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First report of *Cystoisospora belli* parasitemia in a patient with acquired immunodeficiency syndrome

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Abstract

Cystoisospora belli in patients with the acquired immunodeficiency syndrome (AIDS) has been described as cause of chronic diarrhea and disseminated cystoisosporosis. Diagnosis of intestinal cystoisosporosis can be achieved at the tissue level in the villus epithelium of the small bowel. Disseminated cystoisosporosis is diagnosed by microscopy identification of unizoite tissue cysts in the lamina propria of the intestine. We report a case of disseminated cystoisosporosis in a human immunodeficiency virus (HIV)-infected patient with detection of parasitemia. We studied a 39-year old patient with AIDS and chronic diarrhea by analysis of stool and duodenal biopsy samples. Blood samples were also collected and examined by light microscopy and molecular techniques for *C. belli* DNA detection. The unizoite tissue cyst stages were present in the lamina propria, with unsporulated oocysts in feces. Zoites were present in blood smears and DNA of *C. belli* was detected in blood samples. Our study identified a new stage in the life cycle of *C. belli*. Detection of parasitemia is a novel and noninvasive tool for diagnosis of disseminated cystoisosporosis.

Keywords

Cystoisospora belli, parasitemia, AIDS, unizoite tissue cyst, PCR

Introduction

Human cystoisosporosis is caused by the species *Cystoisospora belli* (Lindsay *et al.* 1997). *Cystoisospora belli* is an intracellular protozoan parasite classified in the family Sarcocystidae belonging to the phylum Apicomplexa (Barta *et al.* 2005).

In immunocompetent individuals, infection may be asymptomatic or with a moderate to severe illness characterized by fever, vomiting, diarrhea and abdominal pain. In patients with the acquired immunodeficiency syndrome (AIDS) it has been described as an opportunistic agent that can cause chronic diarrhea, acalculous cholecystitis and cholangiopathy (Velásquez and Carnevale 2015). Reports of disseminated cystoisosporosis with unizoite tissue cysts in the lamina propria of the intestines, lymph nodes, liver and spleen in patients with AIDS have been published (Restrepo *et al.* 1987; Michiels *et al.* 1994; Comin and Santucci 1994; Velásquez *et al.* 2001; Frenkel *et al.* 2003).

The first report described a 38-year old male with AIDS and four episodes of vomiting, diarrhea and weight loss in six month. *C. belli* was identified in feces in the third episode. Finally he died and the autopsy allowed the identification of unizoite tissue cysts in lamina propria of the small intestine, mesenteric and mediastinal lymph nodes (Restrepo *et al.* 1987).

The second case was a 30-year old black female AIDS patient with eight episodes of fever, diarrhea and weight loss during three years. *C. belli* oocysts were identified by parasitological stool examination and unizoite tissue cysts in the lamina propria of the duodenal biopsy samples. After death, the autopsy revealed unizoite tissue cysts in mesenteric and mediastinal lymph nodes, liver and spleen (Michiels *et al.* 1994).

Another case was a 30-year old female AIDS patient with chronic diarrhea who had sexual and asexual stages in epithelium and unizoites in the lamina propria of small intestine (Comin and Santucci 1994).

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In other report we described the histologic presence of unizoite tissue cysts in the lamina propria with negative results in feces of two patients with chronic diarrhea and AIDS (Velásquez *et al.* 2001).

The sixth case was a 26-year old male with AIDS with five years history of *C. belli* diagnosis. In last year he had three episodes of vomiting and diarrhea. *C. belli* oocysts were identified in stool samples. The patient died in a forth similar clinical picture. The autopsy revealed unizoite tissue cysts in mesenteric lymph nodes (Frenkel *et al.* 2003).

To our knowledge, disseminated cystoisosporosis is a disease that is diagnosed when unizoite tissue cysts are identified in histological samples (Velásquez and Carnevale 2015) but there are not reports which demonstrate zoite of *C. belli* in blood of patients with AIDS.

This report describes the clinical manifestations, histological and blood findings of different stages during *C. belli* infection in a patient with AIDS that had features of disseminated cystoisosporosis.

Materials and Methods

Case History

A 39-year-old homosexual man known to be human immunodeficiency virus (HIV)-infected was admitted to the hospital with nausea, vomiting, and diarrhea for five days. The diarrhea was watery in consistency without blood or mucus. He had 7–10 episodes per day. He had been diagnosed of HIV infection one year before. The patient was being treated for HIV infection with abacavir, lamivudine and efavirenz and he continued until now. He denied fever, history of foreign travel or drinking contaminated water.

On physical examination, the patient was with generalized muscle wasting. Laboratory evaluation revealed anemia with hemoglobin level of 11.3 g/dl. Leukocyte count was 7,300/mm^3 and eosinophilia was 4%. Liver function tests were normal. The CD4 lymphocyte count was 50 cells/mm^3. Hepatitis A, B and C serologies were negative. Stool examination showed *C. belli* oocysts. After diagnosis of cystoisosporosis was confirmed, blood samples were obtained. The patient was treated with trimethoprim-sulfamethoxazole and showed improvement in two days. He had two formed stools daily without vomiting.

An upper endoscopy with small intestinal biopsy sampling was performed. He showed marked improvement in clinical and laboratory finding and was discharged from the hospital 15 days after admission.

Stool samples

Stool specimens were collected daily for three days in 5% formalin saline solution. Feces were concentrated by ethyl ether centrifugation and examined by light microscopy. The modified acid-fast stain technique was also applied to smears of the

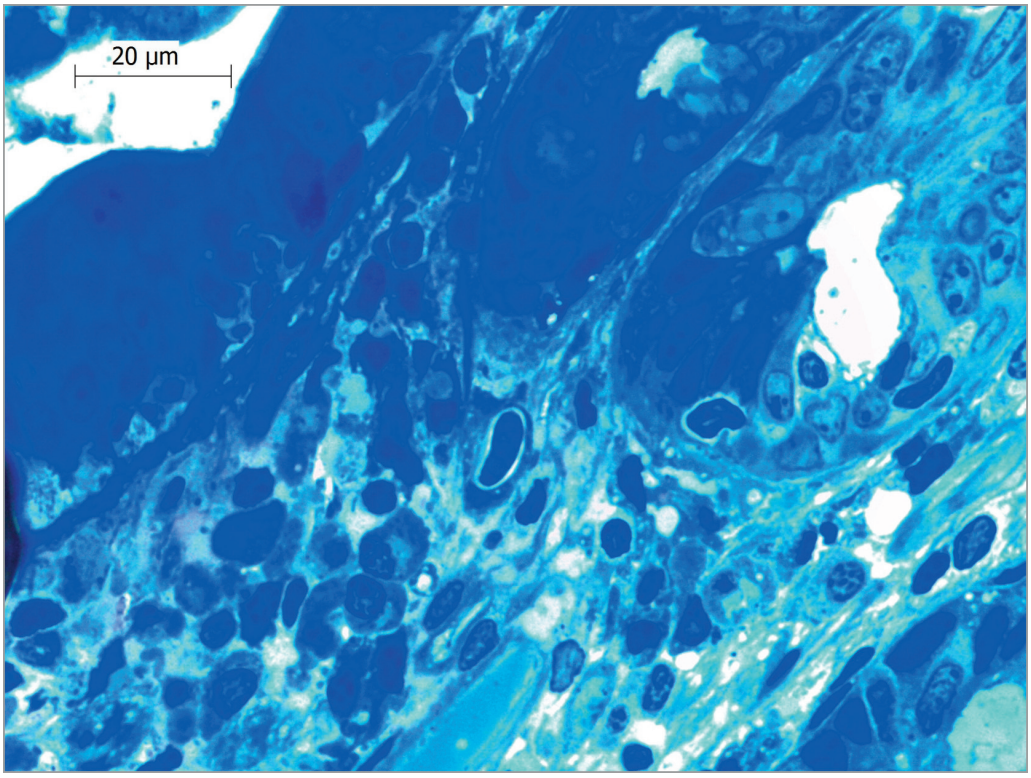

Fig. 1. Unizoite tissue cyst in the lamina propria of the small intestine. Magnification X 1,000

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concentrated samples. Routine stool bacterial cultures were also performed (Velásquez *et al.* 2001).

Duodenal tissues

Five biopsy specimens were obtained from the distal duodenum by flexible fiberglass endoscopy performed with Pentax EPM 2000 equipment. Two tissue samples were fixed in 10% formalin, embedded in paraffin and stained with hematoxylin-eosin and Giemsa. Three specimens were treated with Karnovsky fixative, embedded in polybedaraldite and stained with Azure II (Velásquez *et al.* 2001).

Blood samples

Blood samples were obtained by venipuncture and processed for two purposes.

Thin blood smears were prepared and stained with Giemsa (Shute 1988). They were observed by bright and phase contrast microscopy to investigate the presence of zoites.

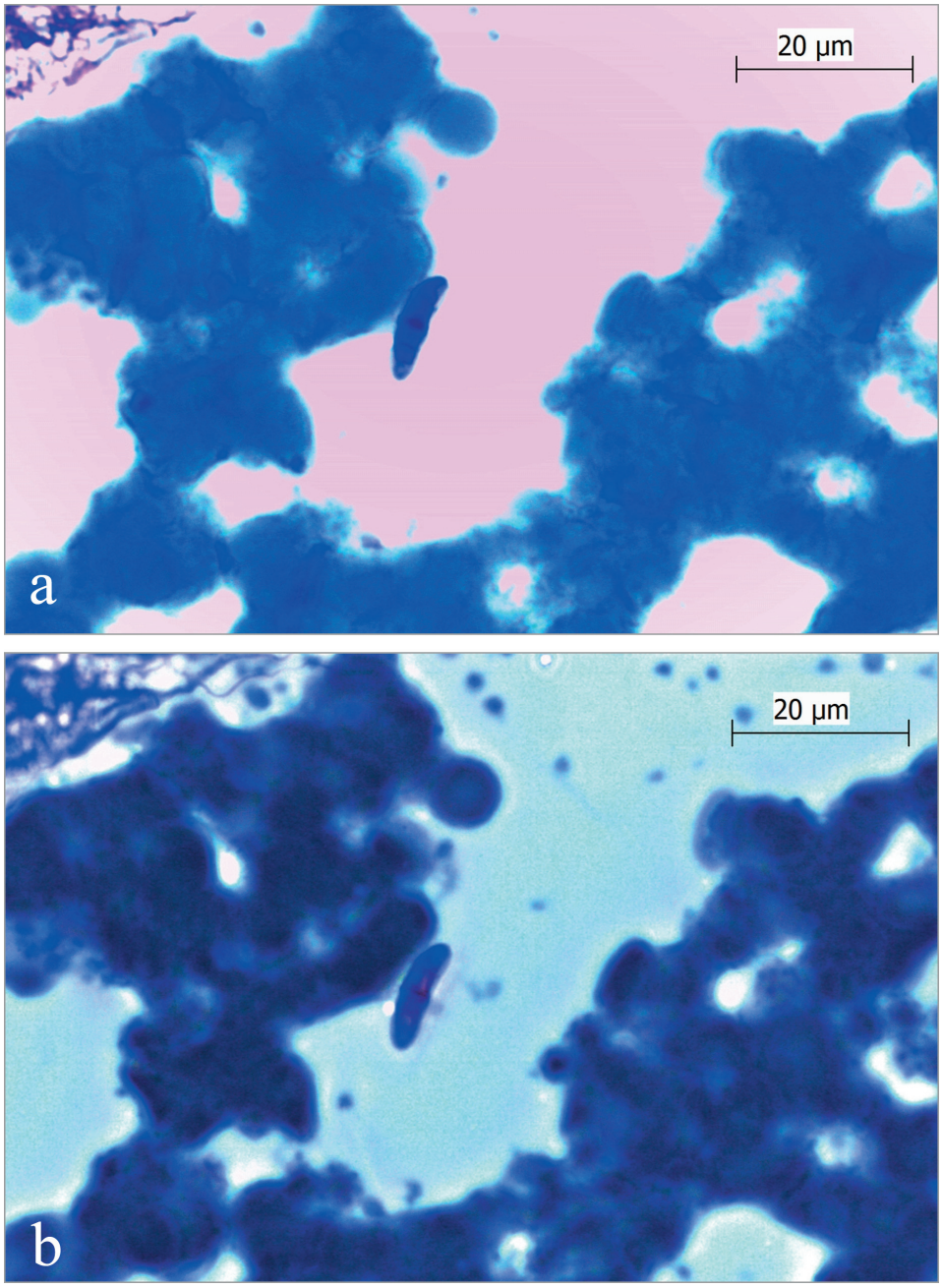

Fig. 2. Zoite of *C. belli* in a blood smear observed by bright field **(a)** and phase contrast **(b)** microscopy. Giemsa stain. Magnification X 1,000

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Whole blood samples were treated with EDTA (1.25 mg/mL of blood) and stored at 4°C until processing for a period no longer than 24 hours. Aliquots of 200 µL were prepared and used for DNA extraction using the QIAamp DNA Blood Mini Kit (QIAGEN, USA) according to the manufacturer´s instructions with the spin protocol. DNA was finally eluted in 200 µL of elution buffer (provided with the kit) and employed for molecular assays.

Molecular analysis

Blood samples were used in order to confirm the presence of *C. belli*. PCR templates corresponded to 2 µL of the purified DNA. Two nested PCR assays were employed. One of them was used to amplify a fragment of the 18S ribosomal RNA gene from *Cystoisospora* sp. and the other for the internal transcribed spacer 1 (ITS-1) of the rRNA genes accordingly to our previous report (Velásquez *et al.* 2011). Twenty microliters of amplicons from the second round were run on ethidium bromide-stained 2% agarose gels and visualized under UV illumination.

To confirm the sequence of the amplification fragment of the ITS-1 rDNA locus, the amplicon was sequenced in both directions by a commercial sequencing service (Macrogen, Korea). The nucleotide sequence obtained was analyzed using the Blast program of the National Center for Biotechnology Information, and aligned with reference sequences retrieved

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R4809          -GATCATTCACAGGTGGCCCTTGAAGCATCTCTAAAACCTTTAGCAACTGAATCCCCATA 59
HM63052.1      -GATCATTCACACGTGGCCCTTGAAGCATCTCTAAAACCTTTAGCAACTGAATCCCCATA 59
DQ060683.2     -GATCATTCACACGTGGCCCTTGAAGCATCTCTAAAACCTTTAGCAACTGAATCCCCATA 59
EU124687.1     ----------------------AAGCATCTCTAAAACCTTTAGCAACTGAATCCCCATA 37
KF686747.1     TGATCATTCGCACGTGGCCCTCGAAGCATCTCTAAAACCTTTAGCAACTGAATCCCCATA 60
                              ************************************

R4809          TTTACGGTCAAA-GGGAACAACCTGCTGAGCAGCAGGGGGAAGCTGTATTTCCCCTTGCC 118
HM63052.1      TTTACGGTCAAA-GGGAACAACCTGCTGAGCAGCAGGGGGAAGCTGTATTTCCCCTTGCC 118
DQ060683.2     TTTACGGTCAAA-GGGAACAACCTGCTGAGCAGCAGGGGGAAGCTGTATTTCCCCTTGCC 118
EU124687.1     TTTACGGTCAAA-GGGAACAACCTGCTGAGCAGCAGGGGGAAGCTGTATTTCCCCTTGCC 96
KF686747.1     TTTACGGTCAAAAGGGAACAACCTGCTGAGCAGCAGGGGGAAGCTGTATTTCCCCTTGCC 120
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R4809          CCAGCTGTTTCCAATGGCCGCTTTCAAGGGCATTAGTCGTTGTCGCTGGTGTATTCTCCT 178
HM63052.1      CCAGCTGTTTCCAATGGCCGCTTTCAAGGGCATTAGTCGTTGTCGCTGGTGTATTCTCCT 178
DQ060683.2     CCAGCTGTTTCCAATGGCCGCTTTCAAGGGCATTAGTCGTTGTCGCTGGTGTATTCTCCT 178
EU124687.1     CCAGCTGTTTCCAATGGCCGCTTTCAAGGGCATTAGTCGTTGTCGCTGGTGTATTCTC-T 155
KF686747.1     CCAGCTGTTTCCAATGGCCGCTTTCAAGGGCATTAGTCGTTGTCGCTGGTGTATTCTCCT 180
               ************************************************************ *

R4809          ACAGGGGACGGTGTAATTCCTCGCGACCTACTCAGAGGGTTCAAACAGAGTACAGGTTGC 238
HM63052.1      ACAGGGGACGGTGTAATTCCTCGCGACCTACTCAGAGGGTTCAAACAGAGTACAGGTTGC 238
DQ060683.2     ACAGGGGACGGTGTAATTCCTCGCGACCTACTCAGAGGGTTCAAACAGAGTACAGGTTGC 238
EU124687.1     ACAGGGGACGGTGTAATTCCTCGCGACCTACTCAGAGGGTTCAAACAGAGTACAGGTTGC 215
KF686747.1     ACAGGGGACGGTGTAATTCCTCGCGACCTACTCAGAGGGTTCAAACAGAGTACAGGTTGC 240
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R4809          TCGCTACTCTTTTCTTGCCCTTTGAACTATGGACGGTGAGAAAGCGGTTGTCCATGTGCG 298
HM63052.1      TCTCTACTCTTTTCTTGCCCTTTGAACTATGGACGGTGAGGAAGCGGTTGTCCATGTGCA 298
DQ060683.2     TCTCTACTCTTTTCTTGCCCTTTGAACTATGGACGGTGAGGAAGCGGTTGTCCATGTGCA 298
EU124687.1     TCTCTACTCTTTTCTTGCCCTTTGAACTATGGACGGTGAGGAAGCGGTTGTCCATGTGCA 275
KF686747.1     TCTCTACTCTTTTCTTGCCCTTTGAACTATGGACGGTGAGGAAGCGGTTGTCCATGTGCA 300
               ** ************************************ ******************

R4809          GGGGAGTCCGCACTGCAGCGATAGCTTCAA-GTGCTCTGCTACACACATTCATTCTCTCC 357
HM63052.1      GGGGAGTCCGCACTGCAGCGATAGCTTCAAAGTGCTCTGCTACACACATTCATTCTCTCC 358
DQ060683.2     GGGGAGTCCGCACTGCAGCGATAGCTTCAA-GTGCTCTGCTACACACATTCATTCTCTCC 357
EU124687.1     GGGGAGTCCGCACTGCAGCGATAGCTTCAAAGTGCTCTGCTACACACATTCATTCTCTCC 335
KF686747.1     GGGGAGTCCGCACTGCAGCGATAGCTTCAAAGTGCTCTGCTACACACATTCATTCTCTCC 360
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R4809          CACTTGGTGGAAGGGGCAGTCTGTTTCCATTGGGGTCGTCACTTGCCCATAAGAGATGTG 417
HM63052.1      CACTTGGTGGAAGGGGCAGTCTGTTTCCATTGGGGTCGTCACTTGCCCATAAGAGATGTG 418
DQ060683.2     CACTTGGTGGAAGGGGCAGTCTGTTTCCATTGGGGTCGTCACTTGCCCATAAGAGATGTG 417
EU124687.1     CACTTGGTGGAAGGGGCAGTCTGTTTCCATTGGGGTCGTCACTTGCCCATAAGAGATGT- 394
KF686747.1     CACTTGGTGGAAGGGGCAGTCTGTTTCCATTGGGGTCGTCACTTGCCCATAAAAGATGTG 420
               ************************************************** ******

R4809          CTCTGTGGATTTGGACGTCGTC 439
HM63052.1      CTCTGTGGATTTGGACGTCGTC 440
DQ060683.2     CTCTATGGATTTGGACGTCGTC 439
EU124687.1     ----------------------
KF686747.1     ----------------------
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Fig. 3. Alignment of the sequence from the amplicon generated by the ITS-1 rDNA nested PCR (named R4809) with *C. belli* ITS-1 sequences available at the GenBank. Asterisks and dashes represent identical residues and deletions, respectively

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from the GenBank with the ClustalW2 multiple sequence alignment program.

Results

Stool findings

Oocysts were identified by parasitological stool examination. The oocyst morphologies were ellipsoidal and unsporulated. A little amount of sporulated oocysts containing two sporocysts were detected. Stool bacterial cultures were negative.

Histological findings

Macrogametes, microgamete and meronts were not observed in the duodenal villus epithelium on biopsy specimens.

Unizoite tissue cysts were observed in the lamina propria of specimens stained with Azure II. The tissue cyst wall was light blue. Unizoites were located in the center of the tissue cyst, surrounded by a clear vacuole. The unizoites were uninucleated (Fig. 1).

Mucosal architecture was abnormal in all biopsy specimens. Villi were reduced in height, and crypts were hypertrophied. The cellularity of the lamina propria was increased in all duodenal biopsies by a mixed infiltrate of neutrophils, plasma cells, lymphocytes and eosinophils.

Blood findings

Crescent-shaped structures that measured about 12–13 by 3–4 μm were detected in thin blood smears stained with Giemsa. These zoites had a light blue cytoplasm with the presence of a central band of azurophilic nuclear chromatin (Fig. 2).

Molecular analysis

Nested PCR amplification of a fragment of the 18S rRNA gene produced an amplicon of the expected size corresponding to 396 bp (data not shown).

The DNA fragment generated from the nested PCR for the ITS-1 contained 439 bp. The sequence of the amplicon differed from the isolates reported by Jongwutiwes *et al.* (2007), and Samarasinghe *et al.* (2008), Velásquez *et al.* (2011) and Shafiei *et al.* (unpublished), with the GenBank accession nos. DQ060683.2 and EU124687.1, HM63052.1, KF686747.1, respectively. It differed from HM63052.1 at three base substitutions and a deletion; from DQ060683.2 at three base substitutions; from EU124687.1 at three base substitutions, a deletion and an insertion; and from KF686747.1 at four base substitutions, a deletion and an insertion (Fig. 3). Identity ranged from 99.09% to 97.62% for previously reported *C. belli* ITS-1 sequences.

The nucleotide sequence of the amplicon was also aligned with reported ITS-1 sequences from *Sarcocystis* sp. As there were no available sequences of the ITS-1 region from *S. hominis* and *S. suihominis*, which correspond to species infecting humans, we used those from *S. cruzi* and *S. neurona* (GenBank accession nos. EF622176.1 and AF098248.1, respectively), which had been described as species infecting cattle and horses respectively in Argentina and in these cases identities were lower than 60%.

Discussion

The prevalence of *C. belli* in AIDS patients with diarrhea in developed countries ranges from less than 1 to 3%. In developing countries the prevalence of *C. belli* in AIDS patient with diarrhea ranges from 1 to 41% (Sorbillo *et al.* 1995; Lindsay *et al.* 1997; Gupta *et al.* 2008; Velásquez *et al.* 2015).

Diagnosis of cystoisosporosis is based on the identification of the oocyst stage of the parasite in fecal samples. The clinical presentation of our patient with nausea, vomiting, and diarrhea with oocysts in parasitological stool examination was consistent with cystoisosporosis diagnosis.

An upper endoscopy with collection of small intestinal biopsy represents an invasive way to diagnose *C. belli* stages and perform a differential diagnosis with other microorganisms. Histologic diagnosis of intestinal cystoisosporosis in biopsy specimens of the small intestine includes the detection of meronts, microgametes, macrogametes and oocysts in epithelial cells.

The identification of oocysts in feces or different stages in intestinal epithelium of *C. belli* is not enough to diagnosis of disseminated cystoisosporosis. Histological diagnosis of disseminated cystoisosporosis is based on the detection of unizoite tissue cysts in lamina propria of the small intestine and this was the finding that we achieved in our patient.

This patient also had crescent-shaped structures in thin blood smears. The differential diagnosis when encountering crescent-shaped structure includes *Plasmodium falciparum* gametocytes, *Toxoplasma gondii* tachyzoites, and merozoites of *Sarcocystis* sp. *P. falciparum* gametocyte is always in a red blood cell and in our case the structure was extracellular. *T. gondii* tachyzoite measures less than 6 μm but in our patient the crescent-shaped structure measured more than 6 μm.

This structure was similar to *Sarcocystis* sp. merozoites. In other report we studied a 31-year old AIDS patient with intestinal plus systemic sarcocystosis with identification of merozoites of *Sarcocystis* sp. in thin blood smears (Velásquez *et al.* 2008). In our actual case microgametes and macrogametes were not detected in lamina propria of the small intestine. Others authors described merozoites of *Sarcocystis* sp. in blood smears of an immunocompetent woman with acute muscular sarcocystosis (Bayani *et al.* 2014).

Although the crescent-shaped structure identified in this case had similar morphology to *Sarcocystis* sp. merozoites, the presence of unsporulated oocysts in feces and the detection of unizoite tissue cysts in lamina propria of the small intestine confirmed a disseminated cystoisosporosis form. The discovery of zoites when examining blood smears is an im-

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portant clue to noninvasive diagnosis of disseminated cystoisosporosis.

To overcome the problem with this study that *C. belli* zoite structure in blood smears was similar to *Sarcocystis* sp. merozoite, we applied the PCR by using sets of primers for amplification of different target sequences of *C. belli* in blood samples as a new tool for diagnosis of disseminated cystoisosporosis.

The detection and/or genetic characterization of *C. belli* based on the 18S rDNA sequence were described for AIDS-patients (Jongwutiwes *et al.* 2007; Velásquez *et al.* 2011). In our case we amplified a fragment of this region with positive results of the expected size, confirming the presence of *Cystoisospora* sp. DNA. In a previous report (Velásquez *et al.* 2011) we had showed that this fragment presented high identity values (over 99%) with the sequences of *Cystoisospora* species other than *C. belli*, and we had employed the ITS-1 region for identification at species level due to the feature of high divergence between species. For the case presented here, we used this last region as a useful target for PCR and sequencing in the blood sample of the patient. We analyzed the DNA fragment generated from the nested PCR for the ITS-1 and the identity was over 97% with previously reported sequences. Although this high identity, there were differences including base substitutions, deletions and insertions with isolates reported by Jongwutiwes *et al.* (2007), Samarasinghe *et al.* (2008), Shafiei *et al.* (unpublished), and including those previously reported from Argentinean patients (Velásquez *et al.* 2011). This minimal sequence variation among isolates cannot be analyzed in correlation with dissemination of the disease, as this information is only available for our previous reported cases, so further information is necessary to elucidate its implications. The molecular tools were of a great value in the case report here to identify at the species level the parasite structures found in blood samples.

In conclusion, unsporulated oocysts in stool examination and unizoite tissue cysts in the lamina propria in duodenal biopsy specimens are present in disseminated cystoisosporosis. Our study identified a new stage in the life cycle of *C. belli* in blood, contributing to elucidate the dissemination way of the parasite. Blood samples with zoites in thin blood smears and DNA detection of *C. belli* by molecular techniques represent a novel and noninvasive approach for diagnosis of disseminated cystoisosporosis.

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