INTESTINAL MICROSPORIDIOSIS IN HIV-POSITIVE PATIENTS WITH CHRONIC UNEXPLAINED DIARRHEA IN RIO DE JANEIRO, BRAZIL: DIAGNOSIS, CLINICAL PRESENTATION AND FOLLOW-UP

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SUMMARY

After the diagnosis of two cases of microsporidial intestinal infection in 1992, in Rio de Janeiro, we have started looking for this parasite in HIV-infected patients with chronic unexplained diarrhea. We have studied 13 patients from Hospital Evandro Chagas, IOC-FIOCRUZ. Fecal specimens from these patients were examined for the presence of Cryptosporidia and Microsporidia, in addition to routine examination.

Spores of Microsporidia were found in the stools of 6 (46.1%) of the 13 patients studied, with 2 histological jejunal confirmations. The Microsporidia-infected patients presented chronic diarrhea with about 6 loose to watery bowel movements a day.

Five infected patients were treated with Metronidazole (1.5 g/day). They initially showed a good clinical response, but they never stopped eliminating spores. After about the 4th week of therapy, their diarrhea returned. Two patients utilized Albendazole (400 mg/day-4 weeks) with a similar initial improvement and recurrence of the diarrhea.

Intestinal Microsporidiosis seems to be a marker of advanced stages of AIDS, since 5 of our 6 infected patients were dead after a 6 month period of follow-up. The present study indicates that intestinal microsporidiosis may be a burgeoning problem in HIV-infected patients with chronic diarrhea in Brazil, which deserves further investigation.

KEYWORDS: Microsporidia, AIDS; Diarrhea; Albendazole therapy; Intestinal infection.

INTRODUCTION

During the last decade human infection caused by species belonging to the Phylum Microspora have been identified with increasing frequency in AIDS patients. These primitive protozoa, called microsporidia, are ubiquitous and occur worldwide as obligate intracellular parasites in most major groups of the animal kingdom. Microsporidia spores, the infective form, present a unique polar coil and lack mitochondria[3].

Five genera have been found so far infecting humans: *Encephalitozoon, Nosema, Pleistophora, Enterocytozoon* and *Septata*. Two species, *Enterocytozoon bieneusi* and *Septata intestinalis*, have been reported as agents of persistent watery diarrhea in human immunodeficiency virus (HIV)-infected patients[29]. *E. bieneusi* is believed to be the most commonly recognized microsporidial agent of diarrhea in AIDS pa-

tients[29]. The second intestinal microsporidia, *S. intestinalis*, recently reclassified as *Encephalitozoon intestinalis*[11], has also been shown to disseminate to the kidneys[4, 22, 26].

Infections caused by these intracellular protozoa present a considerable diagnostic and therapeutic challenge. To date, the reports on the occurrence of these opportunistic agents concerned patients from Europe and North America[4, 5, 9, 17, 21, 27, 28]. Serologic data available from tropical areas suggest that microsporidial infections can be an important problem in such places[6].

In Brazil the first 2 cases of intestinal microsporidial infection were reported in February, 1993 in Rio de Janeiro[20]. Since then, 5 new cases were detected in São Paulo[12] and another 16 were reported from Ceará[31].

The aim of this study was to obtain preliminary data on the occurrence and clinical presentation of intestinal microsporidiosis in AIDS patients under care at Hospital Evandro Chagas, IOC, FIOCRUZ, Rio de Janeiro, Brazil.

PATIENTS AND METHODS

Patients: Thirteen HIV-positive inpatients with chronic unexplained diarrhea, defined as at least 3 loose or watery bowel movements per day, persisting for three to four weeks and with negative routine stool pathogenic bacterial cultures as well as negative tests for ova, cysts and larvae were enrolled in this study. Patients were prospectively evaluated from December 1992 to June 1993 at Hospital Evandro Chagas. They were 6 homosexual males and 7 heterosexual individuals (4 males and 3 females aged 24 to 46 years). HIV infection status was recorded according to the criteria of the Centers for Disease Control, USA[7]. Each patient had a detailed clinical history and physical examination. Blood was drawn for baseline laboratory evaluation which included biochemical and hematologic tests, full blood and CD4+ cell counts, determination of serum β-2-microglobulin, and hepatic enzyme levels.

Diagnosis of intestinal infections: At least three fecal specimens were evaluated for ova, cysts and larvae. Stool samples from each patient were examined for parasites after processing by three different techniques such as thick smear[14], larva recovery[18] and formaldehyde-ether sedimentation[23]. Fecal smears were either stained with fast acid or hot saphranin for *Cryptosporidium*. For microsporidia detection, stools were processed using Weber's chromotrope as per the author's description[28]. Briefly, thin smears of a 1:3 suspension of stools in 10% formalin were prepared, air

dried, fixed in methyl alcohol for 5 minutes and stained with a trichrome-based solution. After differentiation using an alcohol-acid solution, slides were dehydrated in an alcohol series, clarified with xylene, resin-mounted and examined. The specimens containing microsporidia spores were processed for immunofluorescence using anti-*Septata* rabbit serum at 1:400 dilution, as previously described[26]. Standard cultures for detection of Salmonella and Shigella were performed for all patients. Stool samples from some patients were processed for *Campylobacter* and *Mycobacteria* culture.

Colonoscopy, with ileum and colon biopsies, was indicated for one patient. Six patients underwent a jejunal biopsy. Jejunal fragments were fixed in 10% formalin and processed for routine histology. Trichrome staining and the Brown-Brenn method were also used.

Statistical analysis: Statistical analysis was performed using Epi-Info 5.1, a software produced by the Centers for Diseases Control (Atlanta, GA, USA) and World Health Organization. Chi-square analysis, or where this test was inappropriate, Fischer's exact method, was used to determine the significance of differences in frequency distributions.

RESULTS

Patient characteristics: Among the 13 HIV-infected patients with chronic unexplained diarrhea enrolled in the study, 6 presented with microsporidia infection – 3 homosexual and 2 heterosexual males and 1 female – within a period of 6 months. Mean patient age was 35 years (Table 1). The diagnosis of AIDS had been made over a mean period of 42 months before they entered in the study. All patients presented chronic diarrhea, with a mean of 6 loose to watery bowel movements a day, for a mean period of 3.5 weeks. The stools contained no blood or leucocytes. Apart from diarrhea, 5 of 6 patients with microsporidial infection had fever (>38°C) at admission while 4 had cachexia and 5 complained of abdominal pain and anorexia.

Patients submitted to CD4+ lymphocyte counts (3 out of 6 with microsporidial infection) presented a mean pheripheral count of 90 cells/mm^3, ranging from 70 to 129 (normal, 500-1200). All patients fulfilled the CDC criteria for AIDS[7]. In two out of 6 microsporidia-infected patients, serum levels of β-2-microglobulin were measured, and were above normal values in both, 5.5 and 8.4 µl/dl (normal, 1.5-2.5 µl/dl). Patients infected with *Mycobacterium* or *Cryptosporidium* presented with elevated serum alkaline phosphatase levels. The clinical and biological data of the patients studied are summarized in Table 1.

TABLE 1
Summary of clinical features and intestinal pathogens detected in 13 HIV-positive patients
with chronic diarrhea studied in Rio de Janeiro, Brazil.

#	Age	Sex	Abd. pain	Anorexia	Cachexia	Fever	Beta 2 glob	CD4+	Survival (weeks)	Agent
1	35	M	Y	N	Y	N	8.4	–	Alive	Microsporidia
2	33	M	N	Y	N	Y	–	70	Died (6)	Microsporidia
3	46	M	Y	Y	N	Y	–	–	Died (3)	Microsporidia *Cryptosporidium* sp.
4	42	M	Y	Y	Y	Y	5.5	–	Died (2)	Microsporidia *Cryptosporidium* sp. *Isospora belli*
5	35	M	Y	Y	Y	Y	–	129	Died (6)	Microsporidia Mycobacteria
6	24	F	Y	Y	Y	Y	–	72	Died (2)	Microsporidia Mycobacteria
7	35	M	Y	N	N	Y	–	91	Died (4)	Negative
8	35	F	Y	N	N	Y	7.6	–	Alive	Negative
9	34	M	Y	Y	Y	Y	3.8	122	Died (4)	CMV
10	42	M	Y	Y	Y	Y	8	12	Died (3)	Mycobacteria
11	31	M	N	Y	N	Y	11.8	–	Alive	Mycobacteria
12	33	M	N	N	Y	Y	6	41	Died (1)	*Cryptosporidium* sp.
13	36	F	N	N	N	N	–	–	Alive	*Giardia intestinalis*

(N) - absent; (Y) - present

Occurrence of intestinal pathogens: Stool analysis and examination of jejunal and colonic tissues revealed pathogens in 11 (84.6%) of 13 patients. In six (46%) of them microsporidial spores were found. The other 5 patients presented cytomegalovirus (CMV) colitis, intestinal tuberculosis, giardiasis, and cryptosporidiosis (Table 1). Five of the microsporidia-positive patients presented concomitant infections caused by associated pathogens: *Isospora*, *Cryptosporidium*, and *Mycobacterium*. The two patients in whom no intestinal pathogens were found presented spontaneous resolution of the diarrhea during follow-up. All bacterial cultures were negative.

Detection of Microsporidia: Microsporidia spores were found in stool samples from 6 patients; as the sole agent in 2 individuals and associated with other parasites in the 4 remaining positive subjects. In 2 jejunal biopsies, evolutive stages of the parasite were detected as tiny round bodies stained faintly blue by hematoxilyn-eosin in the supranuclear region of epithelial cells (Figure 1). Minor nonspecific inflammatory cell infiltration into the lamina propria and epithelial cells and slight atrophy of villi (villous-to-crypt height ratio about 2:1) were also observed. The two patients shedding microsporidia spores in stools, who underwent jejunal biospy, turned out to be positive for

microsporidia. The anti-Septata serum did not react with any of the microsporidia-positive samples.

Effect of concurrent drugs and tentative theraphy in patients with microsporidiosis: When enrolled in the study, the patients who tested positive for microsporidia were using drugs such as cotrimoxazole, amikacin, ethambutol, ethionamide, ciprofloxacin, pyrazinamide and ketoconazole to maintain the suppression of their previously detected opportunistic infections. Gancyclovir was used for CMV disease and spiramycin for *Cryptosporidium* infection. Six patients, 3 of them with microsporidiosis were receiving zidovudine and 3 of each group were using DDI.

Metronidazole (1.5 g/day for 1 month) was given to 5 microsporidia-positive patients and some improvement of the symptomatology, along with reduction of frequency of bowel movements and number of parasites in the stools, was noticed. However, for all patients, diarrhea always recurred after a period of at least 30 days after the beginning of treatment. Albendazole (400 mg/day for 1 month) was used in two patients after a recurrence with Metronidazole, with a partial response and similar recurrence. In one microsporidia-positive patient, diarrhea resolved spontaneously right after diagnosis, but recurred after 60 days.
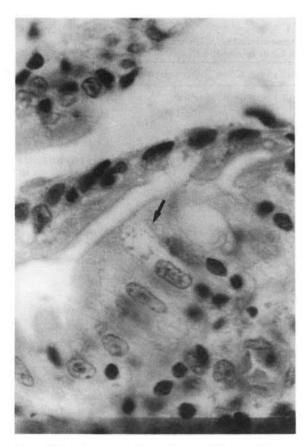
Fig. 1: Histopathology of a jejunal fragment. Microsporidia parasites are seen in a vacuolar space in the supranuclear region of an enterocyte (arrow). Hematoxylin-eosin (X 1000).

Patient follow-up: The group selected for this study consisted of individuals diagnosed with AIDS before diagnosis of microsporidial infection. The time between the first serological anti HIV test, and the intestinal infection ranged from 1 to 6 years. Of the 13 patients analyzed, 8 died between 1 and 6 months after the beginning of the study, and only one of the microsporidia-infected individuals was alive after a 6 month period, always with the presence of microsporidia in the stools despite the therapeutical schedule utilized. Of the 7 patients who were not infected with microsporidia, 2 had their unexplained diarrhea spontaneously resolved, 3 started therapy of their AIDS defining pathology, with an improvement of the symptomatology, and the other 2 died with *Cryptosporidium* and CMV infection.

DISCUSSION

Intestinal microsporidiosis caused by *E. bieneusi* and *S. intestinalis* is being recognized as an emerging opportunistic infection in AIDS patients all over the world. The most common manifestations in people in-fected with *E. bieneusi* and *S. intestinalis* are chronic diarrhea, anorexia and weight loss. The present study documents the existence of intestinal microsporidiosis among HIV-positive patients in Rio de Janeiro, Brazil.

Six patients were found to have microsporidial infection at our Institution within a 6-month period. Heterosexuals were infected as much as homosexuals, indicating that microsporidia infection may probably be waterborne or foodborne.

It is difficult to define precisely the role of microsporidia in the clinical presentation of our patients, since other concomitant opportunistic infections, such as *Isospora*, *Cryptosporidium*, and *Mycobacterium*, were present in 4 of 6 of the microsporidia-infected patients. These agents might also be responsible for the intestinal symptoms, such as the persistent watery diarrhea, anorexia and weight loss observed in the patients.

Interestingly, diarrhea also occurred in the absence of detectable pathogens in 2 patients, who presented spontaneous recovery. This may be related to AIDS enteropathy [15, 16, 24], as a consequence of mucosal alterations due to HIV. One of the patients was infected with *Giardia*, not considered to be an opportunistic agent[25], with consequent treatable gastrointestinal disease. In contrast to previous reports regarding *E. bieneusi*-infected patients[17], in the present study fever was recorded frequently and was attributed to the other associated infections presented by the patients. Elevated serum levels of alkaline phosphatase were found more frequently among the patients infected with *Mycobacterium* and *Cryptosporidium*, even though *E. bieneusi* is now recognized as at least one cause of AIDS-related sclerosing cholangitis[2].

All the patients studied were classified as having AIDS and were severely immunocompromised with a mean CD4+ of 90 cells/mm^3 and/or elevated β-2-microglobulin serum levels (mean of 7.3 1/dl). These findings were consistent with a previous report, since microsporidiosis has been found more frequently among AIDS patients with significantly low CD4+counts[9]. Due to the design of our study, we may have missed milder clinical forms of microsporidia infection, since we only screened patients with chronic diarrhea. In the patients analyzed there was no statistical significance for the parameters studied, either for clinical symptoms or laboratory results.

The six microsporidia-infected patients represented 46% of HIV+ patients with unexplained chronic diarrhea. This frequency of patients infected with

microsporidia was higher than reported in other studies[4,5,9,17,21,27,30] and was probably related to the inclusion criteria, which consisted of performing stool examination using Weber's chromotrope staining only in patients presenting chronic unexplained diarrhea, after previous routine evaluation. However, this higher frequency may be due to the fact that pathogenic protozoa are identified more commonly in people living in developing countries[13,19]. The association of multiple pathogens, mainly *Cryptosporidium* and *Mycobacterium*, might confirm such an epidemiological perspective and might indicate that microsporidia spores should be routinely investigated in all AIDS patients presenting chronic diarrhea, even in those in whom an etiologic agent has been found.

In the present study, a detailed evaluation of each patient, with examination of 3 or more stool samples for each one plus endoscopic procedures, allowed us to determine that 84.6% of the patients had at least one detectable intestinal pathogen, which could be implicated in the etiology of the diarrhea and had not been detected during routine evaluation. Data obtained in this study are similar to those reported by others[1,10,15,25] in developed countries. Microsporidia was the most frequent agent found in our study.

Until recently, Microsporidia infection was diagnosed only after invasive procedures, e.g. duodenal or jejunal biopsies. Electron microscopy is recommended for final species identification[4,22], but was not available in this study. Stool specimen examination using light microscopy allowed the diagnosis of microsporidiosis in all six patients. The modified trichrome staining technique[28] proved to be a valuable tool in the diagnosis of intestinal microsporidiosis. In our study final diagnosis of intestinal microsporidiosis required a careful stool examination. Even though the sensitivity of the technique has not been defined so far, the two patients in whom spores were detected in the stools and who underwent intestinal biopsy presented intracellular microsporidial evolutive forms, which could be observed using light microscopy either on hematoxylin-eosin, trichrome or Brown-Brenn-stained biopsy fragments. The absence of a positive reaction using the anti-*Septata* serum seems to indicate that the species involved in the six cases might be *E. bieneusi*.

Several drugs have been tested for microsporidia infection, but effective therapy for microsporidiosis has not yet been defined. In the present study we observed only transient improvement of the diarrhea using either Metronidazole or Albendazole. All patients presented spores in stools during and after treatment. In contrast to the *Encephalitozoon*-like microsporidian, the species

involved in this study was not susceptible to Albendazole, at 400 mg/day. Other specific antidiarrheic treatments used against the other pathogens detected apparently failed. Antiretroviral therapy did not seem to alter the natural course of the diarrhea. Microsporidia infection may be severe and chronic since available therapy is generally either ineffective or only transiently effective as reported by others when studying infections caused by CMV and *Cryptosporidium*.

The role played by the microsporidia detected in the diarrhea of the present patients is not clear, since they were coinfected with other intestinal pathogens. The present data demonstrate that microsporidiosis occurs in patients with severe immunodeficiency not only in developed areas[8], but also in a tropical developing country such as Brazil. Despite the inclusion criteria used in the study, the mortality rate of microsporidia-infected patients should not be underestimate. This study indicates that intestinal microsporidiosis may be a burgeoning problem in HIV-infected patients with chronic diarrhea in Brazil which deserves further investigation.

RESUMO

Microsporidiose intestinal em pacientes HIV-positivos com diarréia crônica no Rio de Janeiro, Brasil: diagnóstico, clínica e acompanhamento.

Após o diagnóstico, em 1992, de 2 pacientes eliminando esporos de microsporídeos, o presente estudo foi realizado com o objetivo de determinar a ocorrência destes organismos em pacientes HIV-positivos com diarréia crônica sem etiologia definida. O grupo estudado era constituído de 13 pacientes acompanhados no Hospital Evandro Chagas, IOC, FIOCRUZ. Amostras fecais de cada paciente foram examinadas pelos métodos de rotina, além de colorações especiais para a pesquisa de *Cryptosporidium* e de microsporídeos.

Esporos de microsporídeos foram observados nas fezes de 6 (46,1%) dos 13 pacientes. Em 2 a confirmação foi feita por biópsia de jejuno, pela observação de formas evolutivas intracelulares do parasito. Os pacientes infectados por microsporídeos apresentaram diarréia crônica com uma média de 6 evacuações diárias, fezes amolecidas ou aquosas, sem leucócitos ou hemácias. Outros sinais e sintomas incluíram dor abdominal difusa, náuseas, vômitos, anorexia e caquexia.

Em 5 dos pacientes positivos o tratamento com Metronidazol (1,5 g/dia) foi iniciado. Todos apresentaram melhora das manifestações clínicas, in-

clusive com ganho ponderal. Entretanto, todos continuaram eliminando esporos de microsporídeos e houve recorrência da diarréia a partir da 4ª semana de tratamento com Metronidazol. Em 2 dos pacientes foi utilizado Albendazol (400 mg/dia / 4 semanas), com aparente melhora clínica.

A microsporidiose intestinal parece ser doença terminal, pois dos 6 pacientes positivos estudados, apenas 1 continuava vivo após 6 meses de observação. Os dados obtidos sugerem que estas infecções são importantes entre os pacientes com AIDS também no Brasil, de forma semelhante ao que ocorre em outras partes do mundo.

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