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. Early detection of microsporidia could be of benefit for the patients, since the infection is treatable.

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 Phramongkutklao 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-
amined 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 microsporidal 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 microsporidal 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 500 g 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% OsO4 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>
<thead>
<tr>
<th>Parasites</th>
<th>Patient with diarrhea (83)</th>
<th>Patient without diarrhea (58)</th>
<th>Total (141)</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Microsporidia</strong></td>
<td>9 (10.8%)*</td>
<td>1 (1.72%)</td>
<td>10 (7.1%)</td>
</tr>
<tr>
<td><em>Cryptosporidium parvum</em></td>
<td>8 (9.6%)</td>
<td>2 (3.4%)</td>
<td>10 (7.1%)</td>
</tr>
<tr>
<td><em>Blastocystis hominis</em></td>
<td>-</td>
<td>2 (3.4%)</td>
<td>2 (1.4%)</td>
</tr>
<tr>
<td><em>Trichomonas hominis</em></td>
<td>-</td>
<td>1 (1.7%)</td>
<td>1 (0.7%)</td>
</tr>
</tbody>
</table>

*p<0.05 by chi-squre (95% CI).*
### Table 2
Characteristics of patients with microsporidial spore detected.

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

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

Fig 1B–A typical spore of *Enterocytozoon bieneusi* 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 microsporidial-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 bieneusi* were identified in all cases, with the typical characteristic of 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/mm³. In our study, 2 cases with chronic diarrhea who had CD4 counts less than 50 cell/mm³ 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 coinfected 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/mm³ 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 someway.

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.

ACKNOWLEDGEMENTS

We would like to thank all the staff members in the pediatric wards of the Queen Sirikit National Institute of Child Health and Phramongkutklao Hospital for their cooperation. This work was supported by the Phramongkutklao Research Fund.

REFERENCES


