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Quantitative Light Microscopic Detection of *Enterocytozoon bieneusi* in Stool Specimens: a Longitudinal Study of Human Immunodeficiency Virus-Infected Microsporidiosis Patients

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The clinical course of microsporidiosis caused by *Enterocytozoon bieneusi* and the pattern of intestinal shedding of spores have not been correlated, at least in part because detection of *E. bieneusi* in stools is more difficult than detection of other protozoa because of its smaller size and less intense staining. We examined with a modified trichrome stain 124 stool specimens collected over a 2-year follow-up period from 23 human immunodeficiency virus-infected patients with electron microscopic-proven *E. bieneusi* infection and correlated the results with electron microscopic observations from duodenal biopsy specimens taken at the beginning of the study period. *E. bieneusi* was detected in the stool at least once in 74% (17 of 23) of all patients, in 100% (9 of 9) of patients in whose tissue moderate or abundant numbers of parasites were seen, and in 57% (8 of 14) of patients in whose tissue few parasites were seen. In two patients with abundant tissue parasites, many microsporidia were detected in every stool specimen (13 of 13) during the follow-up period, whereas among the patients with few tissue parasites, only 23% (15 of 64) of stool specimens were positive. Furthermore, if spore stages as well as plasmodial stages were detected in tissue, stool specimens were more likely to be positive. Although most of the heavily infected stools were from patients with chronic diarrhea, microsporidia were detected in 33, 28, and 42% of stool specimens from patients with nil, intermittent, and chronic diarrhea patterns, respectively. Although quantitation of *E. bieneusi* spores in stool specimens was closely correlated with quantitation in tissue, it was not correlated with reported patterns of diarrhea.

Several species of microsporidia are becoming increasingly recognized in association with significant disease among human immunodeficiency virus-infected patients (2–5, 7, 12–16, 18). Most reports involve gastrointestinal disease, but *Encephalitozoon hellum* and *Encephalitozoon intestinalis* (formerly *Septata intestinalis* [10a]) have also been associated with extraintestinal infection (4). *Enterocytozoon bieneusi* is the most commonly reported microsporidium that is detected in gastrointestinal specimens (5, 13, 14, 15). Until recently, testing for the presence of these organisms was largely limited to examinations of small bowel biopsy specimens by electron microscopy (EM) (8, 14, 15). Because this is an invasive and costly technique that is not widely available to clinical laboratories, considerable effort has been made to develop simple, reliable stains that can be used to detect microsporidial spores in fecal specimens by light microscopy (1, 6, 7a, 11, 16–20). These efforts have been hampered by the lack of specific stains and the extremely small size of *E. bieneusi* (0.5 to 1.0 by 1.5 μm), which overlaps with the size of bacteria.

Although *E. bieneusi* or other microsporidia may account for between 6.5 and 27% of previously unexplained cases of chronic diarrhea in AIDS patients (2, 15, 19), there have been no longitudinal studies of the pattern of microsporidial spore shedding in stool specimens or the relationship between spore shedding and the appearance of symptoms. We have previously identified a group of 31 human immunodeficiency virus-infected patients with electron microscopically proven intestinal microsporidiosis (14, 15). In the study reported here, we followed 23 of these patients for up to 24 months, quantitatively analyzing 125 stool specimens for microsporidia with a modified trichrome stain (17, 19) and correlating these findings with the pattern of detectable spore shedding, the quantity and stages of the organisms seen in biopsy specimens, the reproducibility of staining results, and the onset of diarrhea.

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MATERIALS AND METHODS

Patients and EM specimens. We studied 23 human immunodeficiency virus-infected patients who had previously been diagnosed with *E. bieneusi* infection by EM examination of duodenal mucosal biopsy specimens. The quantity of microsporidia in the biopsy specimens was categorized as few (organisms detected only after an extensive search), moderate (detected without an extensive search in fewer than one-half of the enterocytes), or abundant (readily detected in more than one-half of the enterocytes) (15). We also categorized specimens as spores plus if spores and plasmodial forms were seen in the biopsy specimens and spores minus if only plasmodial forms were seen. All stool preparations were read without knowledge of the EM results. The biopsies were performed only once at the beginning of the study, and the stool specimens were collected when the patients reported for follow-up studies every 1 to 2 months.

Stool specimens. Stool specimens were collected in Lab Choice commode specimen (20 cm) plastic containers (Vollrath, Gallaway, Tenn.). Immediately on arrival in the laboratory, each specimen was preserved in formalin (one part stool and four parts formalin).

Detection of microsporidia. Slides for light microscopic examination were prepared from a suspension of unconcentrated stool specimen in 10% formalin (1:4 ratio of stool to formalin) by spreading 0.01 ml thinly over a 2-cm area with a 0.01-ml sterile loop to achieve a well-dispersed background. Smears were fixed in methanol for 5 min and were stained by either the Weber modified trichrome method (19) or the trichrome-blue method of Ryan et al. (17) (400501;

TABLE 1. Detection of microsporidia by light microscopy[a]

Patient No.	EM results: quantity	EM results: form	Months After EM Diagnosis: 1	2	3	4	5	6	7	8	9	10	11	12	13	14	15	16	17	18	19	20	21	22	23-25
1	a	s+		(P)				(P)	(P)	(P)		(P)					(P)	(P)				(P)	(P)		
2	a	s+							(P)	(P)		(PP)													
3	m	s+			(P)			N	N		N	N		X											
4	m	s+			[P]N			N		[P]	N														[P]
5	m	s+			(P)				{P}	N			N	N	N	N	N	N	N		N		N		N
6	m	s+		(P)			(P)	(P)																	
7	m	s-				N		N	[P]	N	N	N	N	N		[P]									[P]
8	m	s-							N	N		N	I	N	{P}		N								
9	m	s-	I				[P]																[P]		
10	f	s-	{PP}N			I	{P}		I			{P}													
11	f	s-			N, I		N	I	N	I		{P}	N	N,N		N	N			N			N		
12	f	s-						[P]																	
13	f	s-			N				N			N						N							
14	f	s-		N	[P]							X													
15	f	s-			N					X															
16	f	s-				N	N	N			N	N		N	N	N		N							
17	f	s-				N		X																	
18	f	s-			N	N	N		[P]		N			N						N					
19	f	s-					N											N				[P]			X
20	f	s-										N		N	N										
21	f	s+	N					N										N							
22	f	s+	{P}	I	N	{P}	{P}						X												
23	f	s+				[P]		[P]					[P]												

[a] Abbreviations: N, negative; I, indeterminate; P, positive; X, deceased; m, moderate; f, few; a, abundant; s+, spore and plasmodial stages present; s−, no spores seen; plasmodial stage only; parentheses, many spores; brackets, few to moderate spores; braces, spores rarely seen; light shading, times of chronic diarrhea; dark shading, times of intermittent diarrhea.

TABLE 2. Relationship between quantity of spores detected in stool specimens and stage of microsporidia observed in tissue

Quantity of spores estimated in stools	No. of stool specimens for the following quantity and stage of microsporidia observed in tissue:				
	Abundant, spores seen	Moderate, spores seen	Few, spores seen	Moderate, plasmodia only	Few, plasmodia only
Many	13	5	0	0	0
Moderate-few	0	3	3	5	4
Rare	0	1	3	1	5
None, indeterminate	0	18	6	14	43

Trichrome-blue stain; Meridan, Cincinnati, Ohio), except that the stain was heated to 37°C before use and Paraclear (Alpha-tec Systems, Inc., Irvine, Calif.) was used as the xylene substitute. A culture of *E. hellum* and control slides of *E. bieneusi* and *E. hellum* were obtained from Elizabeth Didier (Tulane University) and were used for quality control in the initial phase. Subsequently, a control slide from patient 1 was stained with each batch. All slides were scanned with the ×100 oil immersion lens for approximately 200 to 250 fields before determining a negative result. A positive result was the observation of pink-red oval structures of about 1 to 2 μm with vacuoles or bands as described previously (6, 10, 16, 17, 19). At least two slides were made per specimen, and each slide was read by at least two people. If the viewers disagreed (all disagreements were only between rare positive and negative readings), the result was scored indeterminate. The following terms are used for quantitation: many (more than 10 spores seen in five fields; they tended to be found in groups), moderate to few (at least one spore seen in 1 to 5 fields), and rare (only an occasional spore seen).

RESULTS

The results for about 300 trichrome-blue-stained samples of the 124 fecal specimens are provided in Table 1. The detection of spores in stool specimens is related to both the presence of spores and the quantity of organisms detected in tissue. *E. bieneusi* was detected in the stool specimens in 100% (9 of 9) of the patients in whose tissue moderate or abundant parasites were seen and in 57% (8 of 14) of the patients in whose tissue few parasites were seen. The relative importance of both the quantity and stage of microsporidia in tissue is described in Table 2. When spores and plasmodia were seen in the tissue, 89% (8 of 9) of the patients and 54% (28 of 52) of the stool specimens were positive, compared with 70% (10 of 14) of the patients and 21% (15 of 71) of the specimens when only plasmodia were seen. Furthermore, when abundant microsporidia, including spores stages, were seen by EM (patients 1 and 2), many spores were detected in all stool specimens.

The pattern of diarrhea was not closely related to the detection of spores or the pattern of shedding (Table 3). Although the two patients with abundant tissue parasites had persistent chronic diarrhea and microsporidia were detected in every stool specimen, there were also five patients with chronic diarrhea and nil or only one positive stool specimen. However, in all five of these patients, only a few microsporidia were observed in their biopsy specimens. Microsporidia were detected in 33, 28, and 42% of the stool specimens from patients having nil, intermittent, and chronic diarrhea, respectively. We noted that two patients (patients 3 and 5) initially had positive stool specimens and then consistently negative specimens in the absence of antimicrobial therapy.

The $CD4^+$ cell counts of our patients have been described previously (15). For 17 patients, the $CD4^+$ (as cells/mm^3) counts were below 200 at the initial visit and remained below 200 when they were measured 5 to 22 months later. Patients 4, 12, and 21 had cell counts above 200, and they remained above 200 when they were measured 8 to 15 months later. Patients 5, 7, and 18 had cell counts which decreased from above 200 to below 200 when they were measured 22 to 25 months later.

TABLE 3. Relationship between the quantity of spores detected in stool specimens and diarrhea pattern

Quantity of spores estimated in stools	No. of stool specimens in patients with the following type of diarrhea:		
	Chronic	Intermittent	None
Many	13	4	1
Rare to moderate	8	11	6
Nil and indeterminate	29	39	13

DISCUSSION

Although for convenience we switched from the Weber's modified trichrome stain to the trichrome-blue stain during the study, new slide preparations of all specimens were made and stained with the trichrome-blue stain (17). The few observed differences in quantity were judged to be related to sampling differences and not technique. Garcia and colleagues (10) have also found that the two stains are comparable. Although some studies indicate that specific antibodies and Uvitex 2B, a fluorochrome giving fewer artifacts than Calcofluor, are slightly more sensitive than modified trichrome methods for the larger non-*E. bieneusi* microsporidia (1, 6, 20), more recently, Didier and colleagues (7a) found Calcofluor and the modified trichrome stain were approximately equivalent and both were more sensitive than the indirect immunofluorescent-antibody stain.

We have shown that the quantity and the frequency of detection of microsporidial spores in stool specimens are related to the detection of spores in tissue. Furthermore, the shedding pattern in patients with abundant organisms (patients 1 and 2) appears to be persistent and easy to detect. For these patients it may be possible to evaluate change in status or treatment effectiveness by detecting spores in stool specimens. However, the apparent natural cessation of infection is not understood: the two patients who had positive specimens and then multiple negative specimens received no antimicrobial treatment. One patient did not have diarrhea, and one patient had only intermittent diarrhea. This may be similar to infections with *S. intestinalis*, in which treatment does not correlate with the shedding pattern (12). Several patients whose biopsy specimens contained only a few microsporidia showed intermittent shedding, probably relating more to sampling differences and the inability to detect few microsporidia than to changes in the parasite burden.

In patients who have multiple possible causes of diarrhea, the assignment of a single cause is difficult. In our patients, microsporidia were detected in proportionally more of the samples from patients with chronic diarrhea than from patients with nil or intermittent diarrhea, which may indicate that microsporidial infection contributed to the chronic diarrhea in these patients.

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