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ORIGINAL PAPER

First detection of *Cryptosporidium* DNA in blood and cerebrospinal fluid of HIV-infected patients

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Abstract

Human cryptosporidiosis is an intestinal infection caused by different species belonging to the genus *Cryptosporidium* in both immunocompetent and immunocompromised individuals. The life cycle of *Cryptosporidium* sp. when affecting the digestive system is well known but the infection of other organs is less studied. Molecular methods are necessary for species and subtypes identification. The goal of this work is to propose a new approach that contributes to the diagnosis of the extra-intestinal dissemination process of *Cryptosporidium* infection. *Cryptosporidium* sp. was detected in stool and biopsy samples of two HIV-infected patients. DNA was extracted from feces, biopsy specimens, blood, and cerebrospinal fluid (CSF). All samples were analyzed by nested PCR-RFLP of the 18S rDNA, real-time PCR, and *gp60* subtyping. *Cryptosporidium* DNA was detected in stool and tissue samples and it was also present in blood and CSF samples. Both cases were characterized as *Cryptosporidium hominis* subtype IeA11G3T3. This is the first report that demonstrates the presence of *Cryptosporidium* DNA in blood and CSF of HIV-infected patients.

Keywords *Cryptosporidium* sp. · Blood · CSF · PCR · AIDS

Introduction

Cryptosporidiosis is caused by protozoa belonging to the genus *Cryptosporidium* that affects the digestive tract of humans and animals. *Cryptosporidium* sp. normally causes self-limiting diarrhea in immunocompetent individuals, but in immunosuppressed patients, such as those infected with human immunodeficiency virus (HIV)/AIDS, it may cause severe long-lasting and even fatal disease affecting the digestive system as well as the pancreas and lungs (Riggs 2002; Amadi et al. 2002; Certad et al. 2005).

The life cycle in the host begins with the ingestion of oocysts, followed by the development of different stages in the microvilli of intestinal epithelial cells and subsequent elimination of oocysts in feces. The life cycle is well known when the digestive system is affected, but the mechanism involved in generating the infection in organs other than those in the digestive system constitutes a field of knowledge little explored.

The gold standard for the laboratory diagnosis of cryptosporidiosis is based on the identification of oocysts in stool samples. The morphology of oocysts, host specificity, or preference at infection sites do not provide sufficient information to identify *Cryptosporidium* species, genotypes, or subtypes (Fall et al. 2003; Jex and Gasser 2010). Many laboratories are using molecular methods for routine diagnosis, based on in-house methods or on commercial platforms in developed and developing countries. Advances in molecular technologies have led to significant improvements in the characterization of genetic variability between and within *Cryptosporidium* species (Jex and Gasser 2010). The amplification and sequencing of one or more genetic loci have been used to characterize species, genotypes, or subtypes of *Cryptosporidium*

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(Xiao et al. 2004; Chalmers et al. 2005; Plutzer and Karanis 2009; Bouzid et al. 2010). Recent work has confirmed the usefulness of mini- and microsatellite DNA markers and sequencing of the *gp60* gene in the study of the population structure of *Cryptosporidium* and in the understanding of the transmission dynamics of infection (Enemark et al. 2002; Cacciò et al. 2005; Smith et al. 2006; Feng et al. 2014).

Currently, 34 species of *Cryptosporidium* are recognized and more than 50 genotypes with an uncertain status of species have been reported (Chalmers and Katzer 2013; Kváč et al. 2014, 2016; Jezkova et al. 2016; Zahedi et al. 2017). At least 15 species (*C. hominis*, *C. parvum*, *C. meleagridis*, *C. felis*, *C. canis*, *C. suis*, *C. muris*, *C. andersoni*, *C. bovis*, *C. cuniculus*, *C. fayeri*, *C. tyzzeri*, *C. viatorum*, *C. wrairi*, and *C. ubiquitum*) are associated with human disease, but the vast majority of human cases of cryptosporidiosis worldwide are caused by two species, namely *C. parvum* and *C. hominis* (Soba and Logar 2008; Ryan et al. 2014). In previous studies (Del Chierico et al. 2011), subtypes of *C. parvum* and *C. hominis* were described in association with clinical manifestations in HIV-positive patients. The understanding of these relationships may represent the starting point for studies on the correlation between species/subtypes and the severity of the symptoms, species subtype, and location according to the affected organ and recognition of relapses and reinfection.

The aim of this work is to propose a new approach based on molecular methods employing fluid samples other than feces that could be useful to the diagnosis of the extra-intestinal dissemination process of *Cryptosporidium* infection.

Materials and methods

We studied two patients that had been diagnosed with *Cryptosporidium* sp. on the basis of oocysts in the feces. Duodenal biopsy specimens, blood, and cerebrospinal fluid (CSF) were obtained for microscopy and DNA purification.

The research protocol was approved by the Ethical Committee for Research, Hospital Francisco J. Muñiz, protocol 323.

Stool samples

Stool specimens were collected daily for 3 days in 5% formalin saline solution and concentrated by ethyl ether centrifugation. Pellets were employed for sucrose flotation (Sheather 1923) and smears were stained with modified acid-fast stain (Henriksen and Pohlenz 1981), modified trichrome (Weber et al. 1992), and Gram-chromotrope (Moura et al. 1996) techniques. These samples were later examined by light microscopy.

Feces in aliquots of 1 ml were also employed for DNA purification according to our previous protocol (Velásquez et al. 2010). Each of these samples was washed twice with saline solution by centrifugation for 3 min at 13000×*g*. The supernatants were discarded.

The final pellet was resuspended in 200 μl of buffer phosphate saline pH 8 with the addition of 20 μl of 5% trypsin. It was incubated overnight at 37 °C while being shaken at 200 rpm and treated by four cycles of freeze-thaw in liquid nitrogen and 90 °C for 15 min each. Then, lysis was performed by the addition of 200 μl of 2X lysis buffer (20 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 4% SDS, 100 mM NaCl), 15 μl of proteinase K stock solution (200 mg/ml), and 14 μl of 1 M dithiothreitol. It was then incubated for 3 h at 58 °C and then overnight at 37 °C.

After lysis, a standard phenol-chloroform extraction was carried out (Maniatis et al. 1989). DNA was precipitated adding isopropanol and 0.25% acrylamide. After being washed with ethanol 70°, the pellet was dissolved in 10 μl of bidistilled water, incubated for 10 min at 55 °C and stored at − 20 °C until later use.

Duodenal tissues

Five biopsy specimens were obtained from the distal duodenum by means of flexible fiberglass endoscopy. Two samples were used for routine histology procedures and stained with hematoxylin-eosin and Giemsa. Two tissue specimens were treated with Karnovsky fixative, embedded in polybedaraldite and stained with Azure II (Velásquez et al. 2001). The fifth collected sample was kept in saline solution and used for DNA extraction by standard methods following our previously reported protocols (Velásquez et al. 2010, 2011). Each sample was centrifuged for 5 min at 15,000×*g*.

The pellet was resuspended in 200 μl of buffer phosphate saline pH 8, with 20 μl of 5% trypsin, and incubated overnight at 37 °C at 200 rpm. Then, 200 μl of 2X lysis buffer (20 mM Tris-HCl pH 8, 20 mM EDTA pH 8, 4% SDS, 100 mM NaCl) and 4 μl of proteinase K stock solution (200 mg/ml) were added. Samples were incubated for 3 h at 58 °C and overnight at 37 °C. After lysis, a standard phenol-chloroform extraction was carried out (Maniatis et al. 1989), and DNA was precipitated in absolute ethanol, dissolved in 10 μl of bidistilled water and kept at − 20 °C until later use.

Blood and cerebrospinal fluid samples

Blood samples were obtained by venepuncture. CSF samples were obtained by lumbar puncture. These samples were processed for DNA extraction.

Aliquots of 200 μl from whole blood samples treated with EDTA (1.25 mg/ml of blood) and CSF 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. DNAs were eluted in

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200 μl of elution buffer (provided with the kit) and employed for molecular assays.

Molecular analysis

Feces, biopsy specimens, blood, and CSF samples were used in order to confirm the presence of *Cryptosporidium* DNA.

Nested PCR-RFLP for the 18S rDNA

Molecular diagnosis was performed to identify the species using a nested PCR-RFLP assay according to the protocol described by Coupé et al. (2005) based on the hypervariable region of the 18S rDNA. The initial amplification was performed with primers SCL1 (5′-CTGGTTGATCCTGC CAGTAG-3′) and CPB-DIAGR (5′-TAAGGTGCTGAAGG AGTAAGG-3′), corresponding to nucleotides 4–23 and 1016–1036. The second-round PCR was performed with primers SCL2 (5′-CAGTTATAGTTTACTTGATAATC-3′) and SCR2 (5′-CAATACCCTACCGTCTAAAG-3′), corresponding to nucleotides 106–128 and 299–318 of the same gene.

Reaction mixtures were prepared according to Velásquez et al. (2010). The first round was carried out in a final volume of 25 μl. The reaction mixture contained 0.2 μM of each primer, 150 μM of each deoxynucleotide triphosphate (dNTP), 1.25 U of *Taq* DNA polymerase, 20 mM $(NH4)_2SO_4$, 75 mM Tris-HCl, 0.01% Tween 20, 2.5 mM $MgCl_2$, 400 ng/μl of bovine serum albumin (BSA), and 2 μl of genomic DNA. For the second round, 5 μl of amplification products was employed in a final volume of 50 μl. The reaction mixture was the one described above, but the final concentration of each primer was 0.4 μM.

Cycle conditions were as follows: initial denaturation at 94 °C for 5 min, 39 cycles of a denaturation step at 94 °C for 30 s, an annealing step at 60 °C (58 °C for the second round) for 45 s, and an extension step at 72 °C for 90 s, with a final extension for 10 min at 72 °C.

Restriction analysis was performed with enzymes *Taq*I (Thermo Scientific) and *Vsp*I (Thermo Scientific), according to the manufacturer's instructions.

Amplification products before and after restriction enzyme digestion were run on 2.5% agarose gel stained with ethidium bromide and visualized under UV transillumination.

Real-time PCR assay

The real-time PCR method was also used using TaqMan™ probes combining a duplex reaction for the detection of *Cryptosporidium* sp. and *C. parvum* and a simple reaction for the detection of *C. hominis* (Jothikumar et al. 2008). To detect *Cryptosporidium* at the genus level, the TaqMan™ probe JVAP18S (5′-Cy5-CGCGCCTGCTGCCTTCCTTA GATG-BHQ-3′) targeting the 18S rRNA and the primers forward JVAF (5′-ATGACGGGTAACGGGGAAT-3′) and reverse JVAR (5′-CCAATTACAAAACCAAAAAGTCC-3′) were employed.

For the *C. parvum* TaqMan™ assay, the oligonucleotides employed were the following: JVAGP2 probe (5′-FAM-ATTTATCTCTTCGTAGCGGCG-BHQ-3′), JVAGF forward primer (5′-ACTTTTTGTTTGTTTTACGCCG-3′), and JVAGR reverse primer (5′-AATGTGGTAGTTGCGGTTGA A-3′).

For the *C. hominis* TaqMan™ assay, the following oligonucleotides were used: JVAGP1 probe (5′-FAM-ATTTATTA ATTTATCTCTT-ACTTCGT-BHQ-3′), JVAGF forward primer (5′-ACTTTTTGT-TTGTTTTACGCCG-3′), and JVAGR reverse primer (5′-ATGTGGTAGTTGCGGTTGAA -3′). These specific oligonucleotides for *C. parvum* and *C. hominis* were designed by Jothikumar et al. (2008) on the basis of GenBank accession number AF190627, which has been identified as being polymorphic, but with an undefined function.

The real-time PCR assays were performed with a 7500 Real-Time PCR System (Applied Biosystems). Each 20 μl duplex reaction contained 10 μl 2X Platinum Quantitative PCR SuperMix UDG (Invitrogen), 100 nM of each probe (JVAP 18S and JVAGP2), 250 nM of each primer (JVAF, JVAR, JVAGF, and JVAGR), and 5 μl of DNA diluted 1:5 in bidistilled water. For the single assay, each 20 μl reaction contained 10 μl 2X SuperMix, 250 nM of each primer (JVAGF and JVAGR), 5 mM $MgCl_2$, 200 nM of the JVAGP1 probe, and 5 μl of DNA diluted 1:5 in bidistilled water. Cycling conditions consisted of a step of 50 °C for 2 min, denaturation at 95 °C for 2 min followed by 45 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 35 s, and extension at 72 °C for 20 s.

Subtyping

DNA samples were used to classify into subtype families by nested PCR amplification and analysis of the *gp60* gene sequence using the subtyping nomenclature system previously proposed to differentiate subtypes within each family (Strong et al. 2000; Glaberman et al. 2002; Sulaiman et al. 2005; Peralta et al. 2016).

The primers used were AL3531 (5′-ATAGTCTCCGCTGT ATTC-3′) and AL3533 (5′-GAGATATATCTTGGTGCG-3′) for the first round of PCR, and AL3532 (5′-TCCG CTGTATTCTCAGCC-3′) and LX0029 (5′-CGAA CCACATTACAAATGAAGT-3′) for the second round. The first round was carried out in a final volume of 25 μl. The reaction mixture contained 200 nM of each primer, 150 μM of each dNTP, 1.25 U of *Taq* DNA polymerase, 20 mM $(NH4)_2SO_4$, 75 mM Tris-HCl, 0.01% Tween 20, 2.5 mM $MgCl_2$, 400 ng/μl of BSA, and 2 μl of genomic DNA. For

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the second round, 5 μl of amplification products was employed in a final volume of 50 μl. The reaction mixture was the one described above, but the final concentration of each primer was 400 nM.

Each PCR reaction was then subjected to 39 cycles of denaturation at 94 °C for 30 s, annealing at 60 °C (58 °C for the second round) for 45 s, and extension at 72 °C for 90 s, with an initial denaturation at 94 °C for 5 min and a final extension at 72 °C for 10 min.

PCR products of the second round were sequenced in both directions by a commercial sequencing service (Macrogen, Korea). The resulting sequences were analyzed using the Blast program of the National Center for Biotechnology Information and aligned with reference sequences retrieved from the GenBank with the ClustalW2 multiple sequence alignment program. All *gp60* sequences obtained in this study have been deposited in the GenBank database.

Results

Case 1

A 46-year-old man known to be infected with HIV was admitted to the hospital with chronic diarrhea. He was on HAART and had a previous diagnosis of cryptococcal meningoencephalitis (8 years) and cryptosporidiosis (1 year).

His CD4 cell count was 9 cells/mm^3 and viral load of 2543 copies/ml. Blood analysis revealed pancytopenia. Abdominal ultrasonography showed cholangiopathy. A bone marrow aspiration was performed and thin smears of the sample stained with Giemsa allowed for the detection of amastigotes confirming visceral leishmaniasis. Due to repeated episodes of a headache, a lumbar puncture was carried out. The physical-chemical analysis of the CSF resulted in normal values. He died due to a multi-parenchymal failure.

Case 2

A 43-year-old man known to be HIV infected was admitted to the hospital with chronic diarrhea, vomiting, and cough. His CD4 cell count was 46 cells/mm^3 and his viral load was of 1,024,086 copies/ml. A coproparasitological examination was positive for *Cryptosporidium* sp. and Microsporidia. Hepatomegaly, steatosis, dilated extrahepatic biliary tract, and multiple vesicular lithiases were observed by means of ultrasonography. A lumbar puncture was performed due to a headache. The CSF showed normal values in the physical-chemical analysis and resulted negative for microorganisms. He developed hospital-acquired pneumonia and died.

Microscopical findings

Cryptosporidium sp. oocysts were identified by parasitological stool examination of Kinyoun-stained smears and the histology revealed *Cryptosporidium* sp. stages in the duodenal epithelium in both cases. Microsporidia spores were detected in stool samples from case 2 and different stages were visualized in the biopsy specimens.

Molecular analysis

Nested PCR amplification of a fragment of the 18S rRNA gene produced amplicons of the expected size corresponding to 214 bp in the stool, biopsy, blood, and CSF samples of both cases. RFLP analysis allowed the identification of *C. hominis* digestion patterns for all samples.

The eight samples corresponding to stool, biopsy, blood, and CSF of cases 1 and 2 were analyzed by real-time PCR assays. All of them were positive for the *Cryptosporidium* sp. assay and the specific *C. hominis* assay. The results confirmed the species determined by the nested PCR-RFLP.

Sequence analysis of a 436-bp fragment of the *gp*60 gene from both blood and CSF identified *C. hominis* subtype IeA11G3T3 in both cases (100% homology). This value was also 100% with previously reported sequences (with 100% coverage) from different geographical localizations including UK (GenBank Accession number GU214354), Japan (AY167593), Kuwait (AY738184), Guatemala, Portugal (AF402288), and 99.77% with Australian samples (FJ839874).

Overall results for blood and CSF samples are shown in Table 1. Molecular methods allowed the detection of *Cryptosporidium* DNA in blood and CSF of these patients. In both cases, the species identified was *C. hominis* and the subtype IeA11G3T3 (GenBank accession numbers MG715500 and MG715501).

Discussion

Molecular methods have been used in patients with cryptosporidiosis for the identification of species, genotypes, and subtypes to answer various epidemiological questions. Molecular data improves the understanding of zoonotic or anthroponotic sources of infection and the relationships between species and subtypes and clinical manifestations. However, most studies rely on fecal samples for diagnosis and typing. The present study identified *Cryptosporidium* DNA in blood and CSF and highlights the need for further research to establish whether *Cryptosporidium* DNA can routinely be detected in blood and what life cycle stages may be present, particularly as Rosales et al. (2005) confirmed the existence of gamont-like extracellular stages of *C. parvum*,

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Table 1 Molecular results for blood and CSF samples

Sample	Nested PCR 18S	RFLP 18S fragment (214 bp)	Real-time PCR *Cryptosporidium* sp. (mean *Ct* value)	Real-time PCR *Cryptosporidium parvum* (mean *Ct* value)	Real-time PCR *Cryptosporidium hominis* (mean *Ct* value)	*gp60* sequencing
Case 1 blood	+	*Cryptosporidium hominis* *Taq*I: 78 bp, 136 bp *Vsp*I: 157 bp, 57 bp	+ (28.07)	–	+ (22.88)	IeA11G3T3 subtype
Case 1 CSF	+	*Cryptosporidium hominis* *Taq*I: 78 bp, 136 bp *Vsp*I: 157 bp, 57 bp	+ (17.83)	–	+ (14.48)	IeA11G3T3 subtype
Case 2 blood	+	*Cryptosporidium hominis* *Taq*I: 78 bp, 136 bp *Vsp*I: 157 bp, 57 bp	+ (19.73)	–	+ (17.92)	IeA11G3T3 subtype
Case 2 CSF	+	*Cryptosporidium hominis* *Taq*I: 78 bp, 136 bp *Vsp*I: 157 bp, 57 bp	+ (18.17)	–	+ (14.78)	IeA11G3T3 subtype

in cell cultures, by light, phase contrast, and transmission electron microscopy.

The use of blood samples and other fluids such as saliva and urine for the identification of parasitic DNA has been reported for the diagnosis of *Plasmodium* sp., *Entamoeba histolytica*, *Toxoplasma gondii*, *Leishmania* sp., and others (Nwakanma et al. 2009; Haque et al. 2010; Siriyasatien et al. 2016), but this strategy has not been yet described for the diagnosis of cryptosporidiosis. To the best of our knowledge, molecular methods had never before been used to detect *Cryptosporidium* sp. DNA in blood specimens of AIDS patients.

Cryptosporidium subtyping at the *gp60* level in HIV-infected patients has been carried out in only a few countries. Among these descriptions, the global distributed *C. hominis* subtype IbA10G2 was described in HIV patients from Portugal, Jamaica, Malaysia, Ethiopia, Peru, Brazil, and Argentina, while the subtype IeA11G3T3 that was identified in the present study had been previously described in AIDS patients from Ethiopia, India, Nigeria, Kenya, Peru, and Argentina (Alves et al. 2006; Cama et al. 2007; Gatei et al. 2008; Lim et al. 2011; Iqbal et al. 2012; Akinbo et al. 2013; Sharma et al. 2013; Adamu et al. 2014; Mbae et al. 2015; Peralta et al. 2016; Ojuromi et al. 2016; Ukwah et al. 2017).

In our work, we have demonstrated for the first time the presence of *Cryptosporidium* DNA in blood and CSF of HIV-infected patients. These findings are of great value for studying the evolution of cryptosporidiosis in AIDS patients and as a basis for understanding the multi-organ dissemination of *Cryptosporidium*.

Compliance with ethical standards

The research protocol was approved by the Ethical Committee for Research, Hospital Francisco J. Muñiz, protocol 323.

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