

Molecular identification of protozoa causing AIDS-associated cholangiopathy in Buenos Aires, Argentina

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Summary

Background. Several species of microsporidia and coccidia are protozoa parasites responsible for cholangiopathy disease in patients infected with human immunodeficiency virus (HIV). The goals of this work were to identify opportunistic protozoa by molecular methods and describe the clinical manifestations at the gastrointestinal tract and the biliary system in patients with AIDS-associated cholangiopathy from Buenos Aires, Argentina. **Material and methods.** This study included 11 adult HIV-infected individuals with diagnosis of AIDS-associated cholangiopathy. An upper gastrointestinal endoscopy with biopsy specimen collection and a stool analysis for parasites were performed on each patient. The ultrasound analysis revealed bile ducts compromise. An endoscopic retrograde cholangiopancreatography and a magnetic resonance cholangiography were carried out. The identification to the species level was performed on biopsy specimens by molecular methods. **Results.** Microorganisms were identified in 10 cases. The diagnosis in patients with sclerosing cholangitis was cryptosporidiosis in 3 cases,

cystoisosporosis in 1 and microsporidiosis in 1. In patients with sclerosing cholangitis and papillary stenosis the diagnosis was microsporidiosis in 2 cases, cryptosporidiosis in 2 and cryptosporidiosis associated with microsporidiosis in 1. In 3 cases with cryptosporidiosis the species was *Cryptosporidium hominis*, 1 of them was associated with *Enterocytozoon bienewisi*, and the other 2 were coinfecting with *Cryptosporidium parvum*. In the 4 cases with microsporidiosis the species was *Enterocytozoon bienewisi*. **Conclusions.** These results suggest that molecular methods may be useful tools to identify emerging protozoa in patients with AIDS-associated cholangiopathy.

Key words. *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Enterocytozoon bienewisi*, chronic diarrhoea, sclerosing cholangitis.

Identificación molecular de protozoarios que causan colangiopatía asociada al SIDA en Buenos Aires, Argentina

Resumen

Introducción. Varias especies de microsporidios y coccidios son parásitos protozoarios responsables de colangiopatías en pacientes infectados con el virus de la inmunodeficiencia humana (VIH). Los objetivos de este

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trabajo fueron la identificación de protozoarios oportunistas por métodos moleculares y la descripción de las manifestaciones clínicas en el aparato digestivo, hígado y vías biliares de pacientes con colangiopatía asociada al SIDA en Buenos Aires, Argentina. **Material y métodos.** El estudio incluyó 11 pacientes adultos VIH positivos, con diagnóstico de colangiopatía asociada al SIDA. A cada paciente se le efectuó una endoscopia digestiva alta con toma de biopsias y un análisis coproparasitológico. Se realizaron ecografías abdominales para detectar compromiso de vías biliares. También se efectuaron colangiopancreatografía retrógrada endoscópica y/o colangiorresonancia. La identificación de los microorganismos a nivel de especie se realizó en muestras de biopsias por métodos moleculares. **Resultados.** Se identificaron microorganismos en 10 casos. Los diagnósticos en pacientes con colangitis esclerosante fueron criptosporidiosis en 3 casos, cistosisporosis en 1 y microsporidiosis en 1. En los pacientes con colangitis esclerosante y estenosis papilar los diagnósticos fueron microsporidiosis en 2 casos, criptosporidiosis en 2 y criptosporidiosis asociada a microsporidiosis en 1. En 3 casos con criptosporidiosis la especie identificada fue *Cryptosporidium hominis*. Un caso estaba asociado con *Enterocytozoon bienewisi* y los otros 2 presentaban coinfección con *Cryptosporidium parvum*. Los 4 casos de microsporidiosis fueron debidos a *Enterocytozoon bienewisi*. **Conclusiones.** Estos resultados sugieren que los métodos moleculares pueden ser herramientas útiles en la identificación de protozoarios en pacientes con colangiopatía asociada al SIDA.

Palabras claves. *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Enterocytozoon bienewisi*, diarrea crónica, colangitis esclerosante.

Abnormalities at the digestive tract level have been recognized as a complication in patients infected with the human immunodeficiency virus (HIV). They include the clinical features of chronic diarrhoea, alitiasic cholecistitis and AIDS-associated cholangiopathy.¹ The cause of AIDS-associated cholangiopathy is related to opportunistic infections produced by *Cryptosporidium* sp, *Enterocytozoon bienewisi*, *Encephalitozoon intestinalis*, cytomegalovirus, mycobacteria and HIV.^{2,3,4}

The genus *Cryptosporidium* is a group of coccidian protozoan parasites that infect a variety of mammals, birds, reptiles, and fish.² Only molecular

methods can be used to accurately differentiate *Cryptosporidium* species and genotypes as their morphologies are indistinguishable. Actually, eight species (*Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium suis*, *Cryptosporidium muris* and *Cryptosporidium andersoni*) and six genotypes (monkey, cervid, chipmunk genotype I, horse, skunk and rabbit) are associated with human disease.^{5,6,7} The most frequent *Cryptosporidium* species that infect humans are *Cryptosporidium parvum* and *Cryptosporidium hominis*.⁵

Microsporidia are intracellular parasites that infect invertebrate and vertebrate hosts. There are more than 1,000 species among which 12 have been reported to infect humans. *Enterocytozoon bienewisi* is recognized as the most prevalent in HIV-infected patients and has also been reported in cats, dogs, chickens, goats, pigs, cattle and others.⁸ The use of molecular methods improves the sensitivity and species-specificity for the definitive diagnosis of *Enterocytozoon bienewisi*.⁹

The goals of this work were to identify opportunistic protozoa by molecular methods and describe the clinical manifestations at the gastrointestinal tract and the biliary system in patients with AIDS-associated cholangiopathy from Buenos Aires, Argentina.

Material and methods

Study population

A prospective study was carried out from March 1997 to March 2011. Molecular biology studies were performed retrospectively. The study included 11 HIV-positive patients, 7 men and 4 women with ages within 23 and 59 years old, with AIDS-associated cholangiopathy, who were selected on the basis of clinical manifestation, serum levels of alkaline phosphatase and ultrasonography abnormalities. Serum alkaline phosphatase levels of at least twice the upper normal value were considered as elevated. Asymptomatic cases were not studied. All patients had CD4 lymphocyte counts lower than 150 cells/mm³ (range 26-126 cells/mm³). Cases 1 to 7 were included in the period 1997-1999 and cases 8 to 11 since in the period 2001-2011.

Each patient was evaluated with a clinical history in order to describe the symptoms and signs of chro-

nic diarrhoea, right upper abdominal quadrant pain, jaundice, nausea and vomiting. Chronic diarrhoea was considered when its duration was more than one month. Routine laboratory analyses were performed, including blood cell count, liver function tests and amylase serum level. CD4 cell count or serum viral load were determined.

The research protocol was approved by the institutional ethical review boards and patients gave their informed consent for this work.

Imaging studies

An abdominal ultrasonography was performed in each patient. Extrahepatic bile duct dilation was considered when its diameter exceeded 4 or 5 mm. Presence or absence of wall thickening was also informed. Wirsung duct dilation was considered when its diameter exceeded 2 mm.¹⁰ In cases with bile duct involvement, an endoscopic retrograde cholangiopancreatography (ERCP) and/or a magnetic resonance cholangiography (MRC) of liver and bile ducts were carried out. Morphological alterations found in the ERCP were interpreted following criteria described by Cello.² This author grouped the observed lesions by endoscopic examination in four types: papillary stenosis, sclerosing cholangitis, association of the former ones and long common bile duct stenosis.

Patients with chronic diarrhoea were studied by upper gastrointestinal endoscopy (UGE) with a *Pentax EPM 2000* equipment. Visualization of duodenal abnormalities by UGE was classified according to Maratka criteria as granular,.jasper or atrophic duodenum.¹¹

Microbiological analyses

Stool specimens were processed for bacteria and parasite analysis. Parasite diagnosis was carried out by serial stool analysis with examination of humid concentrates and slides stained by Kinyoun, Weber trichrome and Gram-chromotrope techniques.^{6,12,13} Bilis samples were not studied. Biopsy specimens from duodenum, peripapillary duodenum and papilla were fixed in formaldehyde, paraffin embedded, and stained with Giemsa and haematoxylin-eosin.^{6,12,13} Other biopsy specimens were placed in Karnovsky fixative, embedded in polybedaralidite and stained with Azur II.¹³ In six cases, biopsy samples were stored in saline solution at -20° C.

Molecular characterization of *Cryptosporidium* spp

The protocol was carried out in cases with etiological diagnosis of *Cryptosporidium* and availability of freeze biopsy samples stored in saline solution. DNA purification was carried out by trypsinization, proteolytic lysis, phenol-chloroform extraction and ethanol precipitation.¹⁴ Nested PCR was carried out essentially as described by Coupe et al,¹⁵ amplifying a fragment of the 18S ribosomal RNA gene from *Cryptosporidium* sp. First round primers were SCL1 (5'-CTGGTTGATCCTGCCAGTAG-3') and CPB-DIAGR (5'-TAAGGTGCTGAAGGAG-TAAGG-3'), corresponding to nucleotides 4-23 and 1,016-1,036. Second round primers were SCL2 (5'-CAGTTATAGTTTACTTGATAATC-3') and SCR2 (5'-CAATACCCTACCGTCTAAAG-3'), corresponding to nucleotides 106-128 and 299-318 of the same gene. Reaction mixture was modified by employing 10X buffer containing (NH₄)₂SO₄ and by the addition of 400 ng/μL of bovine serum albumin. Amplification products from each round were analysed by electrophoresis in agarose gels stained with ethidium bromide and visualised by UV light. The expected amplicons were of 1,032 base pairs (bp) for the first round and 214 bp for the second one. Identification at the species level was carried out by restriction fragment length polymorphism (RFLP) analysis according to Coupe et al,¹⁵ with the following restriction enzymes in a sequential order: *Taq* I, *Vsp* I, *Tru* I, *Bsh*1236 I, *Ssp* I and *Smi* I. Digestion products were analyzed by 2% agarose gel electrophoresis, stained with ethidium bromide and UV visualised. Species were determined according to the restriction patterns.

Molecular identification of microsporidia

DNA purification protocol from freeze samples was performed according to our previous report for cases with etiological diagnosis of microsporidia.¹⁶ PCR amplification for *Enterocytozoon bieneusi* was carried out essentially as described previously,¹⁶ employing the forward primer Eb.gc (5'-TCAGTTTTGGGTGTGGTATCGG-3') and the reverse primer Eb.gt (5'-GCTACCCA-TACACACATCATTTC-3'), corresponding respectively to nucleotides 1-22 and 189-210 of the intergenic transcribed spacer of the rRNA genes of *Enterocytozoon bieneusi* (GenBank Accession number L20290). In order to determine the presence

of *Encephalitozoon intestinalis*, the PCR protocol was carried out essentially as previously described.¹⁷ The primers employed were SINTF1 (5'-TTTC-GAGTGTAAGGAGTCGA-3') and SINTR (5'-CCGTCCTCGTTCTCCTGCCCG-3'), corresponding to nucleotides 362-382 and 861-881 respectively of the small subunit rRNA gene of *Encephalitozoon intestinalis* (GenBank Accession number U09929). The reaction mixture was modified by employing 10X buffer containing $(\text{NH}_4)_2\text{SO}_4$, and by the addition of 400 ng/ μl of bovine serum albumin. Agarose gel electrophoresis and ethidium bromide staining were used to visualise amplification product under UV transillumination.

Results

Clinical manifestations and imaging studies

The 11 cases had criteria of chronic diarrhoea and presented right upper abdominal quadrant pain, vomiting, and elevated alkaline phosphatase levels (twice to ten folds the normal value).

Cases 1 to 7 did not receive highly active antiretroviral therapy. Cases 8, 9 and 11 were treated with zidovudine, lamivudine and efavirenz. Case 10 received lopinavir, ritonavir, amprenavir and efavirenz.

Abdominal ultrasonography showed abnormalities of the biliary tract in all cases: thickening and dilation of common bile duct walls in 6, dilation in 4 and only thickening in 1. The UGE was carried out in all patients and mucosa abnormalities were observed in 9: granular duodenum in 5 and.jasper duodenum in 4. Two cases had no abnormalities.

ERCP and/or MRC were performed in all patients (Figure 1). The diagnoses were sclerosing cholangitis and papillary stenosis in 5 cases, and abnormalities compatible with sclerosing cholangitis in 6.

Microbiological findings

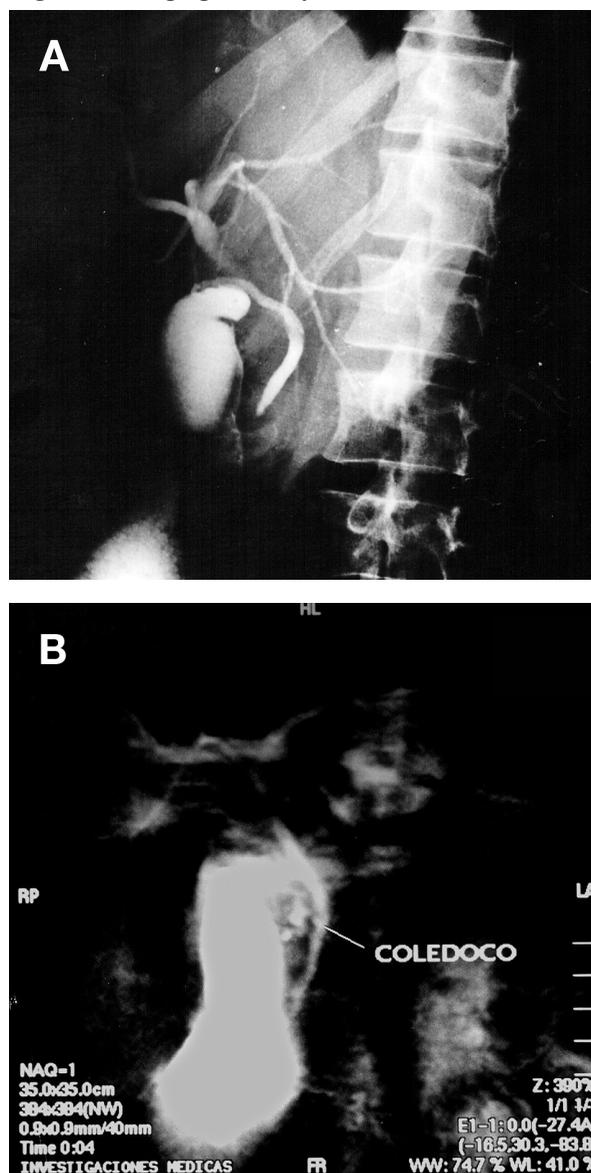
A microorganism was identified in 10 cases. The diagnosis in patients with sclerosing cholangitis was cryptosporidiosis in 3 cases, cystoisosporosis in 1 and microsporidiosis in 1. In patients with sclerosing cholangitis and papillary stenosis the diagnosis was microsporidiosis in 2 cases, cryptosporidiosis in 2 and cryptosporidiosis associated with microsporidiosis in 1 (Figure 2).

Molecular analysis

Cases 1, 9 and 11, with diagnosis of cryptosporidiosis, were studied for molecular characterization. The table 1 shows the results obtained by nested PCR and RFLP. The nested PCR allowed the identification of a 214 bp amplicon in all samples. The RFLP technique showed a restriction pattern compatible with *Cryptosporidium hominis* in case 1 and coinfection of *Cryptosporidium hominis* and *Cryptosporidium parvum* in cases 9 and 11 (Figure 3).

The four cases with microsporidia detected by light microscopy were confirmed as *Enterocytozoon bieneusi* by PCR (Figure 4).

Figure 1. Imaging studies of case 1.



A. ERCP with stenosis and dilation of common bile duct.
B. MRC with stenosis and dilation of common bile duct.

Figure 2. Duodenal biopsy specimen of case 1 stained with Azur II, showing coinfection of *Cryptosporidium* sp and microsporidia (Magnification x 1,000).

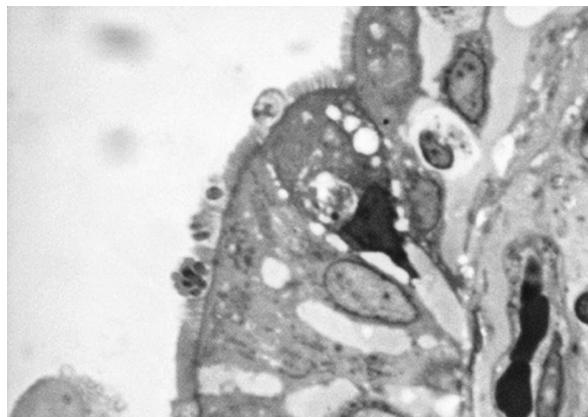
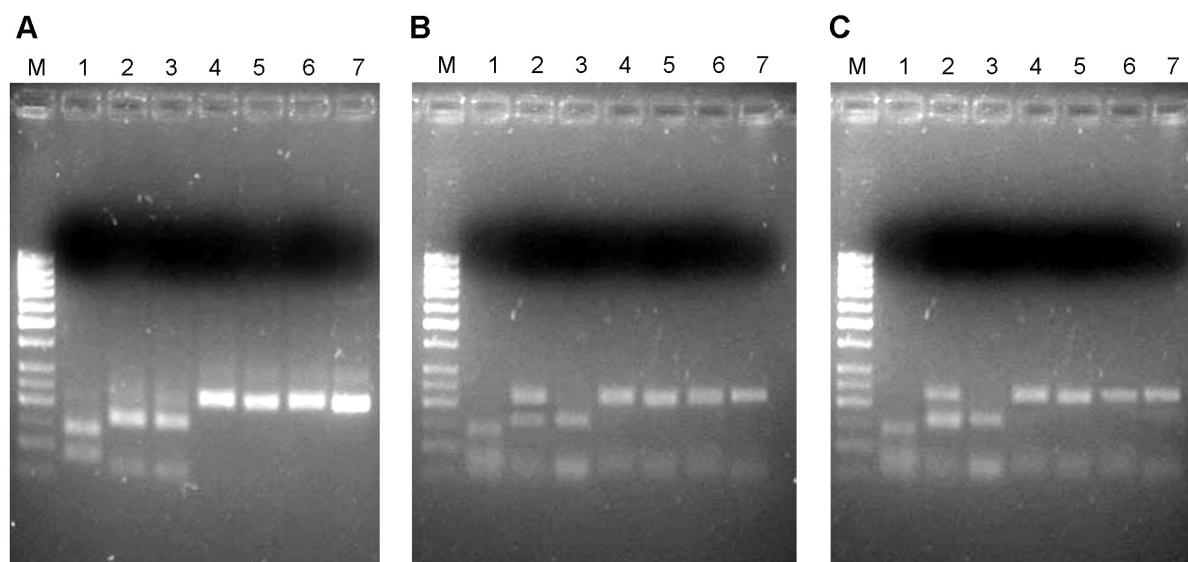


Table 1. Clinical characteristic and diagnosis in the studied group.

Case	Sex/age	Symptoms	CD4	US	ERC/MRC	Histology	Faeces	Molecular identification
1	M/37	RUQ pain, chronic diarrhoea	68	Bile ducts thickening and dilation	SC-PE	<i>Cryptosporidium</i> Microsporidia	<i>Cryptosporidium</i>	<i>C. hominis</i> <i>E. bienewisi</i>
2	F/21	RUQ pain, chronic diarrhoea	7	Bile ducts dilation	SC-PE	Microsporidia	Negative	<i>E. bienewisi</i>
3	M/25	RUQ pain, chronic diarrhoea	48	Bile ducts thickening	SC-PE	Microsporidia	Negative	<i>E. bienewisi</i>
4	F/26	RUQ pain, chronic diarrhoea	25	Bile ducts dilation	SC	Negative	Negative	ND
5	M/24	RUQ pain, chronic diarrhoea	100	Bile ducts thickening and dilation	SC	<i>Cryptosporidium</i>	Negative	ND
6	M/26	RUQ pain, chronic diarrhoea	50	Bile ducts thickening and dilation	SC-PE	<i>Cryptosporidium</i>	<i>Cryptosporidium</i>	ND
7	M/23	RUQ pain, chronic diarrhoea	100	Bile ducts thickening and dilation	SC	<i>Cryptosporidium</i>	<i>Cryptosporidium</i>	ND
8	M/27	RUQ pain, chronic diarrhoea	79	Bile ducts thickening and dilation	SC	<i>Cystoisospora belli</i>	Negative	ND
9	F/33	RUQ pain, chronic diarrhoea	126	Bile ducts dilation	SC	<i>Cryptosporidium</i>	<i>Cryptosporidium</i>	<i>C. hominis</i> <i>C. parvum</i>
10	F/49	RUQ pain, chronic diarrhoea	26	Bile ducts dilation	SC	Microsporidia	Spores	<i>E. bienewisi</i>
11	M/44	RUQ pain, chronic diarrhoea	50	Bile ducts thickening and dilation	SC-PE	<i>Cryptosporidium</i>	<i>Cryptosporidium</i>	<i>C. hominis</i> <i>C. parvum</i>

M: male; F: female; RUQ: right upper quadrant; Alk phos: serum alkaline phosphatase; US: ultrasonography; ERC: endoscopic retrograde cholangiography; MRC: magnetic resonance cholangiography; PE: papillary stenosis; SC: sclerosing cholangitis; ND: not done; *C. Cryptosporidium*; *E. Enterocytozoon*.

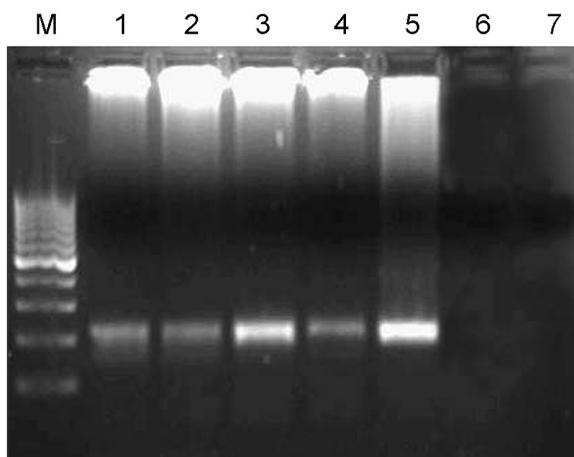
Figure 3. Agarose gel electrophoresis of digestion products from amplicons.

A. Patient 1, showing restriction pattern compatible with *Cryptosporidium hominis*.

B. Patient 9, showing restriction pattern compatible with coinfection of *Cryptosporidium hominis* and *Cryptosporidium parvum*.

C. Patient 11, showing restriction pattern compatible with coinfection of *Cryptosporidium hominis* and *Cryptosporidium parvum*.

Lanes M, 50 bp ladder; lanes 1, digestion with *Taq* I; lanes 2, digestion with *Vsp* I; lanes 3, digestion with *Tru* I; lanes 4, digestion with *Bsh*1236 I; lanes 5: digestion with *Ssp* I; lanes 6: digestion with *Smi* I; lanes 7: amplicon without digestion.

Figure 4. Agarose gel electrophoresis of amplification products after PCR for *Enterocytozoon bieneusi*.

Lane M, 100 bp ladder; lanes 1 to 4, patients 1, 2, 3 and 10 respectively; lane 5, positive control; lane 6, negative control; lane 7, reaction mixture.

Clinical manifestations according to protozoan species

The table 1 describes the species and the more significant characteristics of the group studied.

Discussion

We studied eleven HIV-infected patients who presented chronic diarrhoea and AIDS-associated cholangiopathy, and determined the infecting species. The protozoan species that we found were *Cryptosporidium hominis*, *Cryptosporidium parvum*, *Enterocytozoon bieneusi* and *Cystoisospora belli*.

There are numerous reports of identification of *Cryptosporidium* species in HIV-infected patients with diarrhoea in which *Cryptosporidium hominis* and *Cryptosporidium parvum* are the most frequently found in accordance with the results obtained in our study.^{5,18,19} Other species, such as *Cryptosporidium meleagridis*, *Cryptosporidium felis* and *Cryptosporidium canis*, have also been found in HIV-infected patients but less frequently and none of them were present in our studied group.^{5,18,19}

Until now there are only two reports describing HIV-positive patients with diarrhoea and cholangiopathy in which the species of *Cryptosporidium* were then identified.^{14,20,21}

Cryptosporidiosis in AIDS patients has different clinical manifestations depending on the species causing the infection.²² *Cryptosporidium hominis* is usually associated to asymptomatic forms in patients

with low CD4 lymphocyte counts and it causes diarrhoea.²³ In our study we did not analyse asymptomatic patients. In other report, the compromise of the biliary tract caused by *Cryptosporidium hominis* in patients with AIDS was characterized by a late appearance in the course of the disease, CD4 cell count with values equal or lower than 50 per mm³ and chronic diarrhoea at some point.²⁰ In this report, one case with *Cryptosporidium hominis* infection presented chronic diarrhoea, sclerosing cholangitis and papillary stenosis, but this case was associated with *Enterocytozoon bienewisi* infection.

Cryptosporidium parvum is usually accompanied by diarrhoea and vomiting as described by Cama et al,²² but they did not report *Cryptosporidium parvum* in patients with AIDS-associated cholangiopathy. In our study, two cases with chronic diarrhoea and sclerosing cholangitis presented infection with both species, *Cryptosporidium hominis* and *Cryptosporidium parvum*.

Here, we identified *Cryptosporidium* species from duodenal biopsy samples by the nested PCR-RFLP technique.¹⁵ Currently, there are also molecular methods to characterize species into different populations. *Cryptosporidium hominis* can be categorized in different subtype families by analysis of the GP60 glycoprotein gene sequence and each subtype family of GP60 has multiple subtypes.^{24,25} *Cryptosporidium parvum* can also be categorized in subtype families.^{24,25} The importance of establishing the subtype families for each species is based on the different clinical behaviours characteristic of each species.³ In our study we did not identify the subtype families for the species found but it should be relevant to recognize clinical manifestations in this group of patients.

Differentiation of *Microsporidia* species is helpful in instituting therapy, because dissemination risks and treatment response are not identical for *Enterocytozoon intestinalis* and *Enterocytozoon bienewisi*.²⁷ The microsporidia species most commonly associated with disease in AIDS patients is *Enterocytozoon bienewisi*. The most common clinical presentations of microsporidiosis in AIDS patients are diarrhoea and cholangiopathy.²⁶ In AIDS-associated cholangiopathy and *Enterocytozoon bienewisi* infections, CD4 cell count has values equal or lower than 50 per mm³.²⁷ The studied cases had similar low values of CD4 cells. Molecular methods have shown considerable genetic variation among isolates of *Enterocytozoon bienewisi* by analysis of the ribosomal DNA

internal transcribed spacer (ITS) sequence and more than 70 genotypes have been identified.²⁸ Some of these genotypes have been found to infect humans and animals supporting the likelihood of zoonotic transmission.²⁸ In our study we used a PCR assay based in the amplification of a DNA fragment of the unique rRNA intergenic spacer, but we did not identify the genotypes of *Enterocytozoon bienewisi* present in our positive patients.¹⁶

These results suggest that molecular methods may be useful tools to identify protozoa at the species and genotype levels in patients with AIDS-associated cholangiopathy.

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