in the range of 20 and 50 years, corresponding to 120 males and 23 females, diagnosed with HIV who attended Hospitals Francisco J. Muñiz and José María Penna, Buenos Aires city, Argentina, complaining of chronic diarrhoea (considered when lasting a month or longer) with or without HIV-associated cholangiopathy and CD4 counts less than 500 cells/mm$^3$. Stool samples and duodenal biopsies were studied. Samples were collected since 2012 until 2018.

HIV-associated cholangiopathy was confirmed by endoscopic retrograde cholangiopancreatography (ERCP) and/or magnetic cholangioresonance (CR) of liver and biliary tract. A group of 11 patients who met the inclusion criteria were selected among the 143 studied patients.

The research protocol was approved by the Ethical Committee for Research, Hospital Francisco J. Muñiz, protocol no. 274.

The stool samples were collected during 7 days in 5% formalin saline solution and stored at room temperature until processed.

An upper gastrointestinal endoscopy (UGE) was performed to each patient with a Pentax EPM 2000 equipment.

Endoscopic images of the duodenal mucosa were classified ([63], with modifications) as normal duodenum, granular duodenum when the mucosa presented congestive base with a thin whitish nodularity, jasper duodenum when the folds were flattened with congestive patches, and atrophic duodenum when the folds were deleted and visible vessels were seen by transparency. Five or more biopsy specimens from the distal duodenum were obtained in all cases showing or not macroscopic lesions, and they were stored in 10% formalin, Karnovsky's fixative and at −20 °C in saline solution. In cases with cholangiopathy, peripapillary duodenum samples were obtained.

An ERCP and/or CR were performed in patients with clinical manifestations, humoral pattern of cholangiopathy and bile duct abnormalities by ultrasonography suggestive of cholangiopathy. For the patients who underwent ERCP, a papilla biopsy was obtained and fixed in formalin 10% and Karnovsky fixative. ERCP was performed with an Olympus TJF 100 videoduodenoscope. The bile duct was cannulised and ERCP was performed with contrast medium at low pressure. The site, extension and radiological characteristics of the lesion were determined. The bile duct abnormalities in ERCP and/or CR were classified [22] as papillary stenosis (PS), sclerosing cholangitis (SC), association of the two previous abnormalities and long common bile duct stenosis.

### Microscopy Analysis

Faeces samples were concentrated by ethyl ether centrifugation [93]. Thin smears were prepared from the pellets and observed by light microscopy at a magnification of 1000 after modified trichrome [103] and Gram-chromothrope [68] stains.

Formalin-fixed samples were embedded in paraffin and sliced in 5–10 µm sections. They were stained with hematoxylin and eosin (H&E) and Giemsa for light microscopy observation. In cases where compatible structures with microsporidia were observed, a methenamine silver staining [5] was performed. Samples in Karnovsky's fixative were dehydrated and embedded in polybedaraldita. Semi-thin sections of 1 µm thickness were sliced and stained with Azure II for observation by light microscopy.

The diagnosis of microsporidiosis was carried out by the identification of spores and structures of the merogony and sporogony stages by light microscopy.

In cases that met diagnostic criteria for microsporidiosis, the degree of inflammation and atrophy was determined following the concepts of different authors with modifications tailored to our work [36, 84]. We evaluated well-targeted 5–10 villi where the following characteristics were analysed: mucosal appearance, that may have different morphologies such as a mixed villi pattern (villi have a finger and/or foliaceus and/or ridges-like appearance), cerebriform, convolution, branching or mosaic with flattened mucosa; the villus:crypt ratio, considering a normal ratio of 3:1; description of epithelial infiltrates, including the number of intraepithelial lymphocytes (lower or higher than 30 lymphocytes per 100 epithelial cells), the presence or absence of polymorphonuclear cells or eosinophils; description of infiltrates in the lamina propria, with cell type in the three rows below the villi (considering normal 60% plasmocytes with a lymphocytes, eosinophils and histiocytes remaining). Crypt hyperplasia was considered when an elongation of the same size was observed.

In cases where compatible structures with microsporidia were detected by light microscopy, the samples were processed for TEM as previously described [12] and examined using a Carl Zeiss EM-109 equipment.

### Molecular Diagnosis

Molecular techniques were carried out in those cases with diagnostic criteria of microsporidiosis in stool samples (spores) and/or biopsy specimens (spores, meronts, sporonts).

DNA purification was performed according to previous protocols [96], by standard phenol–chloroform extraction and ethanol precipitation. For biopsy specimens, an initial tripsinization step was added.

A PCR protocol for *E. bieneusi* was applied as previously described [96] for the amplification of a 210-bp fragment corresponding to the internal transcribed spacer (ITS) of the rRNA genes with the primers set Eb.gc::Eb.gt.

The PCR reaction for *E. intestinalis* was carried out [31] for the amplification of a 520-bp region of the SS rDNA employing the primers set SINTF1::SINTR, with modifications as previously described [98].

Samples resulting negative for *E. bieneusi* and *E. intestinalis* were analysed by PCR employing the universal primers set 18f::1492r [106] that amplifies most of the small subunit rRNA of the microsporidia.

Amplification products were analysed electrophoresis in 2% agarose gel stained with ethidium bromide.

## Results

### Prevalence and Pathogenicity

Microorganisms were identified in stool and/or biopsy specimens in 63 cases.

Etiologic agents were: *Crytosporidium* sp. ($n = 22$), microsporidia ($n = 18$), *Cystoisospora belli* ($n = 14$), Cytomegalovirus ($n = 12$), *Giardia duodenalis* ($n = 7$), *Strongyloides stercoralis* ($n = 4$), *Mycobacterium tuberculosis* ($n = 2$), *Histoplasma capsulatum* ($n = 2$), *Mycobacterium avium intracellularis* ($n = 2$), *Cryptococus neoformans*

($n=1$) and *Cyclospora cayetanensis* ($n=1$). Coinfections were detected in 12 cases.

The diagnosis of microsporidia was positive in 18 out of the 143 cases (12.6%). The species in most cases was *E. bieneusi* (16/18 cases) followed by *E. intestinalis* (4/18). All cases with *E. intestinalis* were coinfected with *E. bieneusi*. Out of the 18 cases of microsporidiosis, 4 presented cholangiopathy and they were identified as *E. bieneusi*.

Microsporidia-infected patients were aged 23–51 years; 15 were males and 3 females. Sexual behaviour corresponds to 13 heterosexual and 5 homosexual. Two patients were intravenous drug abusers. Diarrhoea duration was 3–12 months. Most cases had a number of depositions higher than 10 per day (12/18), incontinence (11/18), weight loss (15/18) and vomiting (10/18). Some patients also presented dysphagia (2/18), abdominal pain (2/18), right upper quadrant abdominal pain (4/18) and pancreatitis (1/18).

In patients not receiving antiretroviral therapy (11/18 cases) CD4 count was less than 100 cells/mm$^3$ in 8 cases, between 100 and 200 cells/mm$^3$ in two cases and in one case more than 200/mm$^3$. In patients receiving antiretroviral therapy (7/18 cases) CD4 count was less than 100 cells/mm$^3$ in 1 case, between 100 and 200 cells/mm$^3$ in 5 cases and higher than 200 cells/mm$^3$ in only one case. The endoscopic appearance of the duodenal mucosa presented a granular appearance in 9/18 cases, jasper in 3/18 cases and normal in 6/18 cases. Table 1 summarizes the most significant clinical and microbiological characteristics associated with each of the patients with microsporidosis. Evolution of each patient is described in Table 2.

### Diagnosis by Light Microscopy

Concentrated formalin-preserved faeces were stained with modified trichrome and Gram-chromotrope and observed by light microscopy (Fig. 1a, b), allowing the identification of spores in 13 of 143 cases (9%).

Duodenal biopsy specimens stained with Giemsa, H&E, methenamine silver and Azure II allowed the identification of structures compatible with microsporidia by light microscopy (Figs. 2a–c, 3a, b) in 18 of 143 cases (12.6%). Giemsa, H&E and methenamine silver stains identified structures in 16 of 18 cases and Azure II in all cases.

#### Degree of Inflammation and Atrophy

In all cases that met diagnostic criteria for microsporidiosis the degree of inflammation and atrophy was determined. Most patients presented an aspect of the mucosa with a mixed villi pattern (17/18) with a villus:crypt ratio of 2:1 (17/18). In four cases more than 30 lymphocytes per 100 epithelial cells were found. The lamina propria presented infiltrates with mononuclear predominance in most cases (15/18). In all patients crypt hyperplasia was observed. Among the 10 cases where the identified species was *E. bieneusi* without other microorganisms, in 6 cases there was an increased number of intraepithelial lymphocytes.

### Diagnosis by Transmission Electron Microscopy

Transmission electron microscopy was performed in four patients with microsporidiosis. The species *E. bieneusi* was identified in the 4 cases, one of them in coinfection with *E. intestinalis* (Figs. 2d, 3c).

### Molecular Diagnosis

The PCR technique for the identification of the species *E. bieneusi* and *E. intestinalis* in faecal samples and/or biopsies was used in all patients diagnosed with microsporidiosis [31, 96]. Faecal samples were analysed in 11/18 cases and biopsies in 18/18 cases. The species *E. bieneusi* was identified in 16 patients. In 4 of the 16 patients a coinfection with the species *E. intestinalis* was identified (Table 1). In two patients with spores in stool and structures compatible with microsporidia in duodenal biopsies species *E. bieneusi* and *E. intestinalis* were not identified, but this sample resulted positive employing the universal primers.

## Discussion

More than 100 million people are infected with HIV worldwide. Diarrhoea in patients with HIV may affect 50% of cases in the United States and an even higher percentage in developing countries, reaching 80% with or without antiretroviral therapy [23, 101]. HIV-associated cholangiopathy affected 30% of AIDS patients in the pre-ART era [24]. However, although fewer cases are described due to antiretroviral therapy, they continue appearing [35]. The species of microsporidia *E. bieneusi* and *E. intestinalis* are cause of diarrhoea and HIV-associated cholangiopathy.

With the methodology described here microorganisms were identified in 63/143 cases reaching approximately 44%, in concordance with previous studies [27, 54]. The most common organisms identified in our study were *Cryptosporidium* spp., microsporidia, *Cystoisospora belli*, Cytomegalovirus and *Giardia duodenalis*. *Cryptosporidium* spp. and microsporidia have been the most common causes of chronic diarrhoea in HIV-infected in the pre-ART era in numerous series of works [9, 47, 107]. With the use of ART opportunistic infections with *Cryptosporidium* sp. and microsporidia decreased their prevalence [28, 55, 66]. Many authors agree that diagnosis of microsporidia should be present even with the use of ART in patients with chronic diarrhoea, weight loss and a CD4 cell count of about 100 cells/

**Table 1** Clinical and microbiological characteristics of patients with microsporidiosis

| Case | Sex/age | Symptoms (number of depositions/day) | Incon-tinence | HAART | CD4/mm$^3$ | UGE | US | CR/ERCP | Histology | Faeces | TEM | PCR |
|---|---|---|---|---|---|---|---|---|---|---|---|---|
| 1 | M/40 | CD 15/d | Yes | No | 278 | Granular | ND | ND | *G*, *Cr*, Mc | *G*, *Cr* | *Eb* | *Eb* (DB) |
| 2 | M/35 | CD 15/d | Yes | No | 50 | Granular | ND | ND | Mc | Neg | *Eb* | *Eb* (DB) |
| 3 | M/32 | CD 20/d Ch? | Yes | No | 110 | Granular | ND | ND | Mc | Spores | *Eb* | *Eb* (DB) |
| 4 | M/23 | CD 10/d | Yes | No | 100 | Granular | ND | ND | Mc | Neg | NR | *Eb*, *Ei* (DB) |
| 5 | M/30 | CD 20/d | Yes | No | 50 | Normal | ND | ND | Mc | Spores | NR | *Eb* (DB, F) |
| 6 | M/26 | CD 15/d | Yes | No | < 50 | Granular | ND | ND | Mc | Spores | NR | *Eb* (DB, F) |
| 7 | M/35 | CD 20/d | Yes | No | 50 | Granular | ND | ND | Mc | Spores | *Eb, Ei* | *Eb*, *Ei* (DB, F) |
| 8 | M/34 | CD 5/d | No | Yes | 179 | Normal | ND | ND | Mc | Spores | NR | *Eb* (DB, F) |
| 9 | M/31 | CD 5/d | No | Yes | 194 | Normal | ND | ND | Mc | Spores | NR | *Eb* (DB, F) |
| 10 | M/39 | CD 10/d | Yes | Yes | 100 | Granular | ND | ND | Mc | Spores | NR | *Eb* (DB, F) |
| 11 | M/32 | CD 15/d EAS | Yes | No | 50 | Normal | ND | ND | Mc | Spores | NR | Negative for *Eb*, *Ei*; positive with generic primers (DB, F) |
| 12 | M/29 | CD 15/d | Yes | Yes | 178 | Normal | ND | ND | Mc, *Cb* | Spores, *Cb* | NR | Negative for *Eb*, *Ei*; positive with generic primers (DB, F) |
| 13 | M/51 | CD 15/d | No | Yes | 119 | Jasper | ND | ND | Mc, *Cb* | Spores *Cb* | NR | *Eb*, *Ei* (DB, F) |
| 14 | F/50 | CD 15/d | Yes | Yes | 316 | Normal | ND | ND | Mc | Spores | NR | *Eb*, *Ei* (DB, F) |
| 1C | M/37 | RUQP, CD | No | No | 68 | Granular | D, T | SC-PS | *Cr*-Mc | *Cr* | NR | *Eb* (DB) |
| 2C | F/21 | RUQP, CD | No | Yes | 7 | Granular | D | SC-PS | Mc | Neg | NR | *Eb* (DB) |
| 3C | M/25 | RUQP, CD, pancreatitis | No | No | 48 | Jasper | T | SC | Mc | Neg | NR | *Eb* (DB) |
| 10C | F/49 | RUQP, CD | No | Yes | 26 | Jasper | D | SC | Mc | Spores | NR | *Eb* (DB) |

*UGE* upper gastrointestinal endoscopy, *US* ultrasonography, *ERCP* endoscopic retrograde cholangiopancreatography, *CR* magnetic cholangioresonance (CR), *TEM* transmission electron microscopy, *PCR* polymerase chain reaction, *CD* chronic diarrhoea, *EAS* edematous ascetic syndrome, *Ch?* Cholangitis?, *RUQP* right upper quadrant pain, *SC* scerosing cholangitis, *PS* papillary stenosis, *G Giardia duodenalis*, *Cr Cryptosporidium* spp., *Mc* microsporidia, *Cb Cystoisospora belli*, *E.b Enterocytozoon bieneusi*, *E.i.*, *Encephalitozoon intestinalis*, *Neg* negative, *ND* no data, *DB* duodenal biopsy, *F* faeces, *D* bile ducts dilation, *T* bile ducts thickening

**Table 2** Treatment and evolution of patients with microsporidiosis

| Case | Diarrhoea | Adherence to HAART | Specific treatment | CD4/mm$^3$ value at admission | CD4/mm$^3$ value after treatment | Species identification | Survival |
|---|---|---|---|---|---|---|---|
| 1 | Persisted | No | No | 278 | ND | *G*, *Cr, Eb* | Died at day 35 |
| 2 | Persisted | No | No | 50 | ND | *Eb* | Died at day 38 |
| 3 | Persisted | No | Albendazole | 110 | ND | *Eb* | Died at day 49 |
| 4 | Persisted | No | No | 100 | ND | *Eb*, *Ei* | Died at day 40 |
| 5 | Persisted | No | No | 50 | ND | *Eb* | Died at day 34 |
| 6 | Resolved | No | Albendazole | < 50 | ND | *Eb* | Died at day 100 |
| 7 | Persisted | No | No | 50 | ND | *Eb*, *Ei* | Died at day 12 |
| 8 | Resolved | Yes | Albendazole | 179 | 314 | *Eb* | Discharged at day 7 |
| 9 | Persisted | Yes | No | 194 | ND | *Eb* | Discharged at day 25 |
| 10 | Persisted | Yes | Nitazoxanide | 100 | ND | *Eb* | Discharged at day 25 |
| 11 | Persisted | No | Nitazoxanide | 50 | ND | Mc | Discharged at day 22 |
| 12 | Resolved | Yes | Albendazole, TMS-SM | 178 | ND | Mc, *Cb*, *Sarc* | Died at 1 year |
| 13 | Persisted | Yes | Albendazole, clindamicin | 119 | ND | *Eb*, *Ei*, *Cb* | Discharged at day 37 |
| 14 | Persisted | Yes | Albendazole, nitazoxanide | 316 | 187 and later 396 | *Eb*, *Ei* | Discharged at day 37 |
| 1C | Persisted | No | No | 68 | ND | *Cr*, *Eb* | Died at day 34 |
| 2C | Resolved and relapsed 4 years later | Yes | No | 7 | < 50 | *Eb* | Died at 4 years and 4 months |
| 3C | Persisted | No | No | 48 | ND | *Eb* | Died at day 28 |
| 10C | Persisted | Yes | Nitazoxanide | 26 | ND | *Eb* | Died at day 40 |

*G Giardia duodenalis*, *Cr Cryptosporidum* spp., *Mc* microsporidia, *Cb Cystoisospora belli*, *Sarc Sarcocystis* sp., *Eb Enterocytozoon bieneusi*, *Ei Encephalitozoon intestinalis*, *ND* no data

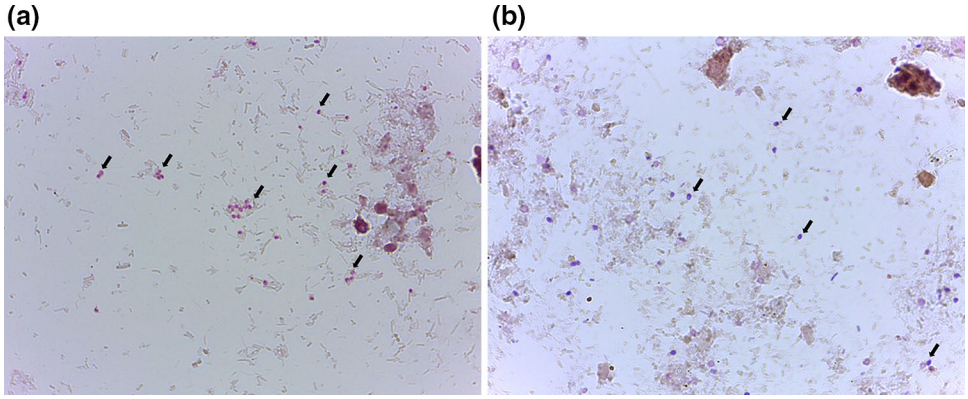


**Fig. 1** Microsporidia spores in faeces observed by light microscopy. **a** Modified trichrome stain showing ovoid, pyriform or bacillar shaped-spores with bright pinkish red stained wall. **b** Gram-chromotrope stain showing spores of microsporidia with the presence of a vacuole or a diagonal band representing the polar tubule. Magnification × 1000

mm$^3$ [3, 7, 27]. Our patients correspond mainly to marginal population, prisoners, intravenous drug abusers, homeless, whose adherence to ART is very low. Due to these characteristics opportunistic infections continue to be frequent in the post ART era and microsporidiosis continues to be a cause of morbidity and mortality.

The diagnosis of microsporidia was positive in 18 out of the 143 cases representing 12.6%, in concordance with previously described prevalence of microsporidia in HIV positive patients that varies between 7 and 50% [104]. A recent meta-analysis [101] showed that the prevalence of microsporidia infection ranged between 0.7 and 81.3% in people with HIV with an estimated pooled prevalence of 11.8% worldwide and 5.6% in Latin America. This last value represents only three studies that were done in Venezuela, Colombia and Perú [25, 41, 90]. Our present report is the first one in Argentina showing the prevalence of intestinal microsporidia infection, particularly in HIV-positive patient.

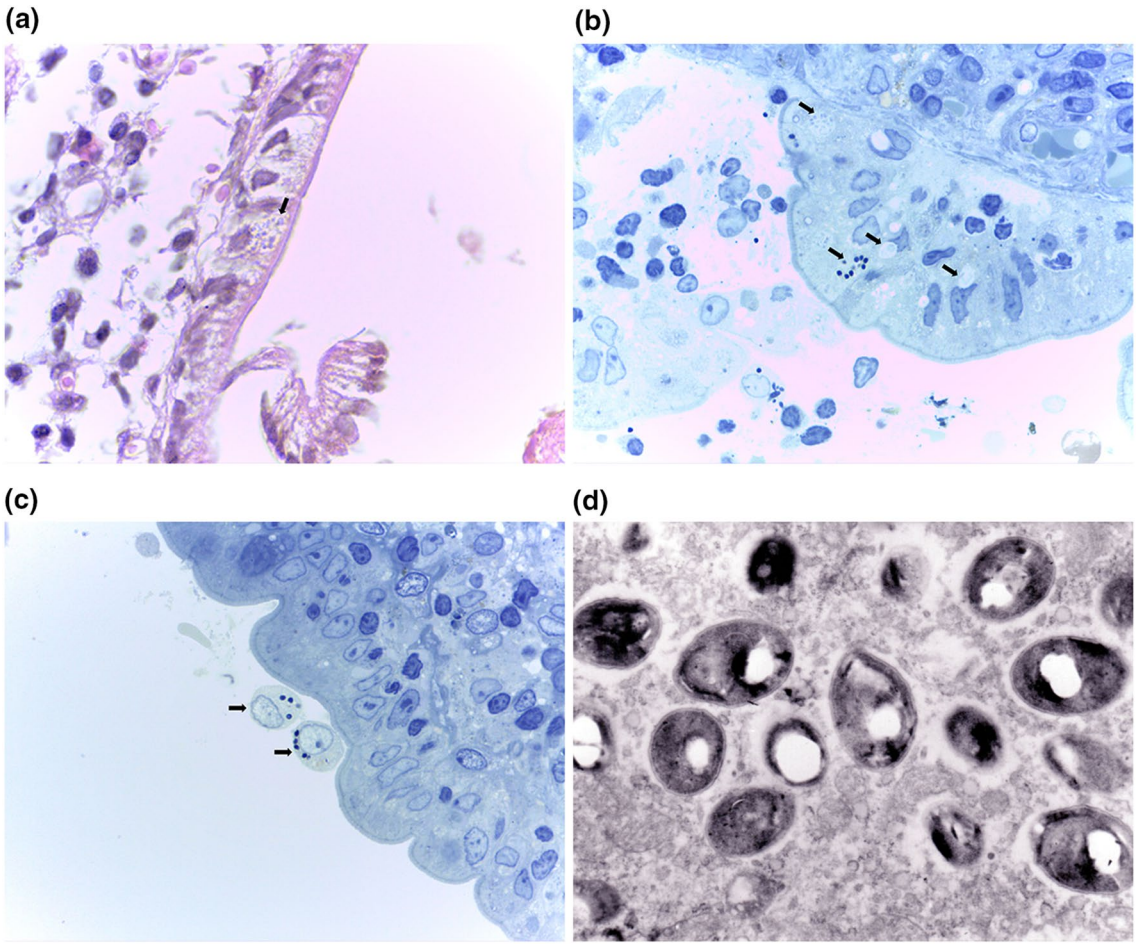


**Fig. 2** *Enterocytozoon bieneusi* in biopsy specimens. **a** Spores with band-shaped or rounded clear structures inside (H&E staining, magnification ×1000). **b** Multiple intraepithelial spores and stages of oval or round shape in direct contact with the cytoplasm of the epithelial cell (Azure II staining, magnification ×1000). **c** Epithelial cell infected with spores leaving the epithelium and reaching the intestinal lumen (Azure II staining, magnification ×1000). **d** Uninucleated spores of *E. bieneusi* in direct contact with the cytoplasm of host cells, in TEM (magnification ×5600)

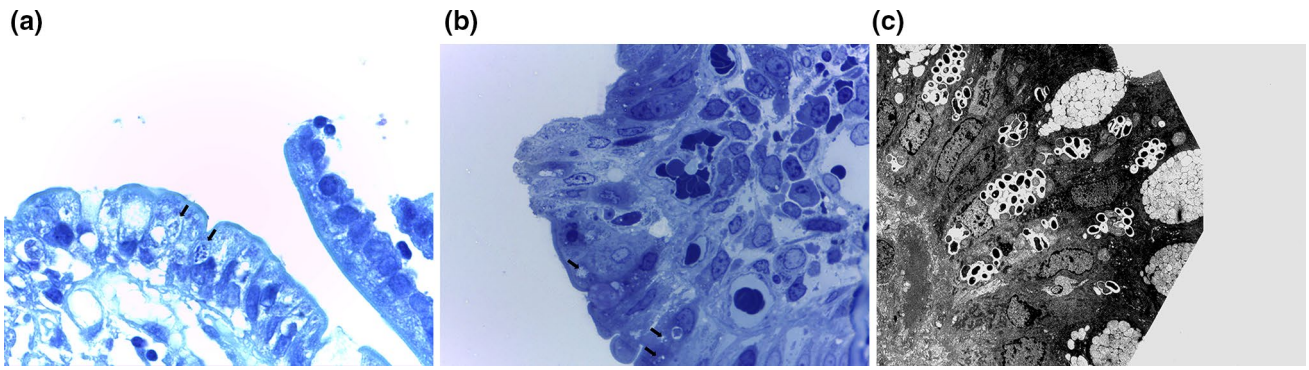


**Fig. 3** *Encephalitozoon intestinalis* in biopsy specimens. **a** Spores with within displaced parasitophorous vacuoles (Giemsa stain, magnification ×1000). **b** Merogony and/or sporogony stages surrounded by vacuoles (Azure II stain, magnification ×1000). **c** Spores of *E. intestinalis* within parasitophorous vacuoles in TEM (magnification ×3300)

Most patients had chronic diarrhoea evolution of 3–12 months with an average of 5 months. Some authors described a broader range of 1–32 months with a higher average of 8 months [4]. The variable number of depositions

per day was higher than that described in other reports which mentioned that most of patients had about five depositions/day [4, 58]. In our cases the number of depositions was ten or more per day. Diarrhoea was accompanied by weight loss in almost all patients studied being a similar finding as described by numerous authors [3, 4, 7, 27, 58]. A symptom that accompanies chronic diarrhoea in most of our patients was incontinence, which is not frequently observed [100].

The endoscopic appearance of the duodenal mucosa presented a granular appearance in 9/18 cases and jasper in 3/18 cases. Changes in the appearance of the duodenal mucosa are not specific to an aetiologic agent since we observed them with different opportunistic infections (data not shown). In 6/18 cases the appearance of the duodenal mucosa was normal and biopsies allowed the identification of microsporidia. In patients with chronic diarrhoea, low CD4 level and normal endoscopic image sampling duodenal biopsy can identify aetiologic agents.

According to our results the diagnosis of microsporidiosis in patients with chronic diarrhoea should be present when it is accompanied by incontinence, weight loss and low CD4 cell count, and although VEDA shows a normal duodenal mucosa aspect, biopsy specimens' collection is also indicated.

HIV-associated cholangiopathy is characterized by abnormalities in the biliary tract in patients with low CD4 level [106]. The diagnosis of HIV-associated cholangiopathy was achieved in 11/143 of the studied patients reaching about 8%. With the described methodology, microorganisms were identified in 10/11 cases. Other authors identified microorganisms only in 60% of cases [80]. The most common organisms identified in our patients with HIV-associated cholangiopathy were *Cryptosporidium* spp. and microsporidia. Microsporidia diagnosis was carried out in 4/11 cases reaching 37%. Other authors identified microsporidia in about 10% of patients [1]. The only species identified in our series was *E. bieneusi.* Abdominal pain, diarrhoea, weight loss, and CD4 cell count was about 100 cells/mm$^3$ or lower occurred in all patients being similar to the description in the literature [80]. Abdominal ultrasonography allows to identify dilation and wall thickening of the common bile duct and other abnormalities in 75% of patients with HIV and cholangiopathy [11] and it was useful to identify abnormalities in all our cases.

In patients with microsporidiosis and cholangiopathy associated with *E. bieneusi,* the pattern of sclerosing cholangitis and papillary stenosis has been described [80]. All our patients had sclerosing cholangitis and two of them had also papillary stenosis.

Summarizing, the diagnosis of microsporidosis in patients with cholangiopathy must be present when vomiting and right upper quadrant pain are accompanied by increased alkaline phosphatase level, abdominal ultrasonography with dilated bile duct and/or thickened walls and CD4 cell count less than or equal 100 cells/mm$^3$. It may be accompanied by chronic diarrhoea.

Stool samples stained with modified trichrome and Gram-chromotrope allowed the identification of spores in 13 of 143 cases constituting about 9% of cases. In similar studies in the United States, Brazil, Sweden and Thailand, prevalence between 9 and 33% was observed [100, 105]. The use of both staining techniques together was very useful since in some samples stained with modified trichrome spores resembled bacteria and yeast and Gram-chromotrope technique allowed us to differentiate.

Duodenal biopsy samples stained with Giemsa, H&E and Azure II helped to identify microsporidia-compatible structures by light microscopy in more than 12% of cases. In Italy, in a study including 21 patients with AIDS and chronic diarrhoea in duodenal biopsies stained with H&E and toluidine blue microsporidia were identified with security in two cases (9.52%) and were suspected in 3 cases [10]. In another study of 59 patients with AIDS and chronic diarrhoea in duodenal biopsies stained with H&E and Giemsa, diagnosis of microsporidia was performed in 8 patients reaching 13% [79]. However, in a study in Rio de Janeiro in Brazil in 40 patients with chronic diarrhoea and AIDS which underwent duodenal biopsies stained with H&E and Giemsa microsporidia were diagnosed in 11 cases reaching 27.5% [13]. Other authors performed the diagnosis of microsporidia in duodenal biopsies in patients with diarrhoea in 30% of cases [81]. In our study identifying structures compatible with microsporidia by light microscopy in 12.6% of cases could be real or underestimated. The variable sensitivity of the light microscopy in different reports could be given by the focal distribution of infected enterocytes and selected sites for biopsies that may or may not correspond to infected cells [13, 65]. Another possibility is the difficulty in identifying structures compatible with microsporidia in cases with low parasite burden [10, 65]. Numerous studies agree that the identification of intracellular stages compatible with microsporidia depends on careful observation and interpretation of histopathology [4, 46, 64]. Some authors suggest re-observation of histological slides if microsporidian suggestive microorganisms are present [4]. This difficulty in identifying compatible parasitic structures with microsporidia using H&E and Giemsa was mentioned by different authors [4, 10, 13, 46, 65, 76]. One reason is that while the spores are easier to identify, it is not so with other parasitic stages given the minimal contrast between the parasitic structure and the cytoplasm of the infected cell [46, 65]. The thickness of the slices is another factor because the better definition of the structures is obtained with thin slices. Some authors suggest that slices of 3 μm in thickness are useful for identifying microsporidia in H&E and Giemsa stained samples [65]. In our work parasitic stages were identified in 14 duodenal

biopsies embedded in polybedaraldita, sliced in semi-thin sections of 1 µm and stained with Azure II. The use of semi-thin sections allowed us to get a better definition of parasitic and cellular stages using light microscopy. Our findings in semi-thin sections were similar to the recommendations of different authors for the identification of microsporidia [46, 65, 74, 76].

A mixed villi pattern was observed in almost all patients (17/18 cases). Villous atrophy and crypt hyperplasia were observed in all patients. Other authors have also described the villus atrophy and crypt hyperplasia as a frequent finding [11, 58, 74, 77, 82]. However, some microsporidosis reported cases presented without villous atrophy [10]. The increased intraepithelial lymphocytic infiltrates were found in some patients (4/14 cases), similar as described by other authors [58, 74, 79, 82]. The infiltrate in lamina propria with mononuclear predominance in most cases is a common finding [58].

Although TEM is the best method to identify structures and differentiate species, it is time-consuming, expensive, with low sensitivity and is not available in all centres precluding its use as routine [56, 74–76]. In this way, molecular methods have gained a main role in the identification of microsporidia at the species level. The target sequences of PCR most commonly used in the diagnosis and identification of microsporidia species that infect humans correspond to fragments of rRNA genes, with greater emphasis on the small subunit and the ITS. The PCR primers were selected for an amplification product of less than 1000 bp, improving diagnosis in formalin-fixed samples [85]. Therefore, we employed the primer set Eb.gt::Eb.gc with an amplification product of 210 bp to identify the species *E. bieneusi* and the set SINTF1::SINTR with an amplification product of 520 bp to identify *E. intestinalis* [31, 96]. The main advantage of using the method of PCR in faecal samples is that it is a noninvasive method that allows the differentiation of species of microsporidia, which is useful in therapy administration. The use of PCR in faecal samples allows detection of infections with more than one species of microsporidia as microscopic observation of spores in faeces does not.

Coinfection of *E. bieneusi* and *E. intestinalis* was first described in a patient in 1992 [8]. In our work four cases coinfected with both species were identified. Other authors showed that coinfection with both species is not uncommon since they identified it in 9.6% of cases [49].

The interpretation of the clinical manifestations, the use of imaging techniques, the application of methods of etiological diagnosis by microscopy in stool and biopsy samples and the use of molecular tools applied to different biological specimens, enabled, by the systematic and integrated employment, the diagnosis and identification of species of microsporidia in patients with AIDS, chronic diarrhoea and/or HIV-associated cholangiopathy.

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