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PREVALENCE AND GENETIC CHARACTERIZATION OF *Cryptosporidium* spp. and *Cystoisospora belli* IN HIV-INFECTED PATIENTS

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SUMMARY

Cryptosporidium spp. and *Cystoisospora belli* are monoxenic protozoa that have been recognized as the causative agents of chronic diarrhea in immunocompromised individuals, especially HIV-infected subjects. The objective of this study was to evaluate the frequency of these intestinal protozoa in HIV-positive patients in the Triângulo Mineiro region of Brazil and to correlate the presence of these infections with clinical, epidemiological and laboratory data of the patients. Oocysts were detected in stool samples of 10 (16.9%) of the 59 patients studied, while *Cryptosporidium* spp. were present in 10.1% (6/59) and *C. belli* in 6.7% (4/59). The frequency of these parasites was higher among patients with diarrheic syndrome and $CD4^+$ T lymphocyte counts < 200 cells/mm^3, demonstrating the opportunistic characteristic of these infections. A significant association was observed between the lack of adherence to antiretroviral therapy and the presence of *Cryptosporidium* spp. and/or *C. belli*. Parasitism with *Cryptosporidium* spp. was more frequent in February and April, the months following the period of high rainfall. The same was not observed for *C. belli*. Genetic characterization of two isolates led to the identification of *Cryptosporidium parvum*, one of the main species associated with the zoonotic transmission of cryptosporidiosis.

KEYWORDS: *Cryptosporidium* spp.; *Cystoisospora belli*; Genetic characterization; HIV; Infectious diseases.

INTRODUCTION

Since the beginning of the AIDS pandemic, opportunistic infections have been recognized as common complications of the HIV infection[8,29]. The rapid dissemination of this virus contributed to the increasing prevalence of opportunistic protozoa among HIV-infected patients, with *Cryptosporidium* spp. and *Cystoisospora belli* being the most relevant species. The clinical manifestations of the disease caused by these protozoa range from self-limited diarrhea, steatorrhea, headache, abdominal pain, fever and weight loss in immunocompetent individuals to chronic diarrhea, cachexy, electrolyte disorders and death in children and adults with immune diseases[14].

Despite their cosmopolitan distribution, intestinal protozoa are more prevalent in tropical and subtropical regions where the climate and sanitary conditions contribute to their maintenance. In Brazil, the prevalence of cryptosporidiosis and cystoisosporosis among HIV-infected patients ranges from 6.4% to 9.1% and from 4.4% to 18%, respectively[1,2,6,7,11,16,18]. OLIVEIRA-SILVA *et al.*[19] investigated the frequency of these parasites in HIV-infected patients living in the Triângulo Mineiro region and found a prevalence of 8.6% for *Cryptosporidium* sp. and of 10.3% for *C. belli*.

Despite the great number of reports about the occurrence of *Cryptosporidium* infection in HIV (+) patients from Brazil, only a few studies have performed the molecular characterization of the isolates found in the fecal samples. Most of these published papers have shown that *C. parvum* and *C. hominis* are the most prevalent species[1,15,25]. With respect to *C. belli*, only one study demonstrated genetic polymorphisms among clinical isolates obtained from HIV(+) patients[23]. Additionally, two isolates were obtained from the same patient with extraintestinal cystoisosporosis, which supports the existence of mixed infection or specific populations which are able to invade and multiply in different host tissues.

The present study evaluated the prevalence of the intestinal protozoa *Cryptosporidium* spp. and *C. belli* among HIV-infected patients who were referred to the hospital of the Universidade Federal do Triângulo Mineiro (UFTM), and the possible association between clinical, epidemiological and laboratory data and these parasitic infections. In addition, clinical isolates were characterized genetically by nested PCR-RFLP of the SSU-rRNA gene of *Cryptosporidium* spp. and by PCR-RFLP of the SSU-rRNA gene of *C. belli*.

MATERIALS AND METHODS

Samples: Between January and August 2005, 120 stool samples

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were collected from 59 HIV-infected patients referred to the hospital of UFTM. For the detection of *Cryptosporidium* spp. and *C. belli* oocysts, the stool samples were concentrated using the formol-ether method[24] and fecal smears were stained using the modified Ziehl-Neelsen technique[13].

Clinical and epidemiological data of the patients, including age, gender, mean $CD4^+$ and $CD8^+$ T lymphocyte count, viral load, use of antiretroviral therapy (ART), and presence of diarrheic syndrome were collected by reviewing the patients' medical records. All patients enrolled in this study received antiretroviral therapy according to Brazilian Ministry of Health's consensus recommendations. The ART regimes were composed of two nucleoside reverse transcriptase inhibitors plus one protease inhibitor or two nucleoside reverse transcriptase inhibitors plus one non-nucleoside reverse transcriptase inhibitor. Data not present in the medical records were excluded from the statistical analysis.

Determination of rainfall indices: The rainfall indices of the municipality of Uberaba, Minas Gerais, were provided by the Instituto de Pesquisas Espaciais (INPE).

Genetic analysis: Genomic DNA was extracted from *Cryptosporidium* spp. and *C. belli* oocysts according to the protocol of BOOM *et al.*[3], modified by PATEL *et al.*[20]. The extracted DNA was purified using the GFX Genomic Blood DNA Purification kit (Amersham Biosciences, Piscataway, NJ, USA) according to manufacturer instructions.

A highly polymorphic region of the SSU-rRNA gene of *Cryptosporidium* spp. was amplified by nested PCR as described by XIAO *et al.*[31]. A first product of approximately 1325 bp was amplified using the primers 18SX1F (5'-AAC CTG GTT GAT CCT GCC AGT AGT C-3') and 18SX1R (5'-TGA TCC TTC TGC AGG TTC ACC TAC G-3'). PCR was carried out in a final volume of 30 µL containing 3mM $MgCl_2$, 200 µM of each dNTP, 2.5 units Platinum *Taq* DNA polymerase (Invitrogen, Carlsbad, CA, USA), 50mM KCl, 200nM of each primer and 5 µL DNA. The amplification conditions were an initial denaturation at 94 °C for three min and 35 cycles of denaturation at 94 °C for 45 s, annealing at 55 °C for 45 s and extension at 72 °C for 40 s, followed by a final extension step for seven min. For amplification of an internal fragment of approximately 825 bp, 2 µL of the first PCR product diluted 1:2 was mixed with the 18SX2F (5'-GGA AGG GTT GTA TTT ATT AGA TAA AG-3') and 18SX2R primers (5'-AAG GAG TAA GGA ACA ACC TCC A-3'). The amplification conditions of the second PCR were the same as those used in the first PCR.

The eukaryote-specific primer 1FPL (5'-GCGGATCCGCGGCC GCTGGTTGATCCTGCCAGT-3') and the universal primer 1520RPL (5'-GCGGATCCGCGGCCGCGGCAGGTTCACCTAC-3') were used to amplify a specific fragment of approximately 1800 bp of the *C. belli* SSU rDNA gene according to previously described protocol[21] modified by RESENDE *et al.*[23]. In short, the reaction was carried out in a final volume of 30 µL containing 2 mM $MgCl_2$, 200 µM of each dNTP, 5% glycerol, 1 unit Platinum® Taq DNA polymerase (Invitrogen, Carlsbad, CA, USA) in a buffer of 10 mM Tris-HCl, pH 8.3, and 50 mM KCl, 670 nM of each primer and 5 µL of DNA. The amplification conditions consisted of an initial denaturation at 95 °C for three min and 40 cycles of denaturation at 94 °C for one min, annealing at 55 °C for one min and extension at 72 °C for two min, followed by a final extension step of 10 min at 72 °C.

Amplification was carried out in a PTC-200 thermocycler (MJ Research, Inc., Watertown, MA, USA) and the amplified products were separated by electrophoresis on 1% agarose gel stained with 0.5 µg/mL ethidium bromide and observed under ultraviolet light.

For analysis of the restriction fragments of *Cryptosporidium* spp., the second PCR product was digested with *Ssp*I and *Vsp*I (New England BioLabs, Inc., Ipswich, MA, USA) according to XIAO *et al.*[31] and for *C. belli*, the 1800 bp fragment of the SSU rDNA gene was digested with *Scr*FI, *Mbo*II and *Rsa*I (New England BioLabs, Inc.) according to RESENDE *et al.*[23]. The restriction fragments generated were separated by electrophoresis on 7.5% nondenaturing polyacrylamide gel and analyzed after silver staining.

Genetic relationships between isolates were determined using the GelCompar II 5.0 program (Applied Maths, Kortrijk, Belgium). The Dice similarity coefficient was used for the calculation of the similarity matrix and the UPGMA method (unweighted pair-group method with arithmetic mean) for dendrogram construction and analysis. For the purpose of comparison, *C. belli* isolate CB14 previously characterized by RESENDE *et al.*[23] was used in the phylogenetic analyses.

Statistical methods: Data were analyzed with the Statistical Package of the Social Sciences (SPSS), version 17.0. The results are reported as absolute and relative frequencies and were compared by Pearson's chi-square and Mann-Whitney tests. A level of significance of $p < 0.05$ was adopted.

RESULTS

In all, 120 stool samples were examined from 59 patients (an average of two samples/patient). Forty-two (71.1%) of the participants were male and 17 (28.8%) were female. The ages of the members of the study group varied from 14 - 56 years with a mean of 35.7 ± 8.9 years.

Oocysts were detected in stool samples of 16.9% (10/59) of these patients, while *Cryptosporidium* spp. were present in 10.1% (6/59) and *C. belli* in 6.7% (4/59). Diarrheic syndrome was observed in 23 (38.9%) of the cases. Of these, 30.4% (7/23) were positive for intestinal protozoa in stool samples, including *Cryptosporidium* spp. in 21.7% (5/23) and *C. belli* in 8.7% (2/23). There was a significant association between diarrheic syndrome and the presence of these protozoa in stool samples ($p = 0.02$). In the group without diarrhea, the prevalence of these protozoa was 8.3% (3/36), with 2.78% (1/36) of the patients being positive for *Cryptosporidium* spp. and 5.56% (2/36) for *C. belli*. No case of mixed infection was observed.

The $CD4^+$ T lymphocyte was quantified in 81.3% (48/59) of the patients included in this study. The mean count was 40 ± 23.7 cells/mm^3 in patients who tested positive for *Cryptosporidium* spp. or *C. belli* and 195.9 ± 201.8 cells/mm^3 in patients with negative stool samples. A significant association was observed between a $CD4^+$ T lymphocyte count < 200 cells/mm^3 and the presence of *Cryptosporidium* spp. or *C. belli* in stool samples ($p = 0.01$). A $CD8^+$ T lymphocyte count could be obtained for 76.2% (45/59) of the patients, but no association was observed between the number of these cells and the presence of these infections ($p = 0.32$).

The viral load was determined in 45.7% (27/59) of the patients

studied, but there was no significant association with cryptosporidiosis or cystoisosporosis (p = 0.21). Thirty-one (51.3%) of the 59 patients regularly used ART. However, only 40% (4/10) of the subjects with these intestinal infections regularly used this therapy. A significant association was observed between the lack of adherence to ART and the presence of *Cryptosporidium* spp. or *C. belli* (p = 0.04).

Analysis of the prevalence of these protozoa according to the time of year and the annual rainfall index showed a higher prevalence of *Cryptosporidium* spp. in February and April, whereas cystoisosporosis mainly occurred in January, February, May and July (Fig. 1).

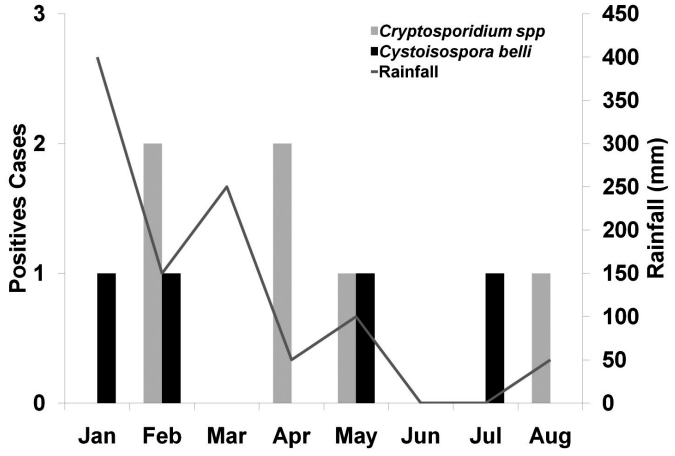

Fig. 1 - The Uberaba rainfall index and seasonal distribution of *Cryptosporidium* spp. and *Cystoisospora belli* in HIV-infected between January and August 2005.

Three (30%) of the 10 clinical isolates were submitted to genetic characterization, including two *Cryptosporidium* spp. isolates and one *C. belli* isolate. In the case of the *Cryptosporidium* spp. isolates, the restriction profiles obtained by digestion with *Ssp*I resulted in fragments of approximately 448, 247, and 106 bp and digestion with *Vsp*I in fragments of 628 and 104 bp. This profile is compatible with *Cryptosporidium parvum* (bovine genotype) (Fig. 2).

The restriction profiles generated by digestion of the specific fragment of *C. belli* with *Scr*FI and *Rsa*I showed 100% similarity with the profile of the isolate used as a control (Fig. 3). Digestion with *Scr*FI produced fragments of approximately 513, 708, and 818 bp, whereas digestion with *Rsa*I resulted in fragments of approximately 181, 339, and 1122 bp (Fig. 3). Digestion of the specific fragment with *Mbo*II generated fragments of approximately 181, 251, 426, 602, and 1013 bp, compatible with restriction profile II[23].

DISCUSSION

In the present study, a high prevalence of *Cryptosporidium* spp. and *C. belli* was observed among HIV-infected patients living in the region of Uberaba and the presence of these parasites was correlated with the occurrence of diarrhea, a reduced CD4+ T lymphocyte count and lack of adherence to ART, demonstrating the opportunistic characteristic of these infections. However, it should be pointed out that even asymptomatic (non-diarrheic) subjects (8.3% of the sample) eliminated oocysts in feces and therefore represent a source of infection for other individuals.

According to OLIVEIRA-SILVA *et al.*[19], low CD4+ T lymphocyte counts are a predisposing factor for protozoal infections. This was demonstrated in the present study in which all patients with cryptosporidiosis or cystoisosporosis had low lymphocyte counts. This finding can be explained through the understanding of immunological disorders in HIV/AIDS patients, who are particularly deficient in a subpopulation of lymphocytes CD4+ T, the main cell population that protects against intracellular parasites[27].

The low rate of adherence to ART observed contributed to the high prevalence of opportunistic infections among the patients studied. Several studies have documented a decline in the prevalence of cryptosporidiosis after the introduction of ART since this therapy suppresses the replication of HIV, leading to an increase in circulating CD4+ T lymphocytes and intestinal repopulation, and subsequently the restoration of mucosal immunity[17,26,28].

Parasitism with *Cryptosporidium* spp. was more frequent in February

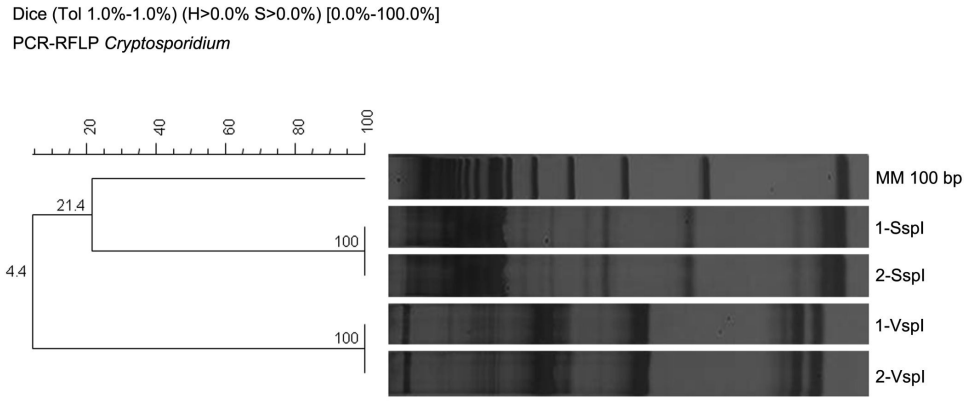

Fig. 2 - Polyacrylamide gel and phenograms of *Cryptosporidium* spp. genetic profiles obtained by *nested* PCR-RFLP. Numbers 1 and 2 correspond to *Cryptosporidium* sp. isolates. The phenograms were created with the software GELCOMPAR II® (Applied Maths) with the Dice coefficient and Unweighted Pair-Group Method Arithmetic. MM - molecular marker.

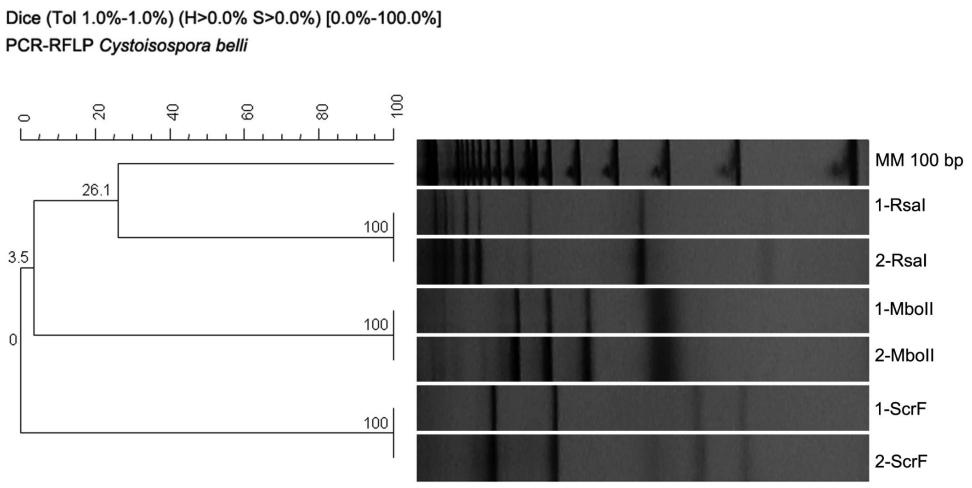

Fig. 3 - Polyacrylamide gel and phenograms of *C. belli* genetic profiles obtained by PCR-RFLP. Lane 1: *C. belli* isolate characterized in the present study; Lane 2: *C. belli* isolate CB14 (included in the analysis for the purpose of comparison). The phenograms were created with the software GELCOMPAR II® (Applied Maths) with the Dice coefficient and Unweighted Pair-Group Method Arithmetic. MM - molecular marker.

and April, the period following the months of high rainfall. Although the occurrence of cryptosporidiosis cases did not differ statistically between seasons, the results are consistent with other studies reporting a higher frequency of cryptosporidiosis in the hotter and more humid months of the year[19,30,32]. This seasonal pattern was not observed for cystoisosporosis, whose chronic character and recurrences are believed to be due to the ability of *C. belli* to infect extraintestinal sites, where the parasite forms tissue cysts that eventually return to the intestine and trigger new pathogenic processes. The prevalence of this infection in HIV (+) may therefore be related more to the immune status of the individual than to the seasonal patterns.

The use of different molecular techniques has led to a better understanding of the epidemiology of cryptosporidiosis, with the identification of different species/genotypes and subtypes of *Cryptosporidium* spp. that infect humans[21]. Although more than five *Cryptosporidium* species/genotypes have been described in humans, *C. parvum* and *C. hominis* are the species responsible for most cases of cryptosporidiosis in the world[4,5,10]. Genetic analysis of the two clinical isolates of *Cryptosporidium* spp. identified them as *C. parvum*, a potentially zoonotic species. The presence of these isolates in HIV-infected patients might be explained by the fact that Uberaba is basically a farming region, which is characterized by a high prevalence of bovine cryptosporidiosis. It is possible that the water reservoirs of the region are contaminated with *Cryptosporidium* spp. oocysts originating from herds found close to the rivers which supply these reservoirs.

Evidence indicates that parasite-related factors such as genotype or differences in virulence between isolates are associated with the intensity of the clinical manifestations of cryptosporidiosis. Variations in the number of excreted oocysts, clinical manifestations and response to treatment are observed in HIV-infected patients with cryptosporidiosis who present similar levels of immunosuppression[12]. In the present study, only one of the patients submitted to genetic characterization presented episodes of diarrhea. Although intraspecies differences might be partly responsible for the variations in clinical manifestations between patients, the lack of adherence to antiretroviral therapy was an important factor. It is known that continuous use of ART suppresses the replication of HIV, leading to an increase in circulating $CD4^+$ T lymphocytes and intestinal repopulation, and subsequently the restoration of mucosal immunity[26].

In the study of RESENDE *et al.*[23], the restriction profiles obtained by digestion of the SSU-rDNA gene of *C. belli* with *Mbo*II demonstrated the presence of genetic heterogeneity among the isolates analyzed. Although in that study it was not possible to correlate the restriction profiles with the clinical manifestations of the patients, the results obtained demonstrate the occurrence of different genotypes infecting humans and open perspectives for the discovery of different routes of transmission of cystoisosporosis. FRENKEL *et al.*[9] suggested a facultatively heteroxenous life cycle for this species, with the formation of extraintestinal tissue cysts in intermediate and/or paratenic hosts. As observed for cryptosporidiosis, the presence of anthropozoonotic transmission would explain the presence of this infection in areas with good sanitary conditions. However, studies investigating a larger number of clinical isolates from different regions are needed in order to determine the extent of heterogeneity between *C. belli* genotypes and the possible relationship of these genotypes with the clinical manifestations and epidemiological characteristics of cystoisosporosis.

RESUMO

Prevalência e caracterização genética de *Cryptosporidium* spp. e *Cystoisospora belli* em pacientes infectados pelo HIV

Cryptosporidium spp. e *Cystoisospora belli* são protozoários monoxenos reconhecidos como agentes causadores de diarréia crônica em indivíduos imunocomprometidos, especialmente aqueles infectados pelo HIV. Os objetivos deste estudo foram o de avaliar a frequência destes protozoários em pacientes HIV - positivos na região do Triângulo Mineiro, Brasil, e correlacionar a presença destas infecções com dados clínicos, epidemiológicos e laboratoriais dos pacientes. Oocistos foram detectados em amostras fecais de 10 (16,9%) dos 59 pacientes estudados,

sendo 10.1% (6/59) das amostras positivas para *Cryptosporidium* spp. e 6,7% (4/59) das amostras positivas para *C. belli*. A frequência destes parasitos foi maior entre pacientes com síndrome diarreica e contagem de linfócitos T $CD4^+ < 200$ cells/mm^3, o que demonstra o caráter oportunista destas infecções. Foi observada uma associação significativa entre a falta de aderência à terapia antiretroviral e a presença de *Cryptosporidium* spp. e/ou *C. belli*. Parasitismo por *Cryptosporidium* spp. foi mais frequente em fevereiro e abril, meses subsequentes ao período chuvoso. O mesmo não foi observado para *C. belli*. A caracterização genética de dois isolados levou à identificação de *Cryptosporidium parvum*, uma das principais espécies associadas com a transmissão zoonótica da criptosporidiose.

AUTHORS' CONTRIBUTIONS

MBOS and DC were responsible for the study design; DCA and DVR participated in the collection of the data and stool samples; DCA, DVR and MCS participated in the processing of samples; DVR was involved in the DNA extraction and molecular analysis; DCA and DVR analyzed and interpreted the data; DCA, MBOS and DVR drafted and revised the manuscript. All authors read and approved the final manuscript. MBOS is guarantor of the paper.

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CONFLICTS OF INTEREST: None declared.

ETHICAL APPROVAL

The study was approved by the Research Ethics Committee of the Universidade Federal do Triângulo Mineiro, Brazil (protocol 712). All subjects provided written informed consent and were included in the study.

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