Original Article

Intestinal Microsporidiosis in Diarrheal Patients Infected with Human Immunodeficiency Virus-1 in Addis Ababa, Ethiopia

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SUMMARY: Intestinal microsporidiosis is most commonly associated with persistent diarrhea in advanced AIDS cases. To determine the prevalence and clinical manifestations of this infection in HIV/AIDS patients, a single fresh stool sample and blood were collected from 243 (214 HIV-positive and 29 HIV-negative) diarrheal patients. The presence of intestinal microsporidiosis in the stool was determined by Uvitex-2B staining and a PCR-based detection method. HIV screening was done by using ELISA, and reactive samples were confirmed by Western blotting. The CD4+ cell count was analyzed using FACScan. Out of 243 diarrheal patients, 39 (16.0%) cases were positive for intestinal microsporidial infection by either of the methods used. Of the 39, only 18 cases positive by microscopy were also positive by PCR. Based on PCR and microscopic analyses the microsporidial parasites were identified as Enterocytozoon bieneusi (30), Ecephalitozoon intestinalis (6), and double infections (3). All microsporidia-positive cases were HIV-positive, and 92.3% had diarrhea for over 4 weeks. The diarrhea was watery in 79.5% of the patients. Weight loss >10% was recorded in 37 (94.9%) cases. The CD4+ cell count was <100 cells/mm³ in 84.4% of subjects, and 59.4% of the patients had a CD4+ cell count of ≤50 cells/mm³, with a mean of 22.8 cells/mm³. This study revealed that intestinal microsporidiosis is a common cause of chronic diarrhea and severe weight loss in advanced AIDS patients in Ethiopia. This condition is attributable mainly to E. bieneusi. Thus, early diagnosis of intestinal microsporidiosis in HIV/AIDS patients would certainly be helpful in the understanding and management of diarrheal illness.

INTRODUCTION

Microsporidiosis, an infection caused by obligate intracellular spore-forming parasites, is frequently reported and well documented in HIV/AIDS patients and immunocompromised individuals worldwide. It is still a major health problem in developing countries (1-7). However, the wide and intensive application of highly active antiretroviral therapy (HAART) with protease inhibitors to treat HIV/AIDS patients in developed countries has substantially decreased the prevalence and incidence of opportunistic intestinal parasites, including Enterocytozoon bieneusi (6,8).

Six genera of microsporidia are known to infect humans: Enterocytozoon, Encephalitozoon, Nosema, Pleistophora, Trachipleistophora, and Vittaforma (1,9). Infection in humans can occur in many tissues, including the small intestine, kidney, liver, and cornea (10,11). Before 1985, reports of clinical disease related to intestinal microsporidial infections in humans were rare. Since then, E. bieneusi has been identified as an etiologic agent of diarrhea (12), and major clinical symptoms associated with intestinal microsporidiosis in HIV/AIDS patients have been frequently reported (1,2,13-15). Chronic diarrhea, malabsorption, and severe weight loss are the most common clinical syndromes associated with these infections in AIDS patients (1,2,10). Different studies have indicated that infection by E. bieneusi and that by Ecephalitozoon intestinalis are the main causes of chronic watery diarrhea and wasting syndrome in AIDS patients when the CD4+ cell count is <100 cells/mm³ (2,6,16,17).

Except for a few published cases, there is insufficient information on the prevalence and magnitude of the clinical importance of microsporidial infections in HIV/AIDS patients in tropical and developing countries. Some studies in Africa suggested that the incidence of microsporidial infections might be less as might be expected given the higher prevalence of immunosuppression due to HIV/AIDS in the continent (6,14,18,19). However, this could be attributed to a lack of adequate diagnostic methods specific to this parasite in that part of the world. The purpose of the present study was to determine the incidence and clinical features of intestinal microsporidiosis in diarrheal patients with HIV/AIDS in Ethiopia.

MATERIALS AND METHODS

Study subjects: The study was conducted from March 2002 to October 2004 in the army hospital, the police hospital, and St. Paul hospital, Addis Ababa, Ethiopia. A total of 243 individuals were selected based on the following criteria: age greater than 14 years; either sex; and either chronic or acute diarrhea (chronic diarrhea was defined as two or more watery or loose stools per day for more than 28 days, while acute diarrhea was defined as two or more loose or watery...
stools per day for less than 28 days). Informed consent was obtained for each subject. Pre- and post-test counselling was given to the volunteer patients for HIV serological tests by trained counsellors. Clinical and basic demographic information was recorded by the attending physicians of the respective hospitals.

Ethical clearance for the project was obtained from the Ethiopian Health and Nutrition Research Institute (EHNRI) and the National Ethical Committee of the Ethiopian Science and Technology Commission (NEC/ESTC).

**Stool sample collection and processing:** A single fresh stool sample was obtained in a labeled cup from each consenting patient selected for the study. A portion of each fresh stool sample was processed for *Cryptosporidium parvum* and *Isospora belli* oocysts by the modified Ziehl Neelsen method. The remaining portion was processed by a water-ether sedimentation method and stained by Uvitex-2B method as described previously (20). Briefly, 1 g of fresh stool was mixed thoroughly with 8 ml of distilled water in a 15-ml conical test tube. After sieving with cotton gauze, 3 ml of ether was added. The mixture was shaken for a minute and centrifuged at 2,000 g for 2 min. From the sediment, a thin smear was prepared on a microscope slide, allowed to air dry, and fixed in methanol for 5 min.

The slides were again allowed to air dry and stained with Uvitex-2B (Ciba-Giegy, Basel, Switzerland) for 10 min. Then the slides were washed in PBS and counterstained with Evans blue (Sigma Chemical, St. Louis, Mo., USA) for 30 s, further washed in PBS for 5 min, and gently rinsed in tap water. Finally, the slides were examined under a fluorescent microscope with a 50-W mercury high-pressure lamp, an water. Finally, the slides were examined under a fluorescent microscope with a 50-W mercury high-pressure lamp, an excitation filter transmitting 355 - 425 nm, and a suppression filter of 460 nm (Leitz Ploemopak Filter Block D; Ernst Leitz) and analyzed with Simulset software (Becton Dickinson) included with the FACScan, following the manufacturer’s instruction.

**Viral load determination:** The HIV-1 viral load was determined by measuring the HIV-1 RNA in plasma samples using Nucleic Acid Sequence-Based Amplification, Nuclisens NASBA (Organan Teknika BV, Bxctel, the Netherlands) and reactive samples were confirmed by Western blot assay (HIV Blot 2.2; Genelabs Diagnostics, The Cavendish Singapore Science Park, Singapore).

**DNA extraction and polymerase chain reaction (PCR) for detection of intestinal microsporidia from stool samples:** DNA was isolated from fresh stool suspension prepared in 200 μl PBS containing 2% polyvinylpolypyrrolidone (PVPP) (Sigma) and heated for10 min in a heat block at 100°C. Sodium dodecylsulphate-proteinase K was added and allowed to incubate in the heat block at 55°C for 2 h. After incubation, DNA was extracted by using the QiAamp Tissue kit spin columns in an Eppendorf microcentrifuge (Qiagen, Hilden, Germany) following the manufacturer’s instructions.

**Amplification:** DNA was amplified by nested PCR as described previously (21). In the first PCR, three outer primers (MSP-1, TGA ATG KGT CCC TGT; MSP-2A, TCA CTC GCC GCT ACT; MSP-2B, GTT CAT TCG CAC TAC ) were used. The second (nested) PCR was run by taking 2 μl of the first PCR product to the mix containing three inner primers (MSP-3, GGA ATT CAC ACC GCC CGT CRY TAT; MSP-4A, AA ARG GGT; MSP-4B, CAA AGC TTA TGC TTA AGT CCA GAGG AG). PCR was amplified in a volume of 40 μl reaction mixture with 10 × PCR buffer 100 M Tris-HCl, pH 9.0, 15 nm MgCl₂, 500 nm KCl, 1% Triton X-100, 0.1% (w/v) gelatin (HT Biotechnology, Cambridge, UK), 200 μl of each of A, T, G, and C nucleotides; 25 pmol of each primer, 1 U of Taq polymerase (Super Taq HC; HT Biotechnolohy) and 2 μl of the DNA samples and run using the GeneAmp PCR system 9600® (Perkin-Elmer Cerus, Norwalk, Conn., USA). The PCR cycling parameters were 25 cycles following initial denaturation of DNA at 94°C for 4 min, followed by denaturation at 94°C for 30 s, primer annealing at 55°C for 45 s, elongation at 72°C for 45 s, and 72°C for 7 min for final extension.

The nested PCR was performed in the same condition with the addition of primers MSP3, MSP4a, and MSP4b on 2 μl of the first PCR product. The final PCR product was resolved by 2% agarose gel electrophoresis with TBE buffer (1 M Tris base, 0.8 M boric acid, 0.01 M ethylenediamine tetra acetic acid, pH 8.0) at 100 v. The gel was read under UV illumination using an instant camera (Polaroid, Waltham, Mass., USA).

### RESULTS

Out of 243 diarrheal stool samples, 39 (16.0%) cases were positive for intestinal microsporidial infections by PCR. Of these, only 18 (7.6%) cases detected by microscopy were also positive by PCR (Table 1). All 39 (18.2%) of the cases were from among the 214 HIV/AIDS diarrheal patients. Based on PCR and microscopic analyses, the microsporidial parasites were identified as *E. bieneusi* (30), *E. intestinalis* (6), and double infections (3) (Figure 1). The spores of *E. intestinalis* were larger than those of the *E. bieneusi* and varied considerably in both shape and size, either broad and rod-like or kidney-shape (Figures 2 and 3).

Of the 39 patients with microsporidial infections, 36 (92.3%) had diarrhea for more than 4 weeks. The majority were male and all were adults, with an age range of 24-48 years (mean 36 years). Severe (>10%) weight loss was recorded in 37 (94.4%) of the cases. Other clinical indications were found, including: anorexia in 29 (74.4%), abdominal pain with cramping in 25 (64.1%), and vomiting in 18 (46.2%), recorded in 37 (94.4%) of the cases. Other clinical indications were found, including: anorexia in 29 (74.4%), abdominal pain with cramping in 25 (64.1%), and vomiting in 18 (46.2%), among others (Table 2).

Concurrent infection with other opportunistic intestinal parasites was also observed. These included *C. parvum* in 4/39 and *I. belli* in 4/39 cases.

The CD4+ cell count was below 100 cells/mm³ in 27/32 (84.4%) of the cases, and 59.4% of the patients had cell counts of less than 200 cells/mm³, with a mean of 22.8 cells/mm³. A few individuals had counts greater than 100

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<th>Microscopy</th>
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</tr>
<tr>
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<tr>
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**Table 1. Results of microscopy and PCR for intestinal microsporidiosis on 243 stool samples**
or less than 200 cells/mm³. The majority (25/28; 89.3%) of diarrhea patients with intestinal microsporidiosis had HIV-1 plasma viral load counts of at least 10,000 copies/ml, with a mean HIV-1 RNA viral load of 22,217 copies/ml, a reflection of low CD4+ levels. The lower detection limit of Nuclisen NASBA was 80 HIV-1 RNA copies/ml.

**DISCUSSION**

Diarrhea is a common clinical feature in HIV-1-infected individuals. In tropical and subtropical countries, HIV-infected individuals had chronic diarrhea associated mostly with significant weight loss (14,16,17,22). The aetiology of diarrhea in HIV/AIDS patients is quite various: bacterial, viral, parasitic, mainly opportunistic intestinal parasites or HIV-induced entropathy (23). Similarly, in this study most of the HIV/AIDS patients with microsporidial infection had severe weight loss (>10% of body weight) and chronic diarrhea lasting more than 4 weeks. In the present study, the majority of the parasites identified were *E. bieneusi* (77%) followed by *E. intestinalis* (15.4%), excluding the 3 cases (7.7%) of double infection. This finding partially deviates in magnitude from previous findings, where 90% of chronic diarrhea in HIV/AIDS patients caused by intestinal microsporidiosis was attributed to *E. bieneusi* (14,18,19). Based on this observation, in Ethiopia the cause of chronic diarrhea in HIV/AIDS patients might not be predominantly one species but could be both species, with a high preponderance of *E. bieneusi* over *E. intestinalis*. Other studies elsewhere have reported that the incidence of *E. intestinalis* in the diarrhea HIV/AIDS patients is rare (20,24). The severity of diarrheal illness is aggravated by co-infection with coccidian intestinal parasites (18,25). In this study, 4 (10.2%) of *C. parvum* and *I. belli* each, were found to be co-infected with intestinal microsporidiosis which might worsen the condition of diarrheal illness in some HIV/AIDS patients.
Like most intestinal parasites, the route of transmission is mainly fecal-oral contamination of food or drinking water. This makes it more difficult to reduce the risk of transmission of intestinal microsporidiosis in developing countries (1,4,6). Where antiretroviral therapy is not accessible, it is essential for HIV-infected individuals to maintain good personal hygiene and to handle drinking water safely (mostly by boiling it before drinking).

It has been reported that the AIDS patients who are at highest risk for intestinal microsporidiosis are those with CD4+ cell counts below 100 cells/mm³ (16). In the present study, more than half of HIV/AIDS diarrheal patients with intestinal microsporidiosis had CD4+ cell counts below 50 cells/mm³, and the majority were below 100 cells/mm³, indicating that infection with intestinal microsporidiosis occurred under a high degree of immunosuppression during the late stage of HIV infection. The majority of the diarrhea patients were categorized as having an HIV-1 viral load ≥10,000 copies/ml, which is directly associated with low CD4+ cell counts. This indicates that intestinal microsporidiosis in HIV/AIDS patients is more apparent when patients are severely immunocompromised.

The major clinical symptoms observed were chronic diarrhea associated with severe weight loss. Another common clinical symptom was anorexia. Similar clinical observations were documented by others elsewhere (2,4).

Based on the World Health Organization clinical staging criteria (26), most of the intestinal microsporidia-infected patients were categorized as stage III or IV. The associated clinical features commonly noted in the late stage of HIV infection with intestinal microsporidiosis were chronic diarrhea for more than 1 month, severe weight loss, and oral candidiasis, and one individual had pulmonary tuberculosis within the previous year.

In Africa, information regarding intestinal microsporidiosis in HIV/AIDS patients is very limited. This is due to a lack of appropriate diagnostic methods and to a lack of experienced and skilled health care workers. In addition, the spores are very small; those of Enterocytozoon bieneusi averaged 1.5 × 1.0 μm and those of Enterobius vermicularis were 2.2 × 1.2 μm (7); these values are consistent with our observation. In the present study, PCR detected more positive microsporidia cases than did light microscopy; this was also similar with a previous study conducted elsewhere (21). Moreover, when stool contained undetectable levels of spores, the light microscope would not have been useful. Similar observation was recorded by Gumbo et al. (19) in Zimbabwe, where microscopy detected intestinal microsporidia in 18% of diarrhea patients, whereas PCR detected them in 51%. In the same country, Van Gool et al. (18) reported 11% of intestinal microsporidia using the Uvitex-2B staining method in AIDS patients. Both studies detected E. bieneusi. The other importance of PCR is that the use of appropriate PCR primers enables species identification (27), so that it could be important for treating E. intestinalis with albendazole (25,28). So far, there is no widely recognized and applied treatment for E. bieneusi.

This study demonstrates that intestinal microsporidiosis (E. bieneusi and E. intestinalis) is an important cause of chronic diarrhea in AIDS patients in Ethiopia. Furthermore, cases of intestinal microsporidiosis appear to be associated with severe immunosuppression, i.e., with CD4+ cell counts below 50 cells/mm³, where E. bieneusi is the dominant species. Thus, it is imperative to treat this kind of parasite along with other opportunistic intestinal parasites by initiating HAART with protease inhibitors, which may help to reduce the risk of developing diarrhea.

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