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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 C*ryptosporidium* 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.

	Gender					
	Female		Male		Total	
Groups	**N**	**%**	**N**	**%**	**N**	**%**
G1	04	28.6	10	71.4	14	100
G2	12	34.3	23	65.7	35	100
G3	21	50.0	21	50.0	42	100
G4	11	78.6	03	21.4	14	100
Total	48	45.7	57	54.3	105	100

G1 = $CD4^+$ T count < 200/mm^3, without anti-retroviral treatment;

G2 = $CD4^+$ T count < 200/mm^3, with anti-retroviral treatment;

G3 = $CD4^+$ T count > 200/mm^3, with anti-retroviral treatment;

G4 = $CD4^+$ T count >200/mm^3, 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/mm^3.

	Lymphocytes ($CD8^+$)		
Groups	**Mean**	**(min–max)**	**Median**
G1	640	(168–1747)	613
G2	694	(187–1728)	611
G3	1049	(300–2000)	892
G4	1008	(521–1851)	986

G1= $CD4^+$ T count < 200/mm^3, without anti-retroviral treatment;

G2 = $CD4^+$ T count < 200/mm^3, with anti-retroviral treatment;

G3 = $CD4^+$ T count > 200/mm^3, with anti-retroviral treatment;

G4 = $CD4^+$ T count >200/mm^3, 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}.

	Plasmatic viral load (log)	
Groups	**Mean**	**Standard Deviation**
G1	4.8	0.9
G2	3.8	1.1
G3	3.6	1.1
G4	3.9	0.9

G1= $CD4^+$ T count < 200/mm^3, without anti-retroviral treatment;

G2 = $CD4^+$ T count < 200/mm^3, with anti-retroviral treatment;

G3 = $CD4^+$ T count > 200/mm^3, with anti-retroviral treatment;

G4 = $CD4^+$ T count >200/mm^3, 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/mm^3.

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

1 AMATO NETO V., BRAZ LMA., DI PIETRO AO., MÓDOLO JR. Pesquisa de oocistos de *Cryptosporidium spp*. em fezes: Comparação entre os métodos de Kinyoun modificado e de Heine. *Rev. Soc. Bras. Med. Trop.,* 1996, 29, 575-8.

2 BLACKMAN E., BINDER S., GAULTIER C., BENVENISTE R., CECILIO M. Cryptosporidiose in HIV-infected patients: Diagnostic sensitivity of stool examination, based on number of specimens submitted. *Am. J. Gastroenterol.*, 1997, 92, 451-3.

3 BLANSHARD C., GAZZARD BG. Gastrointestinal manifestations of HIV infection. *Lancet*, 1996, 348, 379-83.

4 BRADY EM., MARGOLIS ML., KORZENIOWSKI OM. Pulmonary cryptosporidiosis in Acquired Immune Deficiency Syndrome. *J. Am. Med. Assoc.*, 1984, 252, 89-90.

5 BRASIL. Ministério da Saúde. Programa Nacional de DST/AIDS. *Bol. Epidemiol.,* 2002, 1, 14-52.

6 BRASIL. Ministério da Saúde. Secretaria Executiva. Coordenação Nacional de DST e AIDS. Guia de tratamento: recomendações para terapia anti-retroviral em adultos e crianças infectados pelo HIV: 2002-2003. 5.ed. Brasília: Ministério da Saúde, 2003.

7 CAPUANO DM., OKINO MHT., BETTINI MJCB. Freqüência de *Cryptosporidium* sp. e *Isospora belli* em pacientes soropositivos para o HIV na região de Ribeirão Preto, SP – Brasil. *Rev. Inst. Adolf Lutz*, 2001, 60, 11-5.

8 CARR A., MARRIOTT D., FIELD A., VASAK E., COOPER DA. Treatment of HIV-1-associated microsporidiosis and cryptosporidiosis with combination antiretroviral therapy. *Lancet,* 1998, 351, 256-61.

9 CHAPPELL CL., OKHUYSEN P. Cryptosporidiosis. *Curr. Opin. Infect. Dis.,* 2002, 15, 523-7.

10 CIMERMAN S., CASTAÑEDA CG., IULIANO WA., PALACIOS R. Perfil das enteroparasitoses diagnosticadas em pacientes com infecção pelo vírus HIV na era da terapia antiretroviral potente em um centro de referência em São Paulo, Brasil. *Parasitol. Latinoam.,* 2002, 57, 111-9.

11 FISHER LD., BELLE GV. *Biostatistics: a methodology for the health sciences*. New York: Wiley Interscience, 1993. 991p.

12 FLANIGAN T., WHALEN C., TURNER J., SOAVE R., TOERNER J., HAVLIR D., KOTLER D. *Cryptosporidium* infection and CD4 counts. *Ann. Int. Med.*, 1992, 116, 840-2.

13 FOUDRAINE NA., WEVERLING GJ., VAN GOOL T., ROOS MTL., WOLF F., KOOPMANS PP., VAN DEN BROEK P., MEENHORST PL., VAN LEEUWEN R., LANGE JMA., REISS P. Improvement of chronic diarrhoea in patients with advanced HIV-1 infection during potent antiretroviral therapy. *J. Acquir. Immune Defic. Syndr.*, 1998, 12, 35-41.

14 GARCIA LS., BREWER TC., BRUCKNER DA. Fluorescence detection of *Cryptosporidium* oocysts in human fecal specimens by using monoclonal antibodies. *J. Clin. Microbiol.*, 1987, 25, 119-21.

15 GARCIA LS., BRUCKNER DA., BREWER TC., SHIMIZU RY. Techniques for the recovery and identification of *Cryptosporidium* oocysts from stool specimens. *J. Clin. Microbiol.*, 1983, 18, 185-90.

16 GREENBERG PD., KOCH J., CELLO JP. Diagnosis of *Cryptosporidium parvum* in patients with severe diarrhea and AIDS. *Dig. Dis. Sci.*, 1996, 41, 2286-90.

17 GRIFFTHS JK. Human cryptosporidiosis: epidemiology, transmission, clinical disease, treatment and diagnosis. *Adv. Parasitol.*, 1998, 40, 38-85.

18 GROSS TL., WHEAT J., BARTLETT M., O'CONNOR KW. AIDS and multiple system involvement with *Cryptosporidium*. *Am. J. Gastroenterol.*, 1986, 81, 456-8.

19 HENRIKSEN A., POHLENZ JFL. Staining of cryptosporidia by a modified Ziehl-Neelsen technique. *Acta Vet. Scand.*, 1991, 22, 594-6.

20 MAGGI P., LAROCCA AMV., QUARTO M., SERIO G., BRANDONISIO O., ANGARANO G., PASTORE G. Effect of antiretroviral therapy on cryptosporidiosis and microsporidiosis in patients infected with human immunodeficiency virus type 1. *Eur. J. Clin. Microbiol. Infect. Dis.*, 2000, 19, 213-7.

21 MATOS O., TOMAS A., AGUIAR P., CASEMORE D., ANTUNES F. Prevalence of cryptosporidiosis in AIDS patients with diarrhoea in Santa Maria Hospital. *Folia Parasitol.*, 1998, 45, 163-6.

22 MIAO YM., AWAD-EL-KARIEM., FATIH M., FRANZEN C., ELLIS DS., MULLER A., COUNIHAN HM., HAYES PJ., GAZZARD BG. Erradication of cryptosporidia and microsporidia following successful antiretroviral therapy. *J. Acquir. Immune Defic. Syndr.*, 2000, 25, 124-9.

23 MÓDOLO JR., GONÇALVES RC., KUCHEEMBUCK MRG., GOTTSCHALK AF. Ocorrência de criptosporidiose em bezerros na região de Botucatu-SP. *Rev. Bras. Med. Vet.*, 1988, 10, 9-10.

24 OKHUYSEN PC., ROBINSON P., NGUYEN MT., NANNINI EC., LEWIS DE., JANECKI A., CHAPPELL CL., WHITE JR AC. Jejunal cytokine response in AIDS patients with chronic cryptosporidiosis and during immune reconstitution. *J. Acquir. Immune Defic. Syndr.*, 2001, 15, 802-4.

25 ORTOLANI EL. *Padronização da técnica de Ziehl-Neelsen para pesquisa de oocistos de Cryptosporidium: Estudo de alguns aspectos epidemiológicos da criptosporidiose em bezerros de rebanhos leiteiros do Estado de São* Paulo. São Paulo: Universidade de São Paulo, Instituto de Ciências Biomédicas, 1988. 85p. [PhD Thesis]

26 RIGO CR., FRANCO RMB. Comparação entre os métodos de Ziehl-Neelsen modificado e Acid-Fast-Trichome para pesquisa fecal de *Cryptosporidium parvum* e *Isospora belli*. *Rev. Soc. Bras. Med. Trop.*, 2002, 35, 209-14.

27 SHARPSTONE D., GAZZARD BG. Gastrointestinal manifestation of HIV infection. *Lancet*, 1996, 348, 379-83.

28 SOAVE R., JOHNSON JR WD. *Cryptosporidium* and *Isospora belli* infections. *J. Infect. Dis.*, 1988, 157, 225-9.

29 SOUZA LR., RODRIGUES MAM., MORCELI J., KEMP R., MENDES RP. Cryptosporidiosis of the biliary tract mimicking pancreatic cancer in an AIDS patient. *Rev. Soc. Bras. Med. Trop.*, 2004, 37, 182-5.

30 TYZZER EE. A sporozoan found in the pepitic gland of the common mouse. *Proc. Soc. Exp. Biol. Med.*, 1907, 5, 12-3.

31 VAKIL NB., SCHWARTZ SM., BUGGY BP., BRUMMITT CF., KHERELLAH M., LETZER DM., GILSON IH., JONES PG. Biliary cryptosporidiosis in HIV-infected people after the waterbone outbreak of cryptosporidiosis in Milwaukee. *N. Engl. J. Med.*, 1996, 334, 19-23.

32 WALDMAN E., TZIPORI S., FORSYTH JRL. Separation of *Cryptosporidium* species oocysts from feces by using a percoll discontinuous density gradient. *J. Clin. Microbiol.*, 1986, 23, 199-200.