Cryptosporidium sp in HIV-infected individuals attending a Brazilian university hospital

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ABSTRACT: The aim of the current work was to evaluate the occurrence of Cryptosporidium sp in AIDS patients in a region of São Paulo State, Brazil. Patients were divided into groups according to CD4⁺ T lymphocyte count and use of potent antiretroviral treatment. Two hundred and ten fecal samples from 105 patients were fixed in 10% formalin and subjected to centrifuge formol-ether sedimentation. Slides were stained with auramine and confirmed by modified Ziehl-Neelsen. Cryptosporidiosis occurrence was 10.5% with no relationship among gender, age or the presence of diarrhea. The number of oocysts in all samples was small, independent of CD4⁺ T lymphocyte count, HIV plasma viral load, and presence of diarrhea. These results may be due to the reduced prevalence of opportunistic infections in AIDS individuals after the advent of highly active antiretroviral therapy.

KEY WORDS: diarrhea, viral load, HIV, HAART, Cryptosporidium, CD4-positive T-lymphocytes.

CONFLICTS OF INTEREST: There is no conflict.

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INTRODUCTION

Protozoa such as *Cryptosporidium* sp have become important with the advent of AIDS, being the main cause of infectious gastrointestinal tract diseases (17, 18, 28, 31). *Cryptosporidium* sp. was recognized by Tyzzer (30) in 1907. *Cryptosporidium parvum* is a protozoan species in human infections, invading and residing in epithelial cells of the large intestine; it can also be found in epithelial cells of the bile duct, pancreatic duct, esophagus, stomach, and respiratory tract (4, 17, 18, 29, 31). In prolonged infections, it causes liquid diarrhea, dehydration, obstruction of the bile and pancreatic ducts, and pancreatitis, especially in AIDS (17, 18, 29). Vakil et al. (31) and Flanigan et al. (12) have reported more serious cryptosporidiosis in advanced HIV sufferers and with low CD4\(^+\) T lymphocyte counts, generally less than 50 cells/mm\(^3\).

The most common staining techniques for demonstrating *Cryptosporidium* sp oocysts in feces are Giemsa, auramine, modified Ziehl-Neelsen, dimethyl sulphoxide, safranine and Heine, as well as looking for fluorescent mononuclear antibodies (1, 2, 14, 23, 25, 26). The number of slides analyzed and fecal samples collected from each patient varies depending on the author (2, 16, 27). Blackman et al. (2) analyzed between one and five samples per patient and obtained 96% positivity with the first and 100% with the remaining. Blanshard and Gazzard (3), in AIDS patients with diarrhea, identified 80% of the pathogens in three fecal samples.

The control of HIV viremia with anti-retroviral therapy and the consequent increase in CD4\(^+\) T cells are more effective ways to control cryptosporidiosis in AIDS patients (9, 22).

The objectives of this study were to assess the occurrence of cryptosporidiosis in HIV-infected individuals and its relationship with diarrhea, rural or urban zone origin, CD4\(^+\) and CD8\(^+\) T cell counts, HIV plasmatic viral load, and antiretroviral treatment.

PATIENTS AND METHODS

Patients

One hundred and five HIV-infected patients were studied, from July 2002 to September 2003, at the Tropical Diseases Outpatient Clinic, University Hospital, Botucatu Medical School – UNESP, Botucatu, São Paulo, Brazil. The patients were either from urban and rural zones. Mean age was 35.5 years, varying from 18 to 64, with 57 males and 48 females. Patients without determined HIV plasmatic viral load
or CD4\(^+\) and CD8\(^+\) T lymphocyte counts at least three months before or after feces sampling were excluded.

**Methods**

The 105 patients were divided into the following groups:

G1 – 14 individuals with CD4\(^+\) T <200/mm\(^3\), without antiretroviral treatment.

G2 – 35 individuals with CD4\(^+\) T <200/mm\(^3\), on antiretroviral treatment.

G3 – 42 individuals with CD4\(^+\) T >200/mm\(^3\), on antiretroviral treatment.

G4 – 14 individuals with CD4\(^+\) T >200/mm\(^3\), without antiretroviral treatment.

They were randomly assigned to each group depending on their arrival at outpatient examinations.

Two fecal samples were collected on alternate days from each patient in flasks with 5ml of 10% formalin; these were subjected to centrifuge formol-ether sedimentation (32) and each sample was mounted on three slides stained with auramine and examined for immunofluorescence under optical microscope at 400X. The smears that presented shapes suggestive of *Cryptosporidium* sp were treated by the modified Ziehl-Neelsen technique, which is considered the gold standard, using an optical microscope at 1000X (15, 19).

The laboratorial techniques used to demonstrate *Cryptosporidium* were developed by the main author of the present study, who is a biologist at the Tropical Diseases Experimental Laboratory, Botucatu Medical School, UNESP, SP, Brazil.

All patients were evaluated whether they had diarrhea or not during the feces collection period. Diarrhea was defined as increased stool frequency or loose or watery stools.

Counts of T lymphocytes with CD4\(^+\) and CD8\(^+\) markers were by flow cytometry, using FACScount of Becton Dickinson with FACScount\(^{TM}\) Reagents. Results were expressed as absolute number of cells per mm\(^3\) of blood.

Plasmatic quantification of HIV-1 RNA was made by the branched-DNA (b-DNA) method, using Bayer kit HIV-1 RNA 3.0. Results were expressed as absolute number of copies per ml and log\(_{10}\).

Antiretroviral treatment was indicated in accordance with Brazilian Health Ministry National DST/AIDS Program (6). G1 samples were collected before the start of antiretroviral treatment, and G4 patients had not yet been indicated for treatment.
Statistical Analysis
Group comparisons for age, CD8⁺ T, and log of viral count were by Analysis of Variance followed by the Tukey multiple comparisons method; the Fisher Exact test was used for gender and presence of diarrhea and Cryptosporidium sp oocysts. Significance level was considered as 5% (p<0.05) (11).

This research was approved by the Research Ethics Committee of Botucatu Medical School, UNESP, Botucatu, São Paulo, Brazil.

RESULTS
There were statistical differences among groups for gender (p<0.05); G1 and G2 were predominantly male and G4 predominantly female (Table 1).
There were no statistical differences among groups for age group (p>0.05), except that G1 had a lower mean age than the others.
There were statistical differences in CD8⁺ T lymphocyte counts among G1, G2, and G3 (p<0.001); counts in G3 were higher than those in G1 and G2. There was no difference between G3 and G4 or between G4 and G1 or G2 (Table 2).
HIV plasmatic viral load was performed in 102 individuals; it varied from undetectable to 15,092,650 (log 7.18) copies/ml. G1 had the highest viral load (p<0.05), which was intermediate in G4, and not different in the other groups (Table 3).
There were 11 (10.5%) individuals with Cryptosporidium-sp-positive fecal samples, one in G1, three in G2, and seven in G3; there were no significant differences among groups (p>0.05). It is noteworthy that the number of oocysts was small in all individuals who tested positive for Cryptosporidium sp.
Diarrhea was found in 12 (11.4%) individuals. There was no significant difference among groups (p>0.05); however, there was a higher tendency towards diarrhea in G1 and G2. Five out of the 12 individuals with diarrhea tested positive for Cryptosporidium sp. Only seven (6.7%) individuals lived in the rural zone; all cases of Cryptosporidium sp were in feces from urban zone dwellers.
Table 1. Patient distribution according to gender.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Female</th>
<th>Male</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>04</td>
<td>10</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>28.6%</td>
<td>71.4%</td>
<td>100%</td>
</tr>
<tr>
<td>G2</td>
<td>12</td>
<td>23</td>
<td>35</td>
</tr>
<tr>
<td></td>
<td>34.3%</td>
<td>65.7%</td>
<td>100%</td>
</tr>
<tr>
<td>G3</td>
<td>21</td>
<td>21</td>
<td>42</td>
</tr>
<tr>
<td></td>
<td>50.0%</td>
<td>50.0%</td>
<td>100%</td>
</tr>
<tr>
<td>G4</td>
<td>11</td>
<td>03</td>
<td>14</td>
</tr>
<tr>
<td></td>
<td>78.6%</td>
<td>21.4%</td>
<td>100%</td>
</tr>
<tr>
<td>Total</td>
<td>48</td>
<td>57</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>45.7%</td>
<td>54.3%</td>
<td>100%</td>
</tr>
</tbody>
</table>

G1 = CD4⁺ T count < 200/mm³, without anti-retroviral treatment;
G2 = CD4⁺ T count < 200/mm³, with anti-retroviral treatment;
G3 = CD4⁺ T count > 200/mm³, with anti-retroviral treatment;
G4 = CD4⁺ T count >200/mm³, without anti-retroviral treatment.

For male: (G1=G2) > G3 & G4 (p<0.05); for female: G4 > G1, G2 & G3 (p<0.05); Fisher Exact test.

Table 2. Patient distribution according to CD8⁺ T lymphocyte counts/mm³.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Lymphocytes (CD8⁺)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mean</td>
</tr>
<tr>
<td>G1</td>
<td>640</td>
</tr>
<tr>
<td>G2</td>
<td>694</td>
</tr>
<tr>
<td>G3</td>
<td>1049</td>
</tr>
<tr>
<td>G4</td>
<td>1008</td>
</tr>
</tbody>
</table>

G1= CD4⁺ T count < 200/mm³, without anti-retroviral treatment;
G2 = CD4⁺ T count < 200/mm³, with anti-retroviral treatment;
G3 = CD4⁺ T count > 200/mm³, with anti-retroviral treatment;
G4 = CD4⁺ T count >200/mm³, without anti-retroviral treatment.

G3 > (G1 = G2) (p<0.001); Tukey test.
Table 3. Distribution of HIV plasmatic viral load according to means and standard deviations of log_{10}.

<table>
<thead>
<tr>
<th>Groups</th>
<th>Mean</th>
<th>Standard Deviation</th>
</tr>
</thead>
<tbody>
<tr>
<td>G1</td>
<td>4.8</td>
<td>0.9</td>
</tr>
<tr>
<td>G2</td>
<td>3.8</td>
<td>1.1</td>
</tr>
<tr>
<td>G3</td>
<td>3.6</td>
<td>1.1</td>
</tr>
<tr>
<td>G4</td>
<td>3.9</td>
<td>0.9</td>
</tr>
</tbody>
</table>

G1 = CD4⁺ T count < 200/mm³, without anti-retroviral treatment;
G2 = CD4⁺ T count < 200/mm³, with anti-retroviral treatment;
G3 = CD4⁺ T count > 200/mm³, with anti-retroviral treatment;
G4 = CD4⁺ T count > 200/mm³, without anti-retroviral treatment.
G1 > G2 = G3; G4 was intermediate (p<0.05); Tukey Test.

DISCUSSION

In Brazil, AIDS has predominantly affected men since the beginning of the epidemic, a fact also noticed in the current study. However, the percentage of women in this study was much higher (45.7%) than that generally reported in Brazil (28.2%) (5). This reflects the reality at the Center where this study was carried out: the proportion of infected women has been much higher than the national level (5). In recent years, feminization of the AIDS epidemic has been observed in the whole country, with the men-to-women ratio falling from 25:1 in 1985 to 2:1 in 2002 (5).

The prevalence of opportunistic infections has reduced throughout the world with the introduction of highly active anti-retroviral therapy (HAART) (8, 10, 13). This has also been seen in Brazil with cryptosporidiosis (8, 10, 13). Cimerman et al. (10) found it dropped in prevalence from 24.4% to 6.8% after the introduction of HAART, with higher rates in patients with CD4⁺ T cells < 200/mm³.

In a study from July 1990 to June 1997 at Ribeirão Preto, São Paulo State, Brazil, Capuano et al. (7) found a mean positivity of 6.4% for Cryptosporidium. They attributed this low prevalence in their study population of only HIV-infected individuals to the dry climate and satisfactory sanitation resources of that region. Similar results were obtained by Matos et al. (21) in Lisbon, Portugal, where the prevalence of cryptosporidiosis was 8% in AIDS individuals. In the current study, which also
included only HIV carriers, positivity was 10.5%. The very small number of oocysts found can be explained by the use of HAART in 91% of the patients. It was interesting to note that even though cryptosporidiosis is common in calves (23, 25), no patient with parasitosis in this study came from a rural zone.

In the present study, grouping considered the number of CD4$^+$ T cell lymphocytes per mm$^3$ and the presence or absence of HAART. The highest prevalence of Cryptosporidium sp has been noticed in patients with CD4$^+$ T lymphocyte counts <200, especially below 100 cells/mm$^3$, when the infection undergoes chronification (8, 10). Most individuals with Cryptosporidium sp in their feces had more than 200 CD4$^+$ T cells/mm$^3$, which could explain why the infection was self-limited with few oocysts. In this sense, Cimerman et al. (10) found a low prevalence of cryptosporidiosis in AIDS patients with CD4$^+$ T lymphocyte counts >500. In the current study, Cryptosporidium sp was found at a lower proportion in patients with CD4$^+$ T cells <200/mm$^3$; however, 75% of these presented diarrhea, whereas only 28.6% of those with counts >200 cells/mm$^3$ presented diarrhea. This could be explained by findings from literature in which the infection was self-limited in patients with low immunosuppression (8, 10, 12). In relation to CD8$^+$ T lymphocytes, counts below 180 cells/mm$^3$ open the way to a more serious stage, with the infection chronification (8, 12). In the present study population, only in G1 were CD8$^+$ T counts lower than 180 cells/mm$^3$, with much higher mean values in all groups, which agrees with the low occurrence of cryptosporidiosis.

The parasitosis was not associated with the HIV plasmatic viral load in this study. Okhuysen et al. (24), studying seven individuals with AIDS and cryptosporidiosis, observed that during antiviral therapy, when viral load decreases, several intestinal cytokines are expressed in response to chronic cryptosporidiosis, which is associated with the resolution or improvement of Cryptosporidium. Nevertheless, Maggi et al. (20) had reported no effect of antiviral treatment and viral load on cryptosporidiosis resolution.

In the current study, diarrhea was present in less than 50% patients with confirmed cryptosporidiosis. In some situations, patients presenting Cryptosporidium sp oocysts in their feces are asymptomatic and are characterized as healthy carriers (10, 12). The low frequency of diarrhea among patients of the current study could be due to the presence of HAART in most of them (G2 and G3 patients). It is generally
accepted that the treatment improves the immunological status, which could explain the occurrence of asymptomatic infections (9, 22).

According to Chappell and Okhuysen (9), the use of HAART with the consequent increase in CD4\(^+\) T lymphocytes is the most effective intervention for controlling cryptosporidiosis in AIDS patients. Cryptosporidiosis relapsed in those who interrupted this treatment, suggesting that the infection must remain in a dormant state, with the bile duct as a possible reservoir (17, 20).

Finally, further studies are needed to investigate the influence of HAART on the number of oocysts and the severity of signs and symptoms in AIDS patients.

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REFERENCES


