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PROTOZOOLOGY - ORIGINAL PAPER

Cystoisospora belli, liver disease and hypothesis on the life cycle

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Abstract

Cystoisospora belli causes chronic diarrhoea, acalculous cholecystitis, cholangiopathy and disseminated cystoisosporosis in patients with AIDS. Clinical manifestations and histological stages during *C. belli* infection in a patient with AIDS and liver disease were described. It was possible to identify sporozoite-like structures in the villus epithelium of the duodenum, close to the vascularization that underlies the basal membrane and unizoite tissue cysts near to the vascularization in the lamina propria. Unizoite tissue cysts were found inside of sinusoids in the liver communicating with the central vein and with a bile canaliculus and portal spaces. Based on these findings a hypothesis on *C. belli* life cycle could consider that the route of migration of unizoite tissue cysts up the liver is via the portal blood. The unizoite tissue cysts located in hepatic portal vein could migrated via sinusoid to central vein and general circulation through the venous system. The unizoite tissue cysts could also return via bile canaliculus to bile duct to portal triad. This hypothesis allows to understand the presence of unizoite stages in blood, the pathway by which the bile ducts become infected and unizoites in the liver being able to behave like hypnozoites that favour relapses and treatment failures.

Keywords *Cystoisospora belli* · Disseminated cystoisosporosis · Unizoite tissue cyst

Introduction

Cystoisospora belli is a protozoan parasite responsible for human cystoisosporosis (Lindsay et al. 1997). This organism has been described as an opportunistic agent in patients with the acquired immunodeficiency syndrome (AIDS) and is cause of chronic diarrhoea, acalculous cholecystitis and cholangiopathy (Benator et al. 1994; Walther and Topazian 2009). In this group of patients, *C. belli* infection can develop disseminated cystoisosporosis with the presence of unizoite tissue cysts in the lamina propria of the intestines, lymph nodes, liver and spleen (Restrepo et al. 1987; Michiels et al. 1994; Comin and Santucci 1994; Velásquez et al. 2001; Frenkel et al. 2003).

The first report of a patient with AIDS and disseminated cystoisosporosis described a 38-year-old male that had been diagnosed with *C. belli* in faeces and whose autopsy allowed the identification of unizoite tissue cysts in lamina propria of the small intestine, mesenteric and mediastinal lymph nodes. Histopathologic examination of liver was carried out but did not provide any details of unizoite tissue cysts (Restrepo et al. 1987).

The second case was a female AIDS patient of 30-year-old who had eight episodes of fever, diarrhoea and weight loss. Parasitological examination revealed *C. belli* oocysts and histological analysis of duodenal biopsy specimens showed unizoite tissue cysts in the lamina propria. After her death, unizoite tissue cysts in mesenteric and mediastinal

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lymph nodes, liver and spleen were detected in the autopsy (Michiels et al. 1994). The liver had unizoite tissue cysts located in portal areas but not in the biliary tract.

A third case of a 30-year-old female AIDS patient with chronic diarrhoea and sexual and asexual stages of *C. belli* in epithelium also had unizoites in the lamina propria of the small intestine (Comin and Santucci 1994).

Other two cases of AIDS patients with chronic diarrhoea and disseminated cystoisosporosis described the presence of unizoite tissue cysts in the lamina propria but the histopathological examination of the liver was not performed (Velásquez et al. 2001).

A 26-year-old male with AIDS and five-year history of *C. belli* infection was the sixth case reported. The patient died and his autopsy revealed unizoite tissue cysts in mesenteric lymph nodes (Frenkel et al. 2003). The liver histopathology was not performed but there were identified sexual stages in the gallbladder.

Several reports described studies of *Cystoisospora* stages in intestinal, bile ducts or gallbladder abnormalities in patients with AIDS (Dubey et al. 2019; Dubey and Almeria 2019), but there are not descriptions of liver stages except in the report of Michiels et al. (1994). There were several reports describing *C. belli* stages in the gallbladders of patients that are now considered as artefacts and non-protozoal structures (Dubey et al. 2019; Dubey and Almeria 2019).

It is essential to consider that studies of liver disease in patients with disseminated cystoisosporosis are important to increase the knowledge about *C. belli* life cycle, the endogenous stages, the persistent infections in humans and misdiagnosis.

This report describes the clinical manifestations, humoral and histological findings of different stages during *C. belli* infection in a patient with AIDS and liver disease. This knowledge enabled the elaboration of a hypothesis on the life cycle.

Materials and methods

Case description

A 54-year-old male patient, HIV positive for 14 years, not adherent to antiretroviral treatment was admitted for presenting intermittent chronic diarrhoea. His symptoms were associated with nausea and vomiting. As background, he presented recurrent episodes of diarrhoea caused by *C. belli* during the previous 5 years. The patient was hospitalised three times with the same diagnosis 3 years prior to the current hospitalisation. Regarding his clinic history, he always presented eosinophilia (range 7 to 11%), oocysts in faecal matter, low CD4 count (range 97–154 cells/mm^3), liver transaminases with a predominance of alanine transferase (ALT) twice the normal over aspartate aminotransferase (AST), alkaline phosphatase (ALP) twice the normal and negative serology for hepatitis A, B and C. On physical examination, the patient had hepatomegaly and generalised muscle wasting. Laboratory evaluation revealed haemoglobin level of 12.6 g/dl. Leukocyte count was 5500/mm^3 and eosinophilia was 8%. Liver function tests showed normal total bilirubin, ALT 290 U/L (0–41), AST 92 U/L (0–41), ALP 109 U/L (40-129). The CD4 lymphocyte count was 55 cells/mm^3. Abdominal ultrasound was significant for hepatomegaly, isoic lymphadenopathies in hepatic hilum (<10mm) and small mesenteric hypoic lymphadenopathies (<10 mm), normal spleen, normal gallbladder and not biliary ductal dilation. Stool examination showed *C. belli* oocysts. An upper endoscopy with small intestinal biopsy sampling was performed.

The patient was treated with trimethoprim-sulfamethoxazole and showed improvement in 5 days. He had formed stools daily without vomiting. Stool examination did not show *C. belli* oocysts. By day 14 liver function tests revealed normal total bilirubin, ALT 333 U/L (0–41), AST 161 U/L (0–41) and ALP 168 U/L (40–129). Hepatitis A and B serologies were negative but hepatitis C antibody was positive. By day 21, the patient presented diarrhoea (three times a day) again during 6 days despite continuing treatment with trimethoprim-sulfamethoxazole. Stool examination showed *C. belli* oocysts. On the 34th day of hospitalisation, a liver biopsy was performed. He showed marked improvement in clinical, and laboratory finding and was discharged from the hospital 40 days after admission.

Ethical approval

The Ethical Committee for Research of the Hospital Francisco J. Muñiz, approved this research protocol registered under the number 274. Written informed consent from the patient was obtained.

Coprological analysis

Routine stool bacterial culture was carried out. Serial examination for parasites was performed by collection of one stool sample daily for 1 week in 5% formalin. Faeces were concentrated by ethyl-ether centrifugation and stained with the modified acid-fast technique. Stained and unstained specimens were microscopically analysed (Velásquez et al. 2001).

Histological analysis

Duodenal tissues

A flexible fibreglass endoscopy with biopsy sampling was performed with Pentax EPM 2000 equipment. Five

specimens were obtained from the distal duodenum and were processed as follows: two samples were fixed in 10% formalin, embedded in paraffin, and stained with haematoxylin-eosin and Giemsa; other two specimens were preserved with Karnovsky fixative, embedded in polybedaraldite and stained with Azure II, and a fifth sample was stored in saline solution at −20°C (Velásquez et al. 2011).

Liver tissues

Liver biopsy was performed using the percutaneous technique with Tru-Cut needle. Liver samples were fixed in 10% formaldehyde, embedded in paraffin, and stained by haematoxylin-eosin and Giemsa. Other samples were treated with Karnovsky fixative, embedded in polybedaraldite and stained with Azure II for analysis under conventional light microscopy. Another specimen was stored in saline solution at −20°C. Histological examinations considered the identification of unizoite tissue cysts or other stage of *C. belli* and the following hepatic changes: sinusoidal congestion and dilatation; inflammatory alterations including the presence of portal inflammation, central perivenular inflammation, inflammatory cell aggregates within the sinusoids, portal tract granulomas, intralobular granulomas, Kupffer cell hypertrophy and hyperplasia; and hepatocytic injury corresponding to hepatocellular steatosis, ballooning of hepatocytes, hepatocellular necrosis and apoptosis.

Molecular analysis

Frozen duodenal and liver biopsy samples were employed for DNA purification, which was carried out by trypsinization and standard phenol-chloroform extraction according to our previous protocols (Velásquez et al. 2011). DNA was finally dissolved in 10 µl of double-distilled water and kept at –20 °C until use.

A nested PCR assay was used to amplify a fragment of the internal transcribed spacer 1 (ITS-1) of the rRNA genes accordingly to our previous report (Velásquez et al. 2011). Two microlitres of the purified DNAs were used as PCR templates. The procedure was performed employing the primer pairs designed by Samarasinghe et al. (2008). The outer primer pair ITSF (5′-CCGTTGCTCCTACCG ATTGAGTG-3′) and EMR7 (5′-GCATTTCGCTGCGTC CTTCATCG-3′), and the inner primer pair ITSGF (5′-GAT CATTCACACGTGGCCCTTG-3′) and ITSR2 (5′-GAC GACGTCCAAATCCACAGAGC-3′) were employed to sequentially amplify a portion of the ITS-1 rDNA locus of *Cystoisospora* sp. Amplicons were run on GelRed-stained 2% agarose gels and visualised under UV illumination.

Restriction fragment length polymorphism (RFLP) analysis of the products of the second round of amplification was carried out with the *Alu*I restriction enzyme. Digestion products were run on GelRed-stained 3% agarose gels.

Results

Stool findings

Unsporulated, ellipsoidal oocysts that measured about 28–30 µm by 15–17 µm, and a very little amount of sporulated oocysts containing two sporocysts were detected by microscopic stool examination. They were identified in unstained samples and stained smears. Bacterial cultures from faeces were negative.

Histological findings

Duodenal tissues

The unizoite tissue cysts were present in the lamina propria of the duodenum and had a light blue wall in Azur II–stained section. The type of the infected host cells was difficult to determine. Depending on tissue section, the zoites appeared as round, oval, and banana-shaped structures, located in the centre of the tissue cyst, and surrounded by a clear vacuole. They had one rounded nucleus, anteriorly or centrally located (Fig. 1).

In the villus epithelium, meronts were elongated or oval shaped. Merozoites were present singly, in pairs and in groups. Elongated meronts with single merozoites were numerous. Most unique merozoites had a central nucleus with one end rounded and the other pointed. In some unique banana-shaped structures, the nucleus was not located in the centre, and it was possible to identify a structure that resembled a residual or crystalloid body (Fig. 2).

Macrogamonts or microgamonts were not observed in the villus epithelium. An increase in vascularity was observed in the lamina propria. The stages of *C. belli* found in the epithelium were close to the vascularization that underlies the basal membrane. The unizoite tissue cysts were also near to the vascularization in the lamina propria (Fig. 3).

All biopsy specimens revealed an abnormal mucosal architecture, showing villi height reduction and hypertrophied crypts. The lamina propria presented an increased cellularity with a mixed infiltrate of neutrophils, plasma cells, lymphocytes and eosinophils.

In the lamina propria, no oocysts or multinucleated or sexual stages were observed.

Liver tissues

The unizoite tissue cysts were present inside of sinusoids. The sinusoid where the unizoite tissue cysts were identified

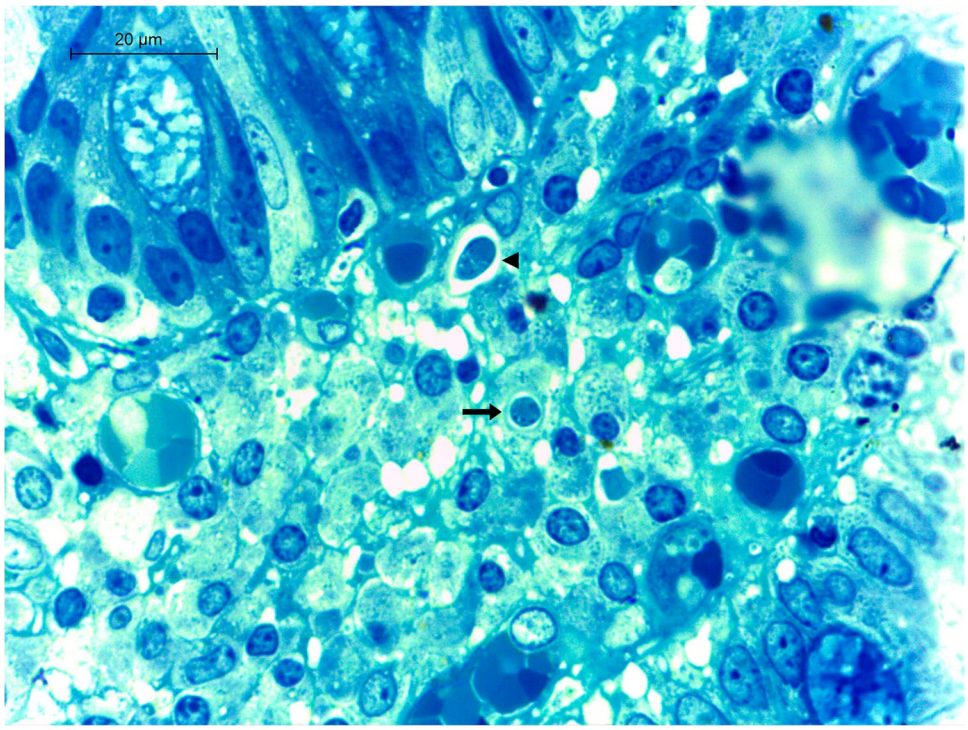

Fig. 1 Unizoites tissue present in the duodenal lamina propria. Banana-shaped (arrowhead) and oval (arrow) forms. Azur II stain

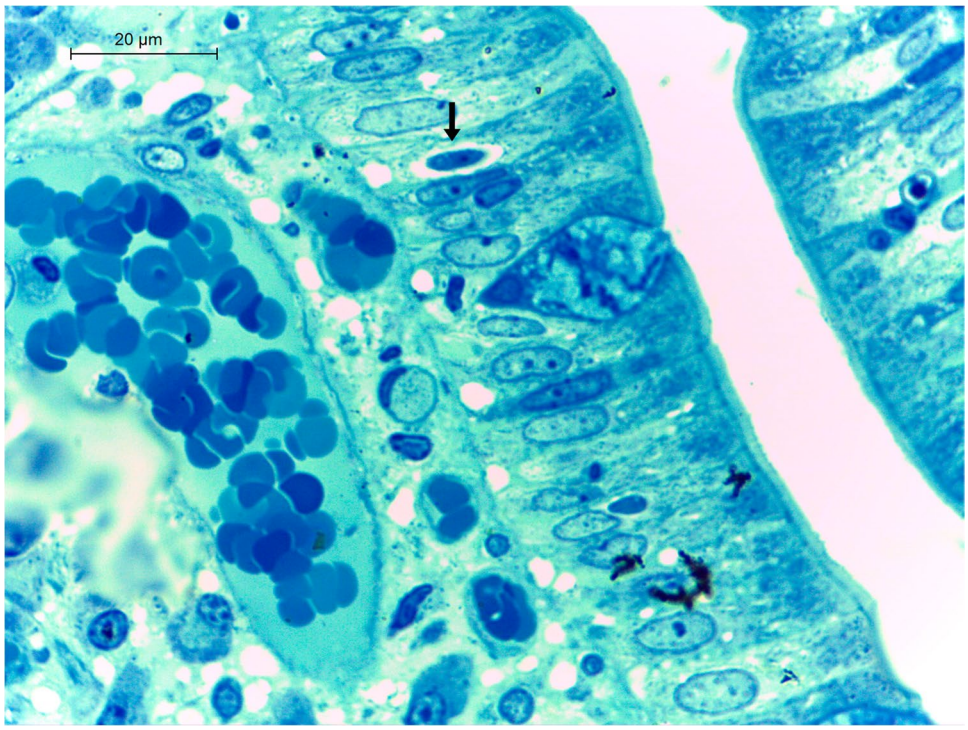

Fig. 2 Banana-shaped form with an internal structure resembling a residual or crystalloid body (arrow). Azur II stain

communicated with the central vein and with a bile canaliculus. The biliary canaliculi were dilated. The unizoite was bordering on Kupffer, mononuclear and polymorphonuclear cells. Pigments located between the unizoite and the surrounding cells could also be observed (Fig. 4).

In other sections, it is possible to identify the unizoite tissue cysts in a location close to the central vein (Fig. 5)

At higher magnification, the unizoite was identified in a space surrounded by pigments and cells, where sinusoids and cuboid cells of intrahepatic bile canaliculus converged.

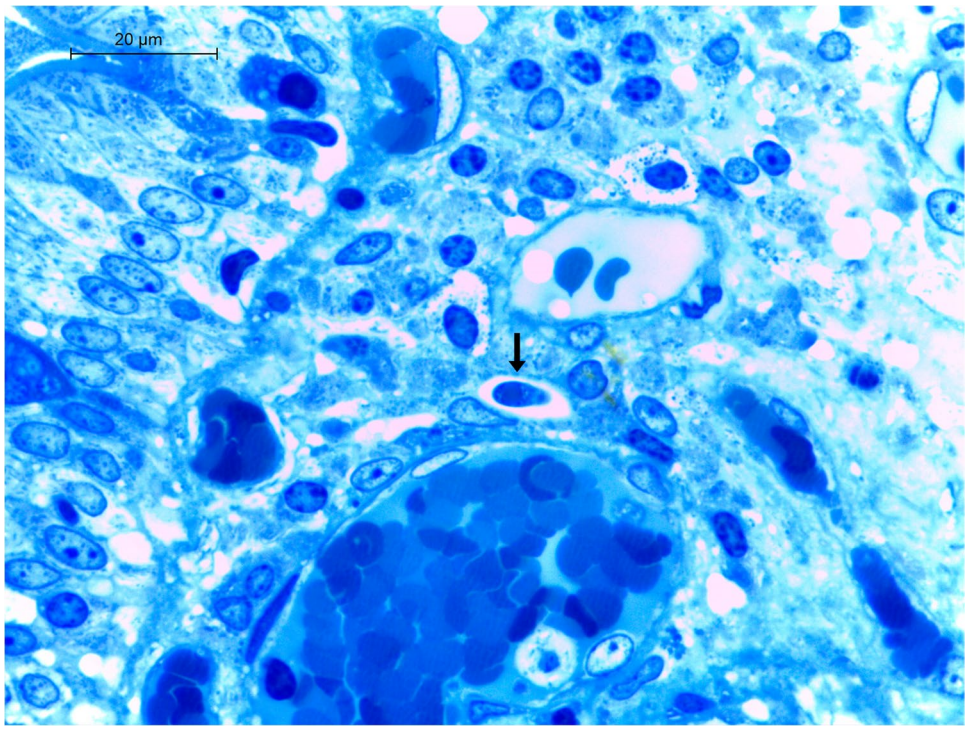

Fig. 3 Unizoite tissue cyst close to the vascularization in the lamina propria (arrow). Azur II stain

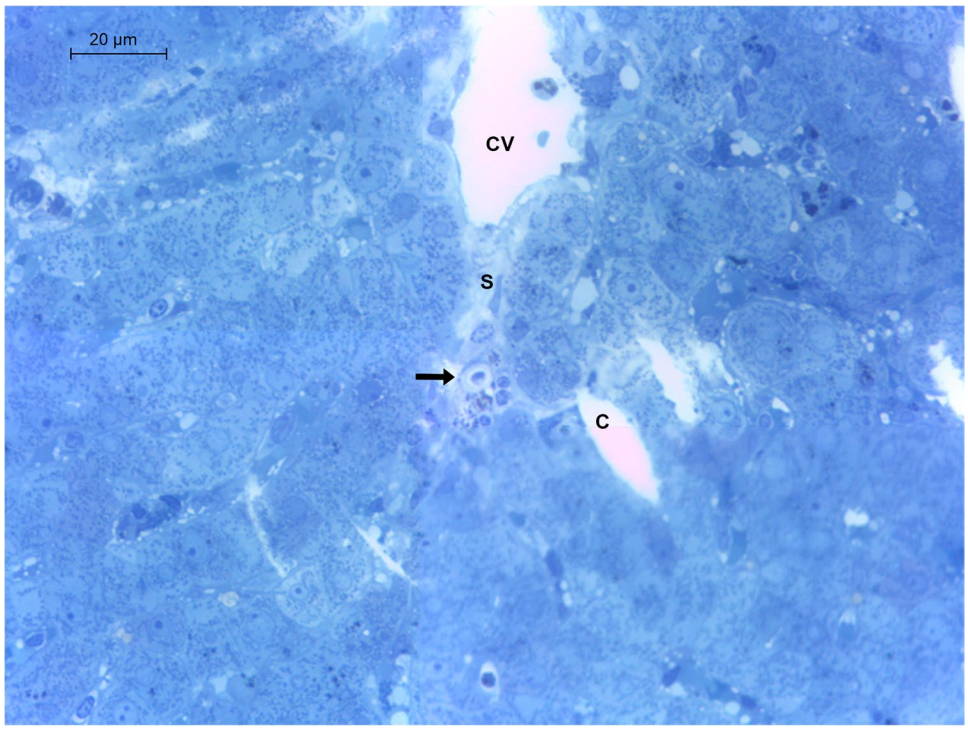

Fig. 4 Unizoite tissue cysts (arrow) present inside of a sinusoid (S) that communicates with the central vein (CV) and with a bile canaliculus (C). Azur II stain

The cuboid cells of intrahepatic bile canaliculus connected with the bile canaliculus (Fig. 6).

At the histological level, morphological changes seen under light microscopy were some binucleated hepatocytes, microvesicular steatosis and areas with ballooning cells. Portal tract was enlarged and contained large numbers of macrophages, lymphocytes and occasionally plasma cells and eosinophils. Portal changes with variable degree of fibrosis and ductular proliferation were observed (Fig. 7).

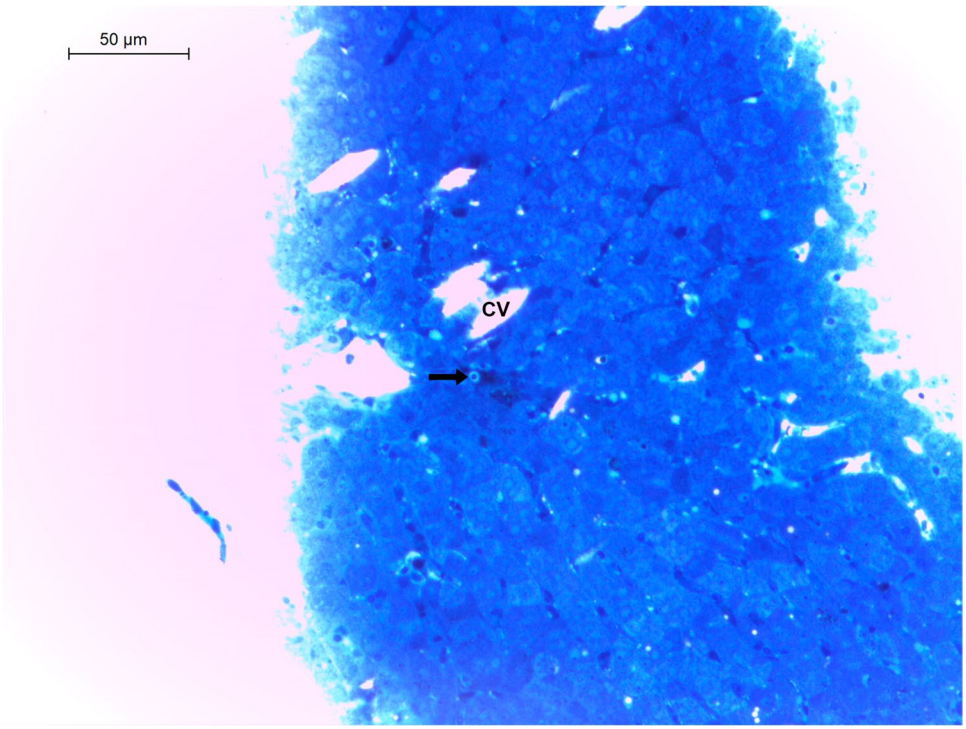

Fig. 5 Unizoite tissue cyst (arrow) in a location close to the central vein (CV). Azur II stain

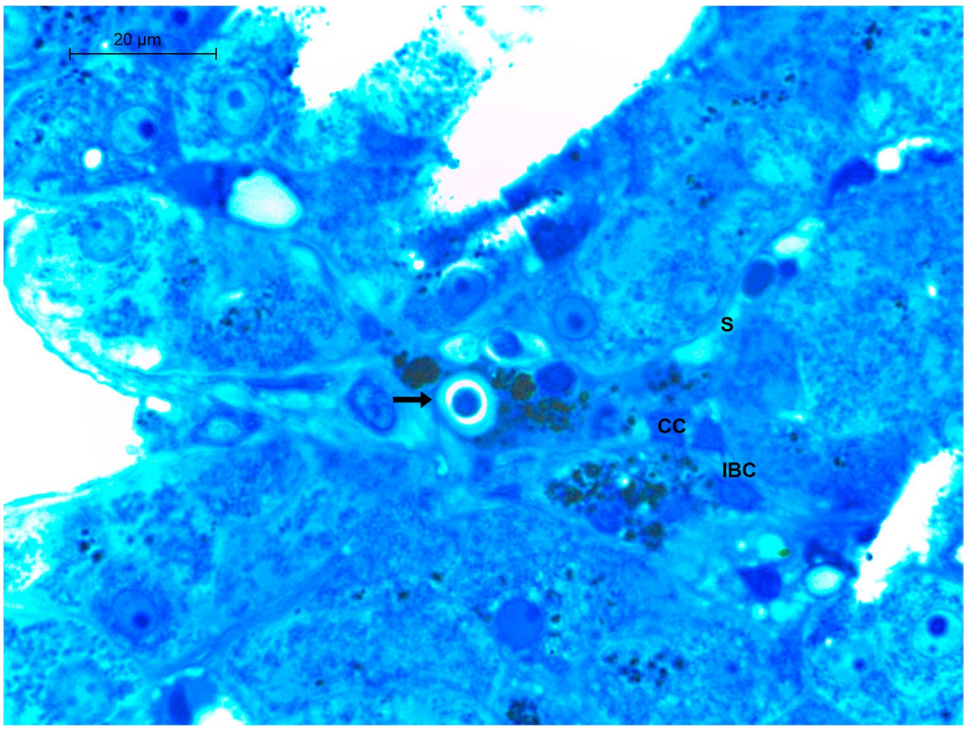

Fig. 6 Unizoite tissue cyst (arrow) in a space where sinusoids (S) and cuboid cells (CC) of intrahepatic bile canaliculus (IBC) converge. Azur II stain

Nested PCR-RFLP analysis

DNA samples obtained from duodenal and liver biopsy specimens were employed for molecular analysis. Both samples generated nested PCR products of 440 base pairs. RFLP fragments were of the sizes expected for *C. belli* species corresponding to 200, 118, 101 and 21 bp (data not shown).

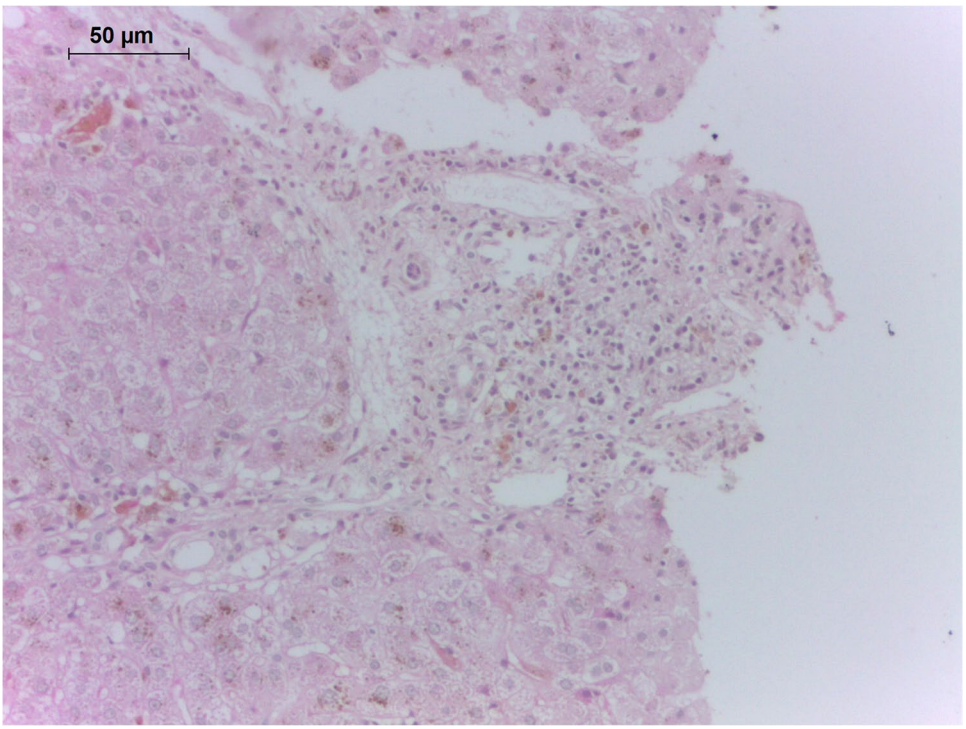

Fig. 7 Portal changes with variable degree of fibrosis and inflammatory cell infiltrate. Haematoxylin-eosin stain

Discussion

The life cycle of *C. belli* consists in endogenous and exogenous development. The endogenous development includes an asexual cycle or merogony and a sexual cycle or gametogony, which take place in the host. The exogenous development is a sporogony which occurs outside the host (Lindsay et al. 1997; Dubey and Almeria 2019). Instead of undergoing the normal developmental cycle in the intestinal tract, some sporozoites may leave the lamina propria, invade extraintestinal sites and produce unizoite tissue cysts in the host. In humans, mesenteric lymph nodes are most often infected but other tissues such as the liver, spleen, and tracheobronchial and mediastinal lymph nodes can also be infected. The unizoite tissue cysts contain a single parasite structurally resembling a sporozoite (Lindsay et al. 1997; Dubey and Almeria 2019). The route by which the sporozoites of *C. belli* reaches the liver and the bile duct epithelium has not been elucidated.

In the patient described in this report, it was possible to identify sporozoite-like structures in the villus epithelium. The stages of *C. belli* found in the epithelium were close to the vascularization that underlies the basal membrane. The unizoite tissue cysts were also near to the vascularization in the lamina propria.

A hypothesis is that sporozoites and/or unizoite tissue cysts invade the epithelial cells of the duodenal mucosa and penetrated to the basement membrane and into the lamina propria. We suppose that the route of migration of unizoite tissue cysts up the liver is via the portal blood although in our case we cannot exclude migration due to lymphatics. Abdominal ultrasound was significant for the diagnosis of lymphadenopathies in hepatic hilum and small mesenteric lymphadenopathies. Lymphadenopathy may occur in hepatitis C or disseminated cystoisosporosis as it was diagnosed in our case (Lindsay et al. 1997; Soresi et al. 1998; Dubey and Almeria 2019).

Another similar case was a 30-year-old female AIDS patient with chronic diarrhoea and cistoisosporosis who had unizoites in the lamina propria of small intestine, some of them located near vessel and within lymphatic channels (Comin and Santucci 1994). The authors supported the possibility that the route of migration of unizoite tissue cysts up the liver was via the lymphatic channels despite closeness to blood vessels.

Knowledge of the biology of coccidian parasites contributes to support our hypothesis. A good model is the life cycle of *Eimeria stiedae* in rabbits that develops in liver and in the epithelium of bile ducts. The route of migration of *E. stiedae* sporozoites between the duodenum and bile ducts of the rabbit was studied in numerous reports (Smetana 1933; Horton 1967; Pakandl 2009). These authors suggested that these sporozoites migrated up the liver and bile duct via the blood or lymph. In our case, it was possible to identify the unizoite tissue cysts of *C. belli* in the liver biopsy specimen. The liver tissues were also positive for *C. belli* by nested PCR-RFLP. This is in concordance with the case reported by Michiels et al. (1994) in which unizoites tissue cysts had

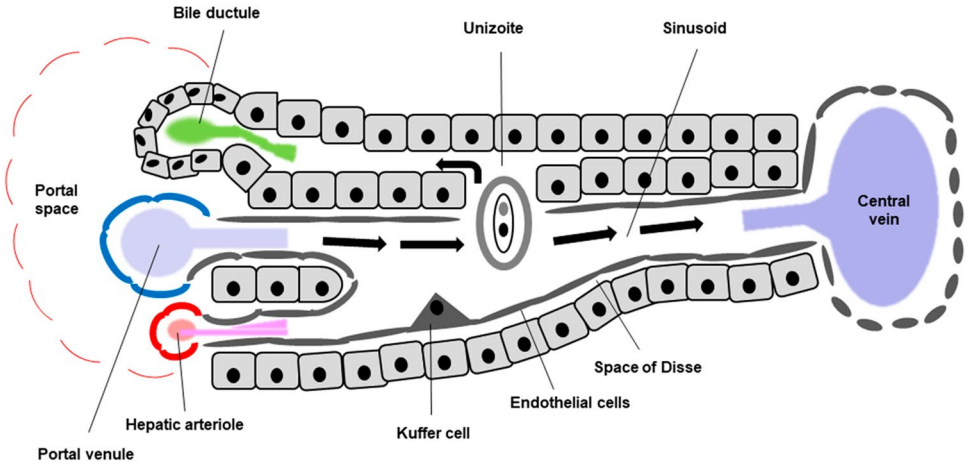

Fig. 8 Model of the route of migration of unizoite tissue cysts up the liver via the portal blood

been identified in the liver parenchyma. The tissue stage observed in the liver was similar to the extraintestinal stage of feline and canine *Cystoisospora* species found in cats and dogs. *C. felis* and *C. rivolta* in cats had unizoites tissue cysts in liver and extraintestinal tissues (Dubey and Frenkel 1972; Dubey 2014, 2018). *C. canis* and *C. ohioensis* in dogs can develop unizoites tissue cysts in extraintestinal tissues including liver in both the canine definitive and paratenic hosts (Dubey 1975; Lindsay 1997). Other report described unizoite tissue cysts of non-determined *Isospora* species in liver of the Scheneider's skink *Eumeces schneideri* (Koudela and Modrý 1999). The identification of unizoites tissue cysts of different species of the genus *Cystoisopora* in the liver suggests the possibility that the stages in the liver are part of the life cycle.

Knowledge of the location of the unizoite tissue cysts in the anatomical structure of the liver is important to interpret the possible participation of the liver in the biological cycle. We described the unizoite tissue cysts present inside of sinusoids. The sinusoid where the unizoite tissue cysts were identified communicated with the central vein and with a bile canaliculus and portal spaces. In the human case reported by Michiels et al. (1994), the unizoites tissue cysts had been also identified in the portal areas of the liver parenchyma.

Our hypothesis is that the route of migration of unizoite tissue cysts up the liver is via the portal blood. The unizoite tissue cysts located in hepatic portal vein could migrated via sinusoid to central vein and general circulation through the venous system. The unizoite tissue cysts could also return via bile canaliculus to bile duct to portal triad (Fig. 8).

The finding of the unizoite bordering on Kupffer, mononuclear and polymorphonuclear cells in liver was in concordance with the case described by Michiels et al. (1994). In our case, histopathology showed moderated steatosis and portal changes. The portal tract enlarged with inflammatory infiltrate and variable degree of fibrosis and ductular proliferation are nonspecific features and may be due for different etiological agents. The finding of unizoitos in the liver parenchyma and a positive nested PCR-RFLP for *C. belli* could be associated with these patterns in the pathological anatomy. Moderate steatosis and cholestasis were observed in the previous case described by Michiels et al. (1994). The diagnosis of hepatitis C in our patient could also show these histological spectrum (Dhingra et al. 2016).

In conclusion, this hypothesis helps to understand the presence of unizoite stages in blood (Velásquez et al. 2016), the pathway by which the bile ducts become infected causing acalculous cholecystitis and cholangiopathy (Benator et al. 1994; Walther and Topazian 2009) and unizoites in the liver being able to behave like hypnozoites that favour relapses and treatment failures (Markus 1991; Montalvo et al. 2013; Silva-Díaz et al. 2017). Further studies are needed to test this hypothesis.

Author contributions All authors contributed equally to this study.

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Declarations

Competing interests The authors declare no competing interest.

Ethical approval The Ethical Committee for Research of the Hospital Francisco J. Muñiz, approved this research protocol registered under the number 274. Written informed consent from the patient was obtained.

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