

## RESEARCH ARTICLE

# Molecular Identification of *Cystoisospora Belli* in Patients Infected with the Virus Human Immunodeficiency

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## ABSTRACT

Infection with *Cystoisospora belli* causes severe diarrhea, bile duct involvement, and Extra-intestinal spread in patients with acquired immunodeficiency syndrome (AIDS). This study included eight adult patients with AIDS and chronic diarrhea diagnosed stool cystosporiasis (oocysts) and / or duodenal biopsies (asexual and sexual stages in epithelium). Identification was carried out at the molecular level using a nested-polymerase chain reaction (PCR) technique that amplifies a fragment of the ribosomal ribonucleic acid (RNA) small subunit gene, using DNA extracted from feces and biopsies. This work allowed us to make the diagnosis of cystosporiasis through analysis coproparasitological, optimize the DNA extraction protocol from samples fecal and implement the nested -PCR technique for the diagnosis of *C. belli* in biological samples from infected patients.

**Keywords:** AIDS, *Cystoisospora belli*, PCR, Oocyst.

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**Conflict of interest:** None

## INTRODUCTION

Cystosporiasis is a cosmopolitan infection in distribution, but it is more common in the tropics, subtropical zones, some areas of the Middle East, Southeast Asia and South America.<sup>1</sup> The Prevalence studies show that these infections can range from 1 to 18%, while that in AIDS patients the prevalence is between 1 and 5% in industrialized countries and 15% in non-industrialized countries. The severity of the disease is variable according to age and condition immunity of patients. Infection with *C. belli* causes self-limited diarrhea in patients immunocompetent, while it can become severe and debilitating in patients immunocompromised.<sup>2,3</sup> It has been reported that it can also cause cholecystitis,<sup>4-6</sup> and in AIDS patients extraintestinal spread.<sup>7-11</sup> The diagnosis of cystosporiasis is generally made by the finding of oocysts on examination of fecal matter and of different stages in the epithelium of the small intestine or bile ducts.<sup>3,6,12</sup> The diagnosis of *C. belli* by molecular techniques has had a minimal development. Until the present, the use of a pair of specific primers and a hybridization probe has been reported complementary to a small subunit region of ribosomal RNA for detection by PCR and Southern blot in samples from infected patients.<sup>13</sup> Also exist Publications of molecular methods for species-level differentiation of in the genus *Cystoisospora*.<sup>14</sup> The objectives of this work were to describe morphologically the stages of *C. belli* sp. in fluids and tissues of AIDS patients and implement a PCR technique

using DNA from *C. belli* extracted from biological samples of infected patients.

## MATERIALS AND METHODS

Eight adult patients with AIDS and chronic diarrhea (more than one month duration) were included in those who were diagnosed with cystosporiasis.

### Morphological Description in Fluids and Tissues

The stool was processed using the modified Telemann, Willis's concentration methods and Sheather<sup>15-17</sup> and spreads of said concentrates were made that were colored with the Kinyoun technique.<sup>18,19</sup>

### Nested PCR for *C. belli*

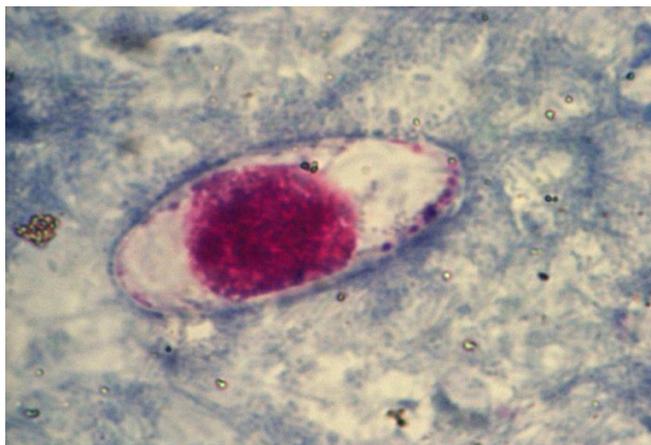
The stool and biopsy samples were processed for DNA extraction, they were applied standard methods of proteinase K digestion, phenol-chloroform extraction, and precipitation in ethanol. Biopsy samples were trypsinized prior to DNA extraction. Tampon lysis used for both materials contained 100 mM Tris-HCl pH 8, 10 mM ethylenediamine tetraacetic acid (EDTA) pH 8, SDS 0.5%, 150 mM NaCl, and 200 µg/mL proteinase K. The DNA samples obtained were used in gene amplification for the diagnosis of *Cystoisospora* according to the protocol described by Müller *et al.*<sup>13</sup> Based on a technique of nested-PCR using primers on the sequence of the gene of the small subunit of the Ribosomal RNA from

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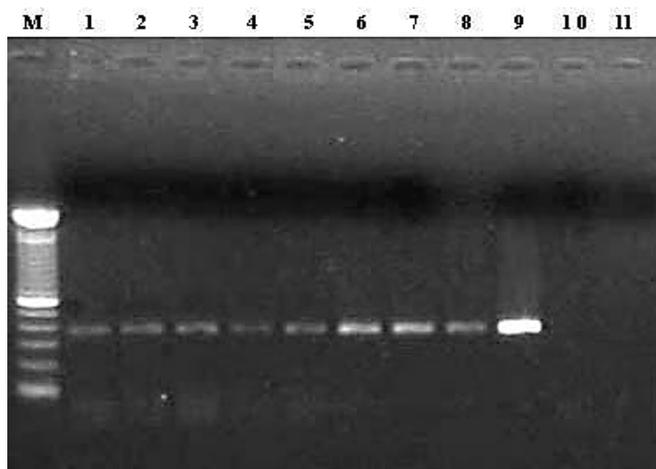
*C. belli* (GenBank accession nos. AF106935 and U94787). The primer set for the first cycle was IsoFO/IsoRO and for the second cycle IsoFI/IsoRI, described by Müller *et al.*<sup>13</sup> used to amplify a 396 base pair fragment (bp). The reaction mixtures were optimized using buffer containing ammonium sulfate.



**Figure 1:** Non-sporulated oocyst of *C. belli* in the concentration pellet of the method Modified Telemann's (400X magnification).



**Figure 2:** Immature oocyst of *C. belli* colored by the Kinyoun technique (1000X magnification).



**Figure 3:** Electrophoresis in agarose gel stained with ethidium bromide, where they are visualized 396 bp amplification products. M Street, 100 bp ladder marker; lanes 1-8, samples from patients, lane 9, positive control; lane 10, negative control; lane 11, reaction mixture.

The amplification steps were those previously described.<sup>13</sup> As a positive control of the amplification reactions, oocyst DNA was used previously purified, human DNA samples were used as a negative control and a reaction control to rule out the possibility of contaminating DNA in each reaction amplification. The amplification products were visualized by electrophoresis in 2% agarose gels, stained with ethidium bromide under UV light. The amplicons of each patient were recovered from the agarose gels and the automatic sequencing of the same in Applied Biosystems equipment.

## RESULTS

In the coproparasitological examination it was possible to identify *Cystoisospora* oocysts. They were presented with their characteristic elliptical shape, 25–30  $\mu\text{m}$  long x 10–19  $\mu\text{m}$  approximately wide and with a sporoblast inside (Figure 1). The sporoblast had a granular appearance, the interior of the oocyst was transparent and with a double wall that was easy to observe.

Recovery was possible by all three concentration methods used. The Visualization in the concentrations by float allowed a more pink hue than the observed in centrifugation. The staining characteristics of *C. belli* in the smears stained with Kinyoun showed a sporoblast from red to fuchsia, with the interior of the oocyst transparent and the double wall (Figure 2).

In the eight cases studied, amplification of the expected 396 bp fragment was possible (Figure 3). The sequence of the 18S gene fragments produced in the 8 cases were identical with those reported for *C. belli*.

## DISCUSSION

*C. belli* is a protozoan belonging to the phylum Apicomplexa, class Coccidia, order Eucoccidiorida, suborder Eimeriorina, family Sarcocystidae.<sup>3,20</sup> The infection is acquired by ingestion of sporulated oocysts that uncyt in the small intestine releasing sporozoites that penetrate epithelial cells. There it occurs asexual (merogonia or sporogony) and sexual (gametogonia) multiplication, giving rise to the formation of oocysts that are eliminated with the feces.<sup>8,20,21</sup> The stool study should be the initial, non-invasive procedure in the search for *C. belli*, taking into account that during the asexual multiplication phase the examination coproparasitological is not suitable. Knowledge of the different concentration techniques and / or coloring allows you to choose, according to its characteristics and available resources, the method appropriate for each diagnostic center.<sup>22</sup> VEDA with taking duodenal biopsies is another method that enables specific diagnosis.<sup>23-25</sup> Histological examination of ultra-thin sections stained with azur II allows visualization of the different stages of the *C. belli* cycle in the cytoplasm of epithelial cells.<sup>26-28</sup> The knowledge and correct identification of these structures is essential because the discovery of any of them is a diagnosis of cystoisosporiasis.<sup>29-33</sup> The VEDA indication is subject to variables such as the general condition of the patient, contraindications, and availability of resources, which may limit their use. This work also allowed us to optimize the DNA extraction protocol from samples fecal

and tissue and implement the nested-PCR technique for diagnosis of *C. belli* in material biology of patients infected with human immunodeficiency virus. The application of different etiologic and molecular diagnostic techniques for the identification parasitic in patients with AIDS improves the prognosis since it enables the administration of specific treatments.<sup>4,34-36</sup>

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