

# INTESTINAL MICROSPORIDIOSIS IN HIV-INFECTED CHILDREN WITH ACUTE AND CHRONIC DIARRHEA

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**Abstract.** A prospective study of intestinal microsporidiosis in HIV-positive children was conducted at the Queen Sirikit National Institute of Child Health and Phramongkutkloa Hospital, Bangkok, Thailand. Hospitalized HIV-positive children with and without diarrhea were enrolled in this study. Microsporidial spores identified by calcofluor fluorescent and gram-chromotrope stain were confirmed by electron microscopy. As well as *Cryptosporidium parvum*, Microsporidia was the most common protozoa found in the present study, each was 7.1%. Microsporidia was significantly more common in those who had diarrhea. Intestinal microsporidiosis was found in HIV-positive children with both acute and chronic diarrhea. This study emphasizes the importance of Microsporidia in HIV-infected children. Early detection of microsporidia could be of benefit for the patients, since the infection is treatable.

## INTRODUCTION

Microsporidia, obligate intracellular protozoan, have gained increasing attention as an opportunistic infection in AIDS patients since the first report of chronic diarrheal case in 1985 (Desportes *et al*, 1985). Of the genera associated with chronic diarrhea, *Enterocytozoon bieneusi* is the most recognized intestinal microsporidiosis while *Encephalitozoon intestinalis* causes both intestinal and disseminated diseases (Cali *et al*, 1993; Orenstein *et al*, 1992). Patients with intestinal microsporidiosis usually present with chronic watery diarrhea. Abdominal cramping and weight loss is also not uncommon. In addition, sclerosing cholangitis, hepatitis and peritonitis in these patients have been reported. The prevalence of intestinal microsporidiosis in HIV-positive patients with chronic diarrhea varies from 10-50% (Canning and Hollister, 1990; Molina *et al*, 1993; Rabenek *et al*, 1993; Kotler and Orenstein, 1994). In Thailand, Wanachiwanawin

*et al* (1998) showed that 33.3% of chronic diarrhea in adult patients with AIDS were caused by microsporidia. Distribution of intestinal microsporidiosis in HIV-infected children in Thailand has not been extensively studied. We conducted a study of parasitic infection emphasis on microsporidia in HIV-infected children with and without diarrhea during 1999-2000.

## MATERIALS AND METHODS

HIV-infected children aged less than 15 years old who were hospitalized in the Queen Sirikit National Institute of Child Health and Phramongkutkloa Hospital were enrolled in this study. Diarrhea was defined as the passage of three or more loose or watery stools in a 24-hour period. The characteristics of each patient concerning age, sex, history of diarrhea and other gastrointestinal symptoms were recorded. Individual data were compared to the result of stool examination.

Stool specimens were collected for 3 consecutive days, examined for any parasitic infections and cultured for routine bacterial pathogens. Fresh stool specimens were ex-

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amed for ova and parasites by using simple smear preparation with saline and lugol iodine solution and formalin-ethyl acetate concentration technique. Stool specimens were preserved in polyvinyl alcohol (PVA) and 10% formaldehyde for special stains.

Stool smears stained with modified acid-fast were used for the detection of oocysts of *Cryptosporidium parvum* and other intestinal coccidia. Calcofluor white M2R (Fluorescent Brightener 28) and gram-chromotrope stain were performed for the detection of microsporidial spores. Calcofluor fluorescent staining was examined under a fluorescent microscope at a wavelength 390-420 nm with a 450 nm wavelength fluorescent filter to reveal fluorescent spores (Vávra *et al*, 1993). Smear slides were also stained with gram-chromotrope to identify microsporidial spores (Moura *et al*, 1996). Each specimen was evaluated with agreement by two experienced investigators for detection of microsporidial spores.

To confirm the diagnosis of microsporidial spores, transmission electron microscopy (TEM) was performed (Visvesvara *et al*, 1991). Fecal debris was removed by straining, washing twice in PBS pH 7.4 and followed by centrifugation at 500g for 5 minutes. The pellets were then preserved with 4% paraformaldehyde. Small aliquots of stool samples were processed for EM. Samples were washed and fixed at 4°C in 2% buffered gluteraldehyde and postfixed for 30 minutes in 1% OsO<sub>4</sub> after repeated rinsing with buffer (pH 7.2). Samples were dehydrated in ethanol and embedded in Spurr-Low medium. Ultrathin section were performed and then stained with saturated uranyl acetate

for 5 minutes in 50% ethanol and for 3 minutes in 0.03% lead citrate. They were examined with an electron microscope and documented on film.

## RESULTS

Between July 1999 to June 2000, 141 HIV-infected children were enrolled in the study. Age range of the children was 1 month to 9 years old, 72 were boys and 68 were girls. 83 patients were admitted in the hospital because of diarrhea while 58 patients had no diarrheal symptom. There was no significant difference concerning age, sex, taking antiretroviral agent and *Pneumocystis carinii* prophylaxis between the groups with and without diarrhea (data not shown). Parasitic infections were detected in 22 of 141 (15.6%) patients. There was no significant difference of prevalence of parasitic infection found between the two groups. However, subacute and chronic diarrhea was significantly higher in the group with parasitic infections ( $p = 0.013$ ). Intestinal parasitic infections in HIV-positive children with and without diarrhea are summarized in Table 1. Two pathogenic protozoa, *C. parvum* and microsporidia were the most common protozoa found in this study, each was 7.1%.

Microsporidia and *C. parvum* were diagnosed in both HIV-positive children with and without diarrhea. *C. parvum* occurred in 9.6% of children with diarrhea and 3.4% of those without diarrhea. Microsporidia were found in 10.8% of children with diarrhea which was significantly higher compared to 1.72% in those without diarrhea ( $p = 0.038$ ). The characterization of those who were positive

Table 1  
Parasitic infections in the enrolled HIV-positive children with and without diarrhea.

Parasites	Patient with diarrhea (83)	Patient without diarrhea (58)	Total (141)
Microsporidia	9 (10.8%)*	1 (1.72%)	10 (7.1%)
<i>Cryptosporidium parvum</i>	8 (9.6%)	2 (3.4%)	10 (7.1%)
<i>Blastocystis hominis</i>	-	2 (3.4%)	2 (1.4%)
<i>Trichomonas hominis</i>	-	1 (1.7%)	1 (0.7%)

\* $p < 0.05$  by chi-square (95% CI).

Table 2  
 Characteristics of patients with microsporidial spore detected.

Case No.	Age (month)	Sex	Percent of CD4	CD4 count	AntiHIV agent	Diarrhea	Duration of diarrhea	Pathogenic bacteria
1	23	M	15	ND	No	Yes	7	+
2	9	F	ND	ND	No	Yes	3	-
3	37	M	1	40	No	Yes	30	-
4	20	M	32	1,061	Yes	No	-	-
5	57	M	ND	ND	No	Yes	5	+
6	28	M	34	1,316	Yes	Yes	6	-
7	9	M	14	1,778	Yes	Yes	1	-
8	24	M	Too low to count	Too low to count	No	Yes	1	+
9	40	F	1	30	No	Yes	30	-

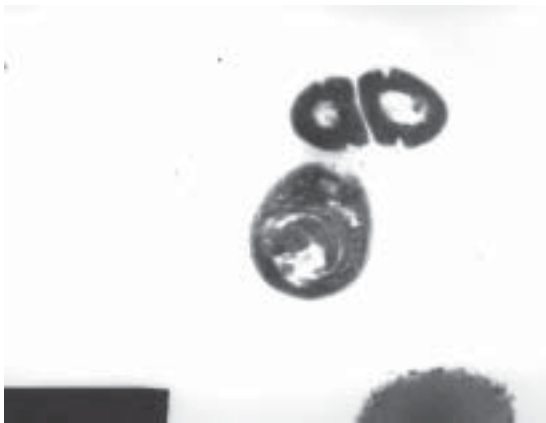


Fig 1A—Transmission electron microscopy of microsporidial spore showing coiled polar filament (original magnification, X 46,200).

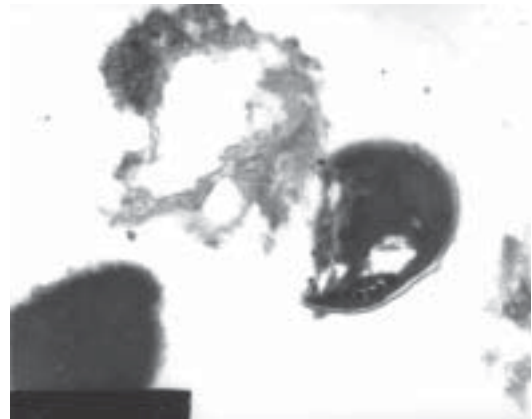


Fig 1B—A typical spore of *Enterocytozoon bienewisi* showing polar filament arranged in 2 rows (original magnification, X 46,200).

for microsporidial spores is shown in Table 2. Unfortunately, the data of one microsporidia-positive diarrheal patient went missing.

A total of 44 smear stools presenting ovoid spores with bright yellowish fluorescence was detected by calcofluor staining. Only ten specimens were found to be positive for microsporidial spores stained by gram-chromotrope. Fig 1A is an electron micrograph showing the ultrastructure of a microsporidial spore that is oval in shape with thick walled

plasma membrane with coiled polar filament. Spores of *Enterocytozoon bienewisi* were identified in all cases, with the typical characteristic *ie* 4 to 7 coils of the polar filament arranged in 2 rows was detected (Fig 1B).

## DISCUSSION

The present study showed that both microsporidia and *C. parvum* were the common parasitic infection found in HIV-positive children.

As previously reported, subacute and chronic diarrhea in these children were significantly associated with parasitic infections (Laughon *et al*, 1988; Smith *et al*, 1988). The clinical outcomes of these parasitic infections have been linked to the status of host immunity (Flanigan *et al*, 1992; Hutin *et al*, 1998). Chronic diarrhea and wasting syndrome found in intestinal microsporidiosis have been clearly correlated to the level of CD4 lymphocyte counts in HIV-positive patients. A case-control study done by Hutin *et al* (1998) found that the association between microsporidiosis in HIV-infected patients and a CD4 lymphocyte count of less than 100 cell/mm<sup>3</sup>. In our study, 2 cases with chronic diarrhea who had CD4 counts less than 50 cell/mm<sup>3</sup> were positive for microsporidia. In contrast to other studies, most of the cases positive for microsporidia had acute diarrheal illness. Some of the cases were coinfecting with pathogenic bacteria so the actual cause of diarrhea may be difficult to determine. However we found intestinal microsporidiosis significantly more common in those who had diarrhea. Microsporidiosis may be associated with acute self-limited diarrhea in the patient with less severe immunosuppression. The clear example of the typical characteristics of opportunistic infection is that found in cryptosporidiosis. Acute self-limited diarrhea has been found in HIV-positive patients with CD4 count more than 180 cell/mm<sup>3</sup> while chronic diarrhea and other severe symptoms were common in the patients who had CD4 count less than this level (Flanigan *et al*, 1992). A case of *Enterocytozoon bieneusi* infection in an immunocompetent patient who had acute diarrhea has also been reported (Sandfort *et al*, 1994). This suggests that microsporidia may be an unrecognized cause of acute self-limited diarrhea. Microsporidiosis might be a common infection in immunocompetent hosts since high seroprevalence of antibodies against *Encephalitozoon* in pregnant French women and Dutch blood donors was detected (van Gool *et al*, 1997). On the other hand, microsporidia found in these patients might only be one of harmless natural human parasites since we also found these parasites in a patient who had no diarrheal illness during admission. In addition, we recently

found 4% of asymptomatic intestinal microsporidiosis in the HIV-negative orphans and child-care workers (Mungthin *et al*, submitted). More studies of prevalence of intestinal microsporidiosis in variety groups of population and clinical manifestations are necessary.

Comparing to the previous study of intestinal microsporidiosis in Thailand which found 33.33% in HIV-positive adult patients with chronic diarrhea, our results are approximately 5 times lower. One of the reasons may be the different group of study population. We included all cases of diarrhea while the previous study did only chronic diarrheal cases. These also explain the wide vary range of prevalence of microsporidiosis reported elsewhere. The other possible explanation is the different risk factors between adult and children. A study by Hutin *et al* (1998) showed that intestinal microsporidiosis was linked to particular behaviors *ie* male homosexual and swimming in the pool. Although there is no study of the risk factor of intestinal microsporidiosis in HIV-positive children, it should be different in some way.

Another important factor determined the variation is the diagnostic method. TEM is still the gold standard, however, it is not practical for the routine diagnosis of microsporidiosis. Several special staining methods such as modified trichrome, gram-chromotrope and chemofluorescence have been developed in order to replace the TEM. However microsporidial spores detected by special stains are difficult to distinguish from bacteria. The acceptable staining method for standard diagnosis has been developed. In this study, we used calcofluor fluorescent stain as the first step for rapid screening of microsporidial spores. Ovoid fluorescing structures were detected under the fluorescence microscope. The technique is simple and quick to perform but calcofluor stain was not specific enough to differentiate microsporidial spores from bacteria, fungus or fecal elements. Gram-chromotrope staining method was chosen to confirm the detection under the light microscope. We experienced that microsporidial spore stained by gram-chromotrope was easy to identify even when presented in small number. The stain could

be done for fresh or preserved stool in 10% formalin or 4% paraformaldehyde. In our experience, screening by calcofluor stain followed by gram-chromotrope stain for the confirmation is a good choice for routine laboratory examination.

In conclusion, our data emphasize that microsporidia is an important cause of diarrhea in HIV-positive children in Thailand. Awareness on the part of clinicians and laboratory technicians is essential for proper diagnosis. Early detection of microsporidia in stool samples are important to HIV-infected patients since the infection is treatable.

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#### REFERENCES

- Cali A, Kotler DP, Orenstein JM. *Septata intestinalis* ng, nsp, an intestinal microsporidian associated with chronic diarrhea and dissemination in AIDS patients. *J Protozool* 1993; 40: 101-12.
- Canning EU, Hollister WS. *Enterocytozoon bieneusi* (Microspora): prevalence and pathogenicity in AIDS patients. *Trans R Soc Trop Med Hyg* 1990; 84: 181-6.
- Desportes I, Le Charpentier Y, Galian A, et al. Occurrence of a new microsporidian: *Enterocytozoon bieneusi* ng, nsp in the enterocytes of a human patient with AIDS. *J Protozool* 1985; 32: 250-4.
- Flanigan T, Whalen C, Turner J, et al. Cryptosporidium infection and CD4 counts. *Ann Intern Med* 1992; 116: 840-2.
- Hutin YJF, Sombardier M-N, Liguory O, et al. Risk factors for intestinal microsporidiosis in patients with human immunodeficiency virus infection: A case-control study. *J Infect Dis* 1998; 178: 904-7.
- Kotler DP, Orenstein JM. Prevalence of enteric pathogens in HIV-infected individuals referred for gastrointestinal evaluation. *Am J Gastroenterol* 1994; 89: 1998-2002.
- Laughon BE, Druckman DA, Vernon A, et al. Prevalence of enteric pathogens in homosexual men with and without acquired immuno-deficiency syndrome. *Gastroenterology* 1988; 94: 984-93.
- Molina JM, Sarfati C, Beanvais B. Intestinal microsporidiosis in human immunodeficiency virus-infected patients with chronic diarrhea: prevalence and clinical and biological features. *J Infect Dis* 1993; 167: 217-21.
- Moura H, da Silva JL, Sodre FC, et al. Gram-Chromotrope: a new technique that enhances detection of microsporidial spores in clinical samples. *J Eukaryot Microbiol* 1996; 43: 94S-5S.
- Mungthin M, Suwannasaeng R, Naaglor T, Areekul W, Leelayoova S. Asymptomatic intestinal microsporidiosis in Thai orphans and child-care workers. (Submitted).
- Orenstein JM, Dieterich DT, Kotler DP. Systemic dissemination by a newly recognized intestinal microsporidia species in AIDS. *AIDS* 1992; 6: 1143-50.
- Rabeneck L, Gyorkey F, Genta RM, et al. The role of microsporidia in the pathogenesis of HIV-related chronic diarrhea. *Ann Intern Med* 1993; 119: 895-9.
- Sandfort J, Hannemann A, Gelderblom H, et al. *Enterocytozoon bieneusi* infection in an immunocompetent patient who had acute diarrhea and who was not infected with the human immunodeficiency virus. *Clin Infect Dis* 1994; 19: 514-6.
- Smith PD, Lane HC, Gill VJ, et al. Intestinal infection in patients with the acquired immunodeficiency syndrome (AIDS). Etiology and response to therapy. *Ann Intern Med* 1988; 108: 328-33.
- van Gool T, Vetter JCM, Weinmayr B, et al. High seroprevalence of *Encephalitozoon* species in immunocompetent subjects. *J Infect Dis* 1997; 175: 1020-4.
- Vávra J, Dahbiova R, Hollister Ws, Canning EU. Staining of microsporidian spores by optical brighteners with remarks on the use of brighteners for the diagnosis of AIDS associated human microsporidiosis. *Folia Parasitol* 1993; 40: 267-72.
- Visvesvara GS, Leitch GJ, Moura H, Wallace S, Weber R, Bryan RT. Culture, electron microscopy, and immunoblot studies on a microsporidian parasite isolated from the urine of a patient with AIDS. *J Protozool* 1991; 38: 105S-11S.
- Wanachiwanawin D, Manatsathit S, Lertlaituan P, et al. Intestinal microsporidiosis in HIV infected patients with chronic diarrhea in Thailand. *Southeast Asian J Trop Med Public Health* 1998; 29: 31-4.