Intensity of Infection in AIDS-Related Intestinal Microsporidiosis

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To quantify intensity of infection in AIDS-related microsporidiosis, 20 patients with known microsporidiosis submitted stools for quantitative spore counts after staining with a calcofluor white stain. Nine patients collected stools for 24 h, for assessment of daily spore excretion, stool-to-stool variation in spore excretion, and patient-to-patient variation in intensity of infection. The number of organisms seen in small bowel biopsy specimens from 7 patients was compared with quantitative fecal spore excretion. Fecal spore concentration in 20 patients ranged from 4.5 × 10³ to 4.4 × 10⁸ spores/mL of stool. There was a strong correlation between fecal spore excretion and duodenal biopsy spore counts (r = .82; P < .024). Microsporidium infections in AIDS patients can be quantified by counting spores in stool and by small bowel biopsy. Variations in intensity of infection from patient to patient are great and are similar to those in AIDS-related Cryptosporidium infection.

Gastrointestinal infection with one of 2 microsporidium species is commonly associated with AIDS-related diarrhea [1]. The frequency of detecting microsporidia in stool is related to the method of staining used, the expertise of the microscopist, the degree of effort put forth in examining the specimens, and the number of specimens submitted. Prior studies have suggested that the intensity of infection may also determine whether a fecal specimen or biopsy [2] is found to be positive. Although intensity of infection has been assessed by electron microscopy [3], methods of quantifying small bowel infection by light microscopy have not been validated.

Intensity of infection has been measured in another spore-forming protozoal infection, Cryptosporidium parvum [4]. In AIDS-related cryptosporidiosis, the number of oocysts found on stool examination correlates with intensity of small bowel infection [5] and with severity of intestinal injury [6, 7]. Quantitative stool microscopy has been used to evaluate effectiveness of chemotherapy for this infection [7, 8].

The objectives of the current study were to develop a reliable and reproducible method for quantifying the number of microsporidium organisms in the stool and in the small bowel of infected AIDS patients and to use the method to define variations in intensity of infection between different patients and from stool to stool in the same patient.

Patients and Methods

Stools from AIDS patients with known microsporidium infection were identified through the services of the Thomas Street Clinic for Immunological Diseases and the Ben Taub General Hospital Microbiology Laboratory, both in Houston. Subjects who were physically able underwent one or more of the following tests: 24-h stool collections, upper gastrointestinal endoscopy, and small bowel biopsy. Subjects were instructed to collect each bowel movement during a 24-h period in a plastic container by use of a commode specimen collector (Lab-Choice; Vollrath, Gallaway, TN). Each container was sealed, dated, timed, and later, weighed and refrigerated until assayed. Concentration of spores in each stool specimen was assayed by quantitative microscopy.

Quantitative fluorescent microscopy on stool. An aliquot of stored stool was diluted 3:1 with 10% formalin. The specimen was mixed well and allowed to stand for 30 min. We mixed 10 µL of formalin-diluted stool with 10 µL of solution A (Fungi-Fluor Solution A; Polysciences, Warington, PA) on each well of a triple-well slide. The smears were air dried at 65°C. One drop of solution A was added to each well, incubated for 30 s, and drained. Four drops (~200 µL) of eosin yellowish solution (Fisher Scientific, Fair Lawn, NJ) were added, incubated for 30 s, and drained. Two drops of solution B (Fungi-Fluor Solution B) were added, rocked briefly, and drained. Two more drops of solution B were added, incubated for 30 s, and drained. The smears were repeatedly dipped into tap water until the specimens no longer released a blue color. The smears were air dried, and a drop of low-fluorescence immersion
oil was placed on the specimen with a coverslip. Smears were examined by a fluorescence microscope (BH2; Olympus, Lake Success, NY), equipped with filter module VGI (excitation 330–380 nm) and a mercury vapor lamp for illumination. Stained specimens were examined at ×500 and ×1000 magnification. Microsporidia spores are 1–3 μm long and have a blue-white fluorescence.

Quantitative spore counts on stool. All spores in 10 randomly selected fields at ×100 magnification were counted in each well. The mean of the 3 counts was converted to spores per milliliter. Accuracy of the assay was assessed by counting serial dilutions of specimens. Reproducibility of the assay was assessed by repeating the assay on the same stool on 3 different days. Multiple specimens from the same patient were used to assess stool-to-stool variation in spore excretion. Mean concentration of all stools passed in 24 h was used to assess patient-to-patient variation in infection intensity.

Stains for small intestine biopsy. Formalin-fixed, paraffin-embedded specimens were cut in 5-μm sections. Four sections from individual biopsies were placed on glass slides and stained with hematoxylin-eosin and the fluorescent stain. For the fluorescent stain, the paraffin-embedded tissue section was deparaffinized. Solution A was added to cover the section, which was incubated at 65°C for 5 min. The fluid was drained and the section was rinsed with tap water. TB auramine M (Difco Laboratories, Detroit) was added to cover the section; incubation followed at room temperature for 5 min. After draining, the section was decolorized with TB decolorizer TM (Difco) for 1 min and rinsed with tap water. The section was again covered with solution A for 1 min and drained. Four drops of eosin yellowish solution were added for 1 min and drained. Two drops of solution B were added, rocked briefly, and drained. Two more drops of solution B were added, incubated for 1 min, and drained. The section was repeatedly dipped in tap water until the section no longer released a blue color. The section was air dried, a drop of low-fluorescence immersion oil was added, a coverslip was applied, and a drop of immersion oil was placed on top of the coverslip. Sections were examined with the fluorescent microscope described above. Stained sections were examined at ×500 and ×1000 magnification. Microsporidia spores demonstrated a blue-white fluorescence.

Quantitative spore counts on small intestine biopsy. Each slide contained four sections from one biopsy taken from the small intestine. All pieces on the slide were assessed. Number of spores from each piece was recorded; the highest number from any piece was designated as the intensity of small bowel infection. Counting of small bowel organisms was done independently and blinded from stool results. The number of organisms was compared with quantitative fecal spore excretion.

Morphologic assessment of small bowel pathology. Hematoxylin-eosin stain was used to evaluate small intestine morphology. Severity of morphologic damage was blindly assessed semiquantitatively for chronic inflammation (0–3) and atrophy (0–3). The four sections of each biopsy on a slide were assessed, and each biopsy was given a score. The average score for all biopsies for each patient was used for analysis.

Results

With the stool-staining technique described, microsporidium spores fluoresce at 360 nm with a brilliant blue-white fluorescence. The technique of counting spores in stool was easily mastered. Morphologic features were used to distinguish fungal spores from microsporidia. Clumping of spores was common but did not adversely affect accuracy or reproducibility of the assay. Dilutions from 1 : 1 to 1 : 16 on 13 stools of different intensity of infection showed proportional fall in spore concentration. The mean number of spores seen on 10 high-powered fields was reduced by ~50% with each dilution, from 337 to 136 to 43 to 16 to 8. Triplicate assays performed on 3 different days on 14 stools showed a mean coefficient of variation of 37%. We assayed positive stools from 20 different patients (all men >45 years old). All had <160/mm³ CD4 cells. Concentration of spores in first stool assayed from these patients ranged from 4.5 × 10³ to 4.4 × 10⁶ spores/mL stool and were normally distributed around a median of 4.9 × 10⁵. There was no cor-
relation between intensity of infection and any observed clinical feature, including age and CD4 cell count ($r = 0.23$). Nine AIDS patients with known microsporidiosis collected each stool for 24 h. Stool numbers were 1–8 per 24 h, and stool weights were 80–1486 g per 24 h. The stool-to-stool variation in spore concentration for each patient was small, about the same as the assay variation (figure 1). Daily spore excretion ranged from $2.9 \times 10^4$ to $6.2 \times 10^5$ (figure 1). Stool volume did not correlate with any measure of infection intensity, including spore concentration ($r = -0.30$), 24-h spore excretion ($r = -0.19$), or biopsy intensity ($r = -0.04$).

Endoscopic duodenal biopsies were taken from 7 patients whose fecal spore concentration ranged from $4.5 \times 10^3$ to $2.5 \times 10^6$ spores/mL (table 1). Histologic features were assessed with hematoxylin-eosin stain. Most patients had normal histology. Mild-to-moderate increase in chronic inflammatory cells was noted in some specimens. Rarely, partial villus atrophy was noted. There was no correlation between severity of histologic abnormalities and the fecal spore concentration. The calcofluor white stain of the biopsy was positive in all cases. The maximum number of organisms seen on a single section of a single biopsy for an individual patient varied from 1 to 63 (table 1). There was a significant correlation between fecal spore excretion and duodenal biopsy spore counts ($r^2 = 0.82; P < .024$). The mean count of all sections correlated with the maximum number seen on any one section ($r = 0.9; P = .001$) and could have been used for analysis without altering the results.

**Table 1. Relationship between intensity of infection in stool and in biopsy.**

<table>
<thead>
<tr>
<th>Patient</th>
<th>Biopsy (organisms/cut)</th>
<th>Stool (spores/mL)</th>
<th>Inflammatory cells</th>
<th>Atrophy</th>
<th>Stool volume (mL) in 24-h period</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>25</td>
<td>$4.5 \times 10^6$</td>
<td>2.3</td>
<td>1</td>
<td>1468</td>
</tr>
<tr>
<td>2</td>
<td>1</td>
<td>$1.3 \times 10^5$</td>
<td>1.0</td>
<td>0</td>
<td>292</td>
</tr>
<tr>
<td>4</td>
<td>25</td>
<td>$3.6 \times 10^5$</td>
<td>1.6</td>
<td>1.2</td>
<td>80</td>
</tr>
<tr>
<td>7</td>
<td>7</td>
<td>$2.5 \times 10^5$</td>
<td>1.0</td>
<td>0.3</td>
<td>114</td>
</tr>
<tr>
<td>8</td>
<td>34</td>
<td>$3.8 \times 10^4$</td>
<td>1.3</td>
<td>0</td>
<td>568</td>
</tr>
<tr>
<td>10</td>
<td>32</td>
<td>$4.9 \times 10^3$</td>
<td>1.0</td>
<td>0</td>
<td>158</td>
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<tr>
<td>19</td>
<td>63</td>
<td>$2.5 \times 10^2$</td>
<td>1.3</td>
<td>0</td>
<td>127</td>
</tr>
</tbody>
</table>

NOTE. Seven patients with AIDS-related microsporidiosis had infection intensity quantified by assessment of fecal spore concentration and numbers of organisms in small bowel biopsy. These 2 methods correlated with one another ($r^2 = 0.82; P < .024$). Small bowel morphology was near normal in most patients and did not vary with infection intensity.

**Discussion**

Our study was designed to develop and validate a method to quantify intensity of infection in intestinal microsporidia. Clarridge et al. [3] used the trichrome stain to perform semiquantitative (rare, moderate, many) assessment of intensity of stool infection in multiple stool examinations of 23 AIDS patients with known intestinal microsporidiosis. We used a modification of the Fungi-Fluor fluorescent stain because of its simplicity, good resolution, and similarity to the antibody-based fluorescent stain used in *Cryptosporidium* studies [4, 5]. Even though the microsporidia spores are much smaller and more easily confused with fecal debris and fungal spores, we found assay results to be reproducible; that is, the stool count repeated on 3 separate days yielded similar results. In addition, the assay was accurate. Serial dilutions of stool had proportionately lower spore counts. In our 20 patients with AIDS-related microsporidiosis, spore excretion ranged from $4.5 \times 10^3$ to $4.4 \times 10^4$ spores/mL. The range of spore concentration was similar to the range of oocyst concentration in *Cryptosporidium* infection ($5 \times 10^{-3} - 9 \times 10^3$ oocysts/mL [5]), although the concentrations are 100 times greater. Of interest, the volume of a microsporidium spore is 25–100 times smaller than that of a *Cryptosporidium* oocyst.

We wanted to assess the intensity of infection in small bowel biopsy and to determine the relationship between stool and small bowel intensity. In previous studies of *Cryptosporidium* infection, fecal and small intestinal intensity correlated well [4, 5]. In the study of microsporidium by Clarridge et al. [3], “heavy” and “light” infections were defined by counting organisms seen on electron microscopy of small bowel mucosal biopsies [3]. Heavy infections were more likely to be associated with frequently positive stool examinations and abundant spores in stool. Previous studies have documented the usefulness of fluorescent chitin stains (collectively known as optical brighteners and including calcofluor white, Fungi-Fluor, Fungiqual A, Uvitex 2B, and Rylux Ba) for the identification of microsporidium organisms in intestinal biopsy specimens [9, 10]. A wide variety of techniques utilizing these stains have shown similar efficacy [10]. We demonstrated that the intensity of intestinal infection correlated with the number of organisms in the stool. This agreement between the two methods suggests that “heavy” and “light” microsporidium infections really do exist and that infection intensity can be assessed by quantitative parasitologic methods.

**References**

5. Genta R, Chappell C, White A, Kimball K, Goodgame R. Duodenal mor-


