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Sensitivity and Specificity of a Monoclonal Antibody-Based Fluorescence Assay for Detecting *Enterocytozoon bieneusi* Spores in Feces of Simian Immunodeficiency Virus-Infected Macaques

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***Enterocytozoon bieneusi* is clinically the most significant among the microsporidia causing chronic diarrhea, wasting, and cholangitis in individuals with human immunodeficiency virus/AIDS. Microscopy with either calcofluor or modified trichrome stains is the standard diagnostic test for microsporidiosis and does not allow species identification. Detection of *E. bieneusi* infection based on PCR is limited to a few reference laboratories, and thus it is not the standard diagnostic assay. We have recently reported the development and characterization of a panel of monoclonal antibodies against *E. bieneusi*, and in this publication we evaluated the specificity and sensitivity of an immunofluorescence assay (IFA), compared with PCR, in simian immunodeficiency virus-infected macaques. The IFA, which correlated with the primary PCR method, with a detection limit of 1.5×10^5 spores per gram of feces, will simplify considerably the detection of *E. bieneusi* spores in clinical and environmental specimens and in laboratory and epidemiological investigations.**

Enterocytozoon bieneusi, an emerging enteric spore-forming protozoan, is clinically the most significant among the microsporidia which are linked to chronic diarrhea, wasting, and cholangitis in up to 30 to 50% of individuals with human immunodeficiency virus (HIV)/AIDS (2, 3, 25) as well as in recipients of organ transplants (17, 18) and malnourished children (23). *E. bieneusi* is occasionally symptomatic in healthy immunocompetent individuals (11, 12, 26) and also may contribute to traveler's diarrhea (10). *E. bieneusi* also infects other mammals, including simian immunodeficiency virus (SIV)-infected macaques, in which lesions, as in humans, are localized in the small intestine, gallbladder, and bile ducts (14).

E. bieneusi infections are difficult to diagnose, primarily because the organisms are indistinguishable in size from bacteria and yeasts in stool. Until recently the diagnosis of intestinal microsporidiosis was based on the microscopic examination of feces stained with fluorochrome Uvitex 2B or by the modified trichrome or calcofluor white stain (6, 7, 13, 24). These methods are nonspecific, as they stain chitin in the endospore layer of the spores, which is also present in some bacteria and yeasts. A more sensitive and specific assay is PCR, but it is not routinely used in clinical diagnosis (4, 5, 8, 9, 16, 22).

The recent derivation and characterization of specific monoclonal antibodies (MAbs) against *E. bieneusi* (21, 28) has made it possible to develop new and much simplified immune-based diagnostic assays. In this communication we have evaluated the sensitivity and specificity of an immunofluorescence assay (IFA) using MAbs against *E. bieneusi* for the detection of spores in fecal samples of SIV-infected macaques, compared with PCR.

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MATERIALS AND METHODS

Fecal samples. Monthly fecal samples were obtained from a cohort of 12 SIV-infected rhesus macaques (*Macaca mulatta*), either experimentally or naturally infected with *E. bieneusi*, for 4 to 8 months to determine the pattern of excretion of *E. bieneusi* spores. The sensitivity and specificity of the IFA were compared with those of PCR, the method that we currently use for detection (Table 1). An additional 232 fecal samples randomly collected from several ongoing studies of SIV-infected macaques were also comparatively tested for the presence of *E. bieneusi* spores by IFA and PCR (Table 2). Fecal samples from SIV-naïve animals were included as controls. All animals were housed at the New England Regional Primate Research Center and were maintained in accordance with the Guide for the Care and Use of Laboratory Animals prepared by the National Research Council.

PCR. (i) DNA extraction. A modified procedure of DNA fecal extraction was used (5). Briefly, 200 μl of 0.5-mm glass beads (BioSpec Products, Bartlesville, OK), 400 μl of digestion buffer (100 mM sodium chloride, 25 mM EDTA, 10 mM Tris [pH 8.0], 2% sodium dodecyl sulfate), 600 μl of Tris-buffered phenol-chloroform (BioExpress, Kaysville, UT), and 200 mg of feces were added to a 2-ml screw-cap tube. The sample was homogenized by the Mini Bead-Beater (BioSpec Products, Bartlesville, OK) at 5,000 rpm (5,500 × *g*) for 2 min. After centrifugation at the top speed for 5 min, 400 μl of aqueous phase was adjusted to 0.7 M sodium chloride. The DNA was extracted using the Geneclean III kit (Bio101, Carlsbad, CA) according to the manufacturer's instructions and stored at −20°C.

(ii) Primary and nested PCR. The first round of PCR (primary PCR) was performed with 1 μl of the DNA preparation described above with primers specific for the *E. bieneusi* ribosomal internal transcribed spacer. The nested PCR was performed with 1 μl of the product from the primary PCR with primers specific for *E. bieneusi* internal transcribed spacer DNA as described elsewhere (4). The sequences of primers were as indicated: outer primers, forward (EBITS3) (5′-GGTCATAGGGATGAAGAGC-3′) and reverse (IBITS4) (5′-TTCGAGTTCTTTCGCGCTCG-3′), and inner (nested) primers, forward (IBITS1) (5′-GCTCTGAATATCTATGGCTAG-3′) and reverse (EBITS2.4)

TABLE 1. Pattern of excretion of *E. bieneusi* spores in a cohort of 12 SIV-infected macaques measured monthly over a period of 8 months, using primary PCR and IFA methods of detection

Macaque	Result at mo[a](PCR/IFA)							
	1	2	3	4	5	6	7	8
1	+/+	+/+	+/+	+/+	+/+	+/+	+/+	+/+
2	+/+	+/+	+/+	+/+	+/+	+/+	+/+	−/−
3	+/+	+/+	+/+	+/+	+/+	−/−	+/+	+/+
4	+/+	+/+	+/+	+/+	+/+	+/+	+/+	ND
5	+/+	+/+	−/−	+/+	+/+	−/−	+/+	ND
6	−/−	+/+	+/+	+/+	−/−	−/−	−/−	ND
7	+/+	+/+	+/+	−/−	ND	ND	ND	ND
8	+/+	+/+	+/+	+/+	ND	ND	ND	ND
9	+/+	+/+	+/+	+/+	ND	ND	ND	ND
10	−/−	−/−	−/−	−/−	ND	ND	ND	ND
11	−/−	−/−	−/−	−/−	ND	ND	ND	ND
12	−/−	−/−	−/−	−/−	−/−	−/−	−/−	ND

[a] +, positive; −, negative; ND, not done.

(5′-ATCGCCGACGGATCCAAGTG-3′). The cycling parameters of primary PCR consisted of 94°C for 3 min; 35 cycles of 94°C for 40 s, 57°C for 40 s, and 72°C for 1 min; and 72°C for 5 min. The cycling parameters of nested PCR consisted of 94°C for 3 min; 30 cycles of 94°C for 40 s, 55°C for 40 s, and 72°C for 1 min; and 72°C for 5 min in a thermocycler (MJ Research, Watertown, MA). The size of the product generated with outer primers (primary PCR) was 435 bp, and the size of the product generated with nested primers was 390 bp (19). The PCR products were visualized by the use of ethidium bromide staining after electrophoretic separation in 1.5% agarose gels. Based on PCR analysis, fecal samples were divided into three groups: positive for primary PCR, positive for nested PCR, or negative for both primary and nested PCR.

IFA. Three MAbs consisting of one immunoglobulin M (IgM) antibody (2G4) and two IgG antibodies (1B7 and 12G8) were evaluated for the detection of *E. bieneusi* spores by indirect IFA. Rabbit polyclonal *E. bieneusi* antibody was used as a positive control (20). 1B7 and 2G4 MAbs were produced against human *E. bieneusi* spores, and 12G8 MAb was produced against monkey *E. bieneusi* spores, as described elsewhere (21, 28). To determine the dilution of the MAbs used, the culture supernatants were titrated on feces positive for *E. bieneusi* spores (data not shown).

One microliter of fecal suspensions, homogenized 1:5 in phosphate-buffered saline (PBS), was mounted on microscopic slides, air dried, and heat fixed over a flame. The slides were incubated with specific MAbs, as undiluted culture supernatants, for 30 min at room temperature. Smears were washed with PBS and incubated with either goat anti-mouse IgM or goat anti-mouse IgG conjugated with Alexa Fluor 488 (Molecular Probes, Eugene, OR) at a dilution of 1:500 in PBS and incubated for 30 min at room temperature. Slides were washed, dried, and mounted with aqueous mounting medium with antifading compound [1,4-diazobicyclo(2,2,2,)-octane (DABCO); Sigma, St. Louis, MO] and examined under a high-power (magnification, ×400) fluorescence microscope (BX40; Olympus Optical Pvt. Ltd., Japan). Purified *E. bieneusi* spores stained with rabbit polyclonal *E. bieneusi* antibody (1/500 dilution in PBS) were used as a positive control.

TABLE 2. Sensitivity of the IFA for the detection of *E. bieneusi*, compared with primary and nested PCR, in 232 feces randomly collected from SIV-infected macaques

PCR result	No. of samples (n = 232)		
	Total	IFA positive	IFA negative
PCR negative	81	0[a]	81
Primary PCR positive	89	89[a]	0
Nested PCR positive	62	42[b]	20

[a] Specificity of IFA was 100%.
[b] Sensitivity of IFA compared to nested PCR was 87%.

RESULTS

IFA. All three *E. bieneusi*-specific MAbs reacted with the simian *E. bieneusi* spore wall but not with other microsporidia, as shown elsewhere (21, 28). The MAb of IgM had a titer of 1:4, whereas the two IgG antibodies had titers of >1/1,000, as determined by indirect IFA on feces of SIV-infected macaques containing *E. bieneusi* spores. Of the three MAbs evaluated (Table 3), the IgM 2G4 gave the brightest fluorescence and was selected for further studies (Tables 1 and 2).

Cohort study. Table 1 summarizes the pattern of excretion of spores in 12 macaques sampled monthly over 4 to 8 months. Nine of the 12 SIV-infected macaques were positive by both primary PCR and IFA, confirming the chronicity of infection through the consistent excretion of *E. bieneusi* spores in this subpopulation, as well as the sensitivity of the IFA compared with PCR. Animals 10 to 12 were also consistently negative by both assays.

Eighty-nine of 232 fecal samples which were reacted in the primary PCR were also strongly reactive by IFA, demonstrating again the excellent correlation between the two assays, as the specificity of IFA was 100% compared to primary PCR (Table 2). Of the 62 fecal samples which failed to generate a primary PCR signal but which did so by nested PCR, only 42 of the 62 samples were positive by IFA, indicating that, while IFA was more sensitive than the primary PCR, as it detected 42 positive samples which were negative with primary PCR, it was less sensitive (87%) than the nested PCR (Table 2). Samples negative by both primary and nested PCR were also negative by IFA.

Relative sensitivity of IFA and PCR. Feces containing *E. bieneusi* spores were twofold diluted to 1:2,048 (Table 3). The sensitivities of the IFA, using three different MAbs, were compared at each dilution with each other and with primary and nested PCRs. Table 3 demonstrated that, although IgM MAb appeared to detect approximately two- to threefold-more *E. bieneusi* spores than IgG MAbs till 1/32 dilution of feces, all three MAbs were equally effective, detecting similar numbers of spores from 1/64 till 1/256 dilution, and corresponded well with the primary PCR results, with a detection limit of ~150,000 spores per gram of feces. The nested PCR was again more sensitive, with a detection limit of approximately 70,000 spores per gram of feces.

DISCUSSION

We have evaluated a panel of three MAbs against *E. bieneusi* as reagents for IFA, and all appeared to be equal to the task, as shown in Table 3. We have selected 2G4, an IgM MAb which gave the brightest fluorescence, for the studies described in Tables 1 and 2, and we believe this MAb to be best suited for clinical diagnosis and for other laboratory and epidemiological investigations of *E. bieneusi* infections in humans and in other mammals. With other mammalian microsporidia, specific polyclonal and monoclonal antibodies show strong cross-reactivity by IFA (1, 15, 27, 29). None, however, cross-reacted with *E. bieneusi* spores, while the MAbs generated against *E. bieneusi* appear to reciprocate by being genus specific too, as they failed to react with other microsporidia that infect humans (21, 28).

Both assays reflected the chronicity of *E. bieneusi* infections

TABLE 3. Comparative sensitivities of IFA (using three different MAbs) and PCR in serially diluted feces containing *E. bieneusi* spores

Serial dilution	Result		Spore count/10 microscopic fields[a]			Estimated no. of spores/g of feces
	Primary PCR	Nested PCR	IgG IFA (IB7; IgG2a)	IgG IFA (12G8; IgG2b)	IgM IFA (2G4; IgM)	
Undiluted	+	+	597	480	1,537	3.8×10^7
1:2	+	+	453	415	1,280	1.9×10^7
1:4	+	+	281	224	845	9.5×10^6
1:8	+	+	181	124	442	4.8×10^6
1:16	+	+	138	107	328	2.4×10^6
1:32	+	+	87	58	118	1.2×10^6
1:64	+	+	30	29	38	6.0×10^5
1:128	+	+	26	19	26	3.0×10^5
1:256	+	+	15	9	20	1.5×10^5
1:512	−	+	0	0	0	7.5×10^4
1:1,024	−	−	0	0	0	3.8×10^4
1:2,048	−	−	0	0	0	1.9×10^4
Control	−	−	0	0	0	0

[a] At a magnification of ×400.

in SIV-infected macaques on the one hand and the consistency and equal sensitivity of the IFA and the primary PCR. When nested PCR was performed, the sensitivity was higher. Both assays have shown that the detection limit was approximately 1.5×10^5 spores per gram of feces and approximately half this number by nested PCR. The PCR, the nested PCR in particular, is a time-consuming assay, requiring sophisticated equipment and skilled labor, and consequently is limited to investigative laboratories. The IFA in contrast should make it possible to include the detection of *E. bieneusi* together with other protozoa in routine clinical diagnosis, as it is simple, is more rapid to perform (2 h versus 16 h), and does not require expensive reagents and equipment. The IFA will also make it possible to determine how common *E. bieneusi* is in the environment, particularly in drinking water, and in other mammalian species. The monitoring of *E. bieneusi* should specifically benefit children with malnutrition in developing countries (23) and individuals with immunodeficiencies who are at the greatest risk.

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