Spore shedding pattern of *Enterocytozoon bieneusi* in asymptomatic children

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Stool samples from seven human immunodeficiency virus (HIV)-negative and two HIV-positive children with asymptomatic *Enterocytozoon bieneusi* infections were daily examined to quantify spore shedding using Gram-chromotrope staining under light microscopy. The spore shedding pattern and intensity in these children was variable. Mean spore concentrations in the stool samples from these children ranged from $2.4 \times 10^2$ to $1.2 \times 10^5$ spores per gram. Light microscopy could detect spores in stool specimens for 9–33 days, while PCR was able to detect *E. bieneusi* in stools for 3–40 days longer. This suggests that light microscopy may not detect low levels of spore shedding. Considering that the asymptomatic group are a potential source of infection, detection methods with a higher sensitivity should be used.

INTRODUCTION

*Enterocytozoon bieneusi* is one of the common causes of chronic diarrhoea in AIDS patients, especially in those with CD4+ cell counts less than 100 mm$^{-3}$ (Weber et al., 1994; Kotler & Orenstein, 1998; Franzen & Müller, 2001). *E. bieneusi* can also cause diarrhoea in patients with other immunosuppressive conditions such as organ transplantation (Gumbo et al., 1999a), and in immunocompetent hosts (Sandfort et al., 1994; Sobottka et al., 1995).

It is assumed that these organisms are transmitted via the faecal-oral route since their spores are shed via stools. Possible modes of transmission, including human-to-human (Hutin et al., 1998; Gumbo et al., 1999b), animal-to-human (Dengiel et al., 2001; Sadler et al., 2002; Sulaiman et al., 2003) and waterborne transmission (Hutin et al., 1998; Dowd et al., 1998; Fournier et al., 2000), have been suggested in several studies. The role of human-to-human transmission of intestinal microsporidiosis was indicated in a case-control study which showed that homosexuality was one of the risk factors of intestinal microsporidiosis (Hutin et al., 1998). A study of *E. bieneusi* infection in human immunodeficiency virus (HIV)-positive patients in Zimbabwe also showed that those who had a history of contact with a diarrhoeal patient had a 1.9-fold greater risk of getting the infection (Gumbo et al., 1999b). Considering this mode of transmission, humans are the important source of this organism.

Variation of spore shedding intensity of *E. bieneusi* in HIV-positive patients was shown in two recent studies (Clarridge et al., 1996; Goodgame et al., 1999). However, to our knowledge there have been no reports on the spore shedding pattern of *E. bieneusi* in asymptomatic cases, the potential source of the infection. Therefore we aimed to study the pattern of *E. bieneusi* spore shedding in asymptomatic children.

METHODS

Stool specimens were collected from seven HIV-negative and two HIV-positive children with asymptomatic *E. bieneusi* infections who lived at an orphanage in Bangkok, Thailand. The surveys of *E. bieneusi* infection in these children were conducted between October 2001 and October 2002. The study was approved by the Ethics Committee of the Royal Thai Army Medical Department.

Screening for microsporidal spores in stool specimens was performed by Gram-chromotrope staining (Moura et al., 1996). Species-specific PCR was used for the identification of *E. bieneusi* (Katzwinkel-Wladarsch et al., 1996). Stool examination was performed daily until negative for *E. bieneusi* by Gram-chromotrope staining for 2 months. A quantitative assay for *E. bieneusi* spores was performed using light microscopy. After vigorous mixing of each stool specimen, 0.2 g of stool sample was diluted in 600 µl of PBS. The diluted sample was then thoroughly agitated until a completely homogeneous suspension was achieved. Ten microlitres of each diluted stool specimen was smeared onto a slide for Gram-chromotrope staining. Three slides were made for each specimen. *E. bieneusi* spores were defined as violet oval structures of 1–2 µm with vacuole or band as described previously (Moura et al., 1996). Slides were examined at a magnification of $\times 100$ with oil immersion for the whole smear by an experienced microscopist. For
each specimen the mean of the counted spores from the three slides was calculated as spores per gram to represent the amount of spores in the specimen.

Stool specimens that were negative when examined by light microscope were processed for PCR detection of *E. bieneusi*. Extraction of genomic DNA was performed by using the QIaAmp DNA Stool Mini Kit (QIAGEN) following the manufacturer’s instructions. Genomic DNA of each sample was kept at −20 °C until use. PCR amplification was performed using the primer pair (MSP3 and MSP4B) to amplify a 508 bp fragment of *E. bieneusi*, containing 122 bp of the 3’ end of the small-subunit rRNA gene, 243 bp of the intergenic transcribed spacer and 143 bp of the 5’ region of the large-subunit rRNA gene, with the conditions as described by Katzwinkel-Wladarsch *et al.* (1996). The PCR products and molecular markers were electrophoresed in 2% agarose gel (FMC Bioproducts) with 1.5% Tris/borate/EDTA (TBE) buffer using a gel electrophoresis device (Bio-Rad). Bands were visualized after staining with ethidium bromide under UV light and documented on high-density printing paper using the UVsave gel documentation system I (Uvitech).

**RESULTS AND DISCUSSION**

The spore shedding pattern of *E. bieneusi* was examined in seven HIV-negative and two HIV-positive children aged between 9 and 23 months. The two HIV-positive children were on zidovudine (AZT) and didanosine (ddI) treatment; unfortunately we have no record of their CD4 lymphocyte count. Four of the children were girls and five were boys. None of them had diarrhoeal symptoms during the study.

The number of stools collected from each child is shown in Table 1. During the period in which spores could be detected by light microscopy, the percentage of days on which stool samples were successfully collected from each child varied between 33 and 100. After a negative result by light microscopy, stool specimens were collected daily for 2 months, with the response rate between 55 and 95%. According to the outcomes in infection examined by tissue biopsy were well correlated. In comparison, the specimens from asymptomatic HIV-positive and -negative children in the present study contained lower spore concentrations, ranging from 2.4 × 10^2 to 1.2 × 10^3 spores per gram. The maximal spore concentration reached 5.7 × 10^5 spores per gram in one child who was HIV-negative. Quantitative spore counting by Goodgame *et al.* (1999) showed spore concentrations ranging from 4.5 × 10^5 to 4.4 × 10^8 spores per gram in stool specimens of 20 patients. They also showed that the quantity of spores excreted in the stool and the intensity of the infection examined by tissue biopsy were well correlated. In comparison, the specimens from asymptomatic HIV-positive and -negative children in the present study contained lower spore concentrations, ranging from 2.4 × 10^2 to 1.2 × 10^3 spores per gram. However, the intensity of infection alone may not be enough to explain the different clinical outcomes in *E. bieneusi* infection.

The amount of oocyst excretion in stools has been shown to be correlated with the clinical outcomes in cases of cryptosporidiosis. The study of *Cryptosporidium* oocyst excretion

**Table 1. Duration of *E. bieneusi* spore shedding in each child as detected by light microscopy and PCR**

<table>
<thead>
<tr>
<th>Case no.</th>
<th>HIV status</th>
<th>Detection by light microscopy</th>
<th>Duration of PCR-positive, light microscopy-negative period (days)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Duration of spore-positive period (days)</td>
<td>No. positive/total specimens during spore-positive period (%)</td>
</tr>
<tr>
<td>1</td>
<td>Negative</td>
<td>33</td>
<td>22/26 (84.6)</td>
</tr>
<tr>
<td>2</td>
<td>Positive</td>
<td>22</td>
<td>15/18 (83.3)</td>
</tr>
<tr>
<td>3</td>
<td>Negative</td>
<td>17</td>
<td>3/12 (25)</td>
</tr>
<tr>
<td>4</td>
<td>Negative</td>
<td>9</td>
<td>5/9 (55.5)</td>
</tr>
<tr>
<td>5</td>
<td>Positive</td>
<td>16</td>
<td>7/12 (50.8)</td>
</tr>
<tr>
<td>6</td>
<td>Negative</td>
<td>9</td>
<td>5/5 (100)</td>
</tr>
<tr>
<td>7</td>
<td>Negative</td>
<td>13</td>
<td>6/6 (100)</td>
</tr>
<tr>
<td>8</td>
<td>Negative</td>
<td>12</td>
<td>4/4 (100)</td>
</tr>
<tr>
<td>9</td>
<td>Negative</td>
<td>12</td>
<td>5/5 (100)</td>
</tr>
</tbody>
</table>
patterns in healthy volunteers showed that those who had diarrhoeal illness excreted 50-fold more oocysts than those who had mild enteric symptoms or no symptoms (Chappell et al., 1996). However, AIDS patients with diarrhoeal illness caused by Cryptosporidium parvum might excrete both low and high numbers of oocysts (Goodgame et al., 1993). A study of the spore shedding pattern of E. bieneusi in HIV-positive patients also showed the same picture. Semi-quantitative examination of stool specimens from 23 HIV-positive patients by Clarridge et al. (1993) showed no correlation between the numbers of spores shed and diarrhoeal patterns. In another study, it was also shown that the intensity of spores in stool specimens was not correlated to the 24-hour stool volume (Goodgame et al., 1999). This might indicate that factors other than the intensity of infection contribute to symptoms. Thus, the relationship between the intensity and clinical outcomes of the infection should be further studied in different groups of patients including immunocompetent and immunocompromised patients with different clinical manifestations.

Marked variation of E. bieneusi spore density occurred in each stool specimen from each child, as shown in Fig. 2. Stool-to-stool variability of spore shedding showed 7- to 411-fold differences in each child. Thus it appears that spore production is not a constant process. The number of spores shed tended to decrease after a period of time. This might indicate the spontaneous cessation of infection in these children.

The length of time over which spores could be detected by light microscopy for each child was measured as the number of days from the first day of spore detection to the last day of positive spore detected, and ranged between 9 and 33 days. However, during this time, not all specimens were positive for E. bieneusi by light microscopy. In one child, only 25% of the stained smear gave positive results when examined by an experienced microscopist; the period between two positive stool specimens was from 1 day to 2 weeks. Using a PCR method for the detection of E. bieneusi, all the Gram-chromotrope-negative stool specimens that occurred in-between Gram-chromotrope-positive ones showed a positive specific band. PCR was also used to detect E. bieneusi in the negative stool specimens collected after the duration of E. bieneusi positivity by light microscopy.

Table 1 shows the duration of E. bieneusi-positive PCR, which could detect E. bieneusi in the stool specimens for 3-40 days longer. The lowest spore concentration detected by light microscopy was approximately $3 \times 10^2$ spores per gram. This might reflect the sensitivity of light microscopy for the detection of E. bieneusi spores in stool specimens. PCR could detect E. bieneusi in the Gram-chromotrope-negative stool specimens during and after the duration of E. bieneusi positivity by light microscopy. Low sensitivity of light microscopy has been shown in several studies (Rinder et al., 1998; Menotti et al., 2003). A multicentre study showed that PCR detected as little as $10^2$ spores per gram while a detection limit of $10^4$ spores per gram of stool was shown by light microscopy (Rinder et al., 1998). More recently, a real-time PCR method has been developed in order to accurately quantify E. bieneusi DNA in stool specimens (Menotti et al., 2003). Real-time PCR showed a much higher sensitivity for the detection of E. bieneusi spores; it could detect as little as $\sim 40$ spores per gram. Light microscopy could detect E. bieneusi spore with a sensitivity of $\sim 40\,000$ spores per gram.

In conclusion, lower E. bieneusi spore concentrations in HIV-positive and -negative asymptomatic children have been shown compared to the previous study of HIV-positive patients with intestinal microsporidiosis. The spore shedding pattern and intensity in these children was variable. We found that light microscopy may not detect low levels of spore shedding. Thus, the role of the asymptomatic group as the source of infection is important and might be underrated. Using detection methods with a high sensitivity, such as PCR, should be useful in providing more epidemiological data. Until we have enough information for the determination of prevention and control strategies, in an orphanage where E. bieneusi infection could spread easily universal precautions should be taken.

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REFERENCES


