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Detection and Identification of *Enterocytozoon bieneusi* and *Encephalitozoon* Species in Stool and Urine Specimens by PCR and Differential Hybridization

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Several species of microsporidia can cause disease in humans in both immunocompromised and immunocompetent individuals. *Enterocytozoon bieneusi* and *Encephalitozoon intestinalis* are most commonly associated with chronic diarrhea. All *Encephalitozoon* species, including *E. intestinalis*, *E. hellem*, and *E. cuniculi*, also cause disseminated infections. As distinctive treatment options are available for the different genera, identification is clinically important. We evaluated a PCR with primers directed to a conserved region of the small subunit rRNA gene of microsporidia. Hybridization with a generic microsporidium probe and specific probes for each of the four different species was used for identification. Probes were labeled with ruthenium and detected by electrochemiluminescence. The sensitivity of the assay was tested with plasmids containing the region of interest from each of the four different species and *Vittaforma corneae* as a control. In addition, the assay was tested with feces spiked with cultured spores from each of the three *Encephalitozoon* species and *V. corneae*. An analytical sensitivity of 3.5×10^2 to 3.5×10^3 spores per g of feces, corresponding to 17 to 170 gene copies per PCR, was found, which is several orders of magnitude more sensitive than microscopy after Uvitex 2B fluorescent staining. Stool samples from 22 microscopically diagnosed patients and from 61 uninfected controls were evaluated, showing a sensitivity of at least 95% and a specificity of 100% compared to microscopy. The method was further tested by spiking urine samples with spores of the different *Encephalitozoon* species.

Several species of microsporidia can cause disease in humans (17). *Enterocytozoon bieneusi* and the *Encephalitozoon* species *E. intestinalis*, *E. hellem*, and *E. cuniculi* have been described as opportunistic pathogens in human immunodeficiency virus (HIV)-infected patients and other immunocompromised patients such as transplant recipients (11, 18, 24, 29, 34, 36). Infections with microsporidia in immunocompetent individuals such as travelers have also been described (31, 35). In HIV-infected patients, *E. bieneusi* and *E. intestinalis* can cause a severe, persistent diarrhea, and the species have frequently been isolated from stool specimens (11, 16, 17, 34, 39). Furthermore, *Encephalitozoon* species are associated with rhinosinusitis, keratoconjunctivitis, nephritis, hepatitis, and disseminated infections (9, 17, 18, 24, 29). The *Encephalitozoon* species have been isolated from different clinical specimens such as urine and respiratory excretions (10, 16, 17), and *E. hellem* and *E. cuniculi* have occasionally been found in stool specimens (18, 29, 31).

Routine diagnosis is generally performed with microscopy after feces samples are stained by using fluorescent stains with optical brightening agents such as Uvitex 2B or Fungifluor or by using chromotrope-based stains (16, 40). However, microscopy requires experienced personnel, as distinction among the different species can be difficult, and the three *Encephalitozoon* species cannot be differentiated from each other by light microscopy (16). Correct identification is of clinical importance, as treatment of microsporidiosis depends upon the infecting species: the *Encephalitozoon* species can be treated with albendazol, whereas for *E. bieneusi*, efficacy of treatment with fumagillin has recently been shown (8, 27, 30).

Several studies on the diagnosis of intestinal microsporidiosis by PCR-based methods have been published (13, 14, 21, 25, 27, 32, 33, 35, 42). However, the reported assays either do not include differentiation of *E. bieneusi* and all three *Encephalitozoon* species or require laborious sequencing or restriction fragment length polymorphism analysis for species differentiation. To our knowledge, our study is the first report of a method for detection and identification of the four medically most important microsporidial species with a single PCR followed by hybridization with species-specific probes, allowing rapid differentiation between *E. bieneusi* and each of the *Encephalitozoon* species. Although several case reports on microscopic detection of spores of the *Encephalitozoon* species in urine or renal tissue have been published, only a few PCR-based studies have evaluated urine as a clinical sample (6, 18, 20, 23, 24, 26, 28, 29).

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MATERIALS AND METHODS

Microsporidial cultures. *E. intestinalis*, *E. hellem*, and *E. cuniculi* were cultured in RK 13 rabbit kidney cell monolayers (39). Spores were harvested by centrifugation, washed with phosphate-buffered saline (PBS), resuspended in PBS, and counted microscopically. *E. bieneusi*, the most frequently encountered species of microsporidia in stools of HIV-positive patients, cannot be grown in long-term cultures (41). As a control which was expected to test positive with the general probe and negative with the species-specific probes, spores from a culture with the microsporidial species *Vittaforma corneae* were used.

To reconstruct feces samples with known numbers of spores, 10-fold serial dilutions were made for each cultured microsporidial species, and these were added to feces from uninfected subjects. Suspensions of 1 g of feces in 8 ml of water were made, and 3.5×10^2, 3.5×10^3, 3.5×10^4, and 3.5×10^5 spores were added per sample, respectively. Fifty microliters of each spiked suspension was added to 900 μl of guanidium thiocyanate-containing lysis buffer, and DNA was extracted as described below, resulting in 17, 1.7×10^2, 1.7×10^3, and 1.7×10^4 gene copies per PCR.

For light microscopy, spores were concentrated from the remaining fecal suspensions with ether according to the Ridley method. Approximately 20 μl of the 100-μl pellet was stained with fluorochrome Uvitex 2B and examined with fluorescence microscopy at ×1,250 magnification (38, 40). The number of spores was quantified as sporadic (one or two spores per microscopy slide), few (a few spores per slide), some (one spore per high-powered field [HPF]), several (2 to 10 spores per HPF), many (10 to 25 spores per HPF), or very many (>25 spores per HPF).

DNA extraction. DNA was extracted from feces according to the method of Boom et al., with a slightly modified procedure (5). In short, approximately 200 mg of feces was suspended in 700 μl of guanidium thiocyanate-containing lysis buffer, and 50 μl of this suspension was added to 900 μl of lysis buffer. This mixture was heated for 10 min at 80°C and briefly centrifuged. Fifty microliters of silica suspension was added to the supernatant. The DNA, bound to silica, was washed with guanidium thiocyanate-containing buffer, ethanol, and acetone, and the DNA was dissolved in 50 μl of Tris-EDTA buffer. For extraction of the spiked feces samples, 1 g of feces was dissolved in 8 ml of water, and 50 μl of this suspension was added to 900 μl of lysis buffer. After heating for 10 min at 80°C and a short centrifugation, 50 μl of silica suspension was added to the supernatant. The DNA, bound to silica, was further washed and eluted as described above.

For extraction of DNA from cultured spores, counted spores were added to 900 μl of lysis buffer with 20 μl of silica suspension. The DNA, bound to silica, was further washed and eluted as described above.

Spiked urine samples. Three dilutions of counted spores from each of the three *Encephalitozoon* species were added to different portions of urine samples from two healthy laboratory workers and compared to spiked PBS. Spiked samples were incubated for 3 h at 37°C to mimic an in vivo situation and kept overnight at 4°C. For DNA isolation, 100 μl of the urine or PBS was added to 900 μl of lysis buffer with 20 μl of silica suspension. The DNA was further washed and eluted as described above.

PCR. The target used most frequently for PCRs is the microsporidial small subunit rRNA gene (16). *E. cuniculi*, the only microsporidial species for which the genome has been fully sequenced to date, contains 44 copies of the gene per diploid genome, thereby substantially increasing the sensitivity of the PCR (22). Primers used for this study were forward primer FP (positions 4 to 23 of *E. bieneusi* [GenBank accession no. AF023245], 5′-CAGGTTGATTCTGCCTG ACG, and reverse primer RP (positions 263 to 244 of *E. bieneusi*), 5′-ATCTC TCAGGCTCCCTCTCC, which was 5′ biotinylated.

The uracil-*N*-glycosylase system was used to prevent false-positive reactions due to carryover of amplimers. The final reaction mixture (50 μl) contained 10 μl of DNA eluate; 200 ng of each primer; 2.5 U of Ampli-Taq DNA polymerase (Perkin-Elmer); 0.5 U of uracil-*N*-glycosylase (Applied Biosystems, Foster City, Calif.); 5 μg of bovine serum albumin (Boehringer-Mannheim B.V., Almere, The Netherlands); 20 μg of α-casein (Sigma); 10 mM Tris-HCl (pH 8.3); 50 mM KCl; 4.25 mM $MgCl_2$; dATP, dGTP, and dCTP at concentrations of 200 μM each; and 400 μM dUTP (Applied Biosystems) (5). The PCRs were performed with a Perkin-Elmer 9600 thermocycler as follows: 2 min at 50°C and 5 min at 95°C, followed by 35 cycles each consisting of 20 s at 95°C, 20 s at 63°C, and 1 min at 72°C, followed by 5 min at 72°C.

Hybridization. A general microsporidium probe was used (positions 20 to 39 of *E. bieneusi* [GenBank accession no. AF023245]), 3′-GACGTR(A/G)GATG CTAK(G/T)TCTCTG, directed to a conserved region of the small subunit rRNA gene of microsporidia. The following specific probes were used for identification of the different species: for *E. bieneusi* (positions 143 to 162), 5′-TGTGGCTA AAAGCGGAGAAT; for *E. intestinalis* (positions 149 to 168 [GenBank accession no. L39113]), 5′-GGGGGCTAGGAGTGTTTTTG; for *E. cuniculi* (positions 148 to 167 [GenBank accession no. L39107]), 5′-ATAGTGGTCTGCCCC TGTGG; and for *E. hellem* (positions 154 to 173 [GenBank accession no. AF177920]), 5′-TCTGGGGGTGGTAGTTTGTA. Probes were 5′ labeled with Tris-(2,2′-bipyridine)-ruthenium(II) chelate and detected by electrochemiluminescence (ECL) with an M8 Analyzer (Igen International, Gaithersburg, Md.). Prior to hybridization, excess primers were removed from the PCR products according to the method of Boom et al. (4). Hybridization with the general probe and each species-specific probe was performed for separate reactions by adding 20 μl of probe to 30 μl of purified PCR product, followed by incubation of the mixtures for 2 min at 95°C and 5 min at 55°C with a 9600 thermocycler (Perkin-Elmer). Next, 10 μl of streptavidin-coated magnetic bead (Dynal Biotech, Hamburg, Germany) solution (3 μl of bead suspension with 7 μl of PCR II buffer [Perkin-Elmer]) was added, followed by incubation for another 15 min at room temperature. Fifty microliters of the bead-hybrid suspension was added to 100 μl of water, and the ECL signal, expressed in luminosity units (LU), was measured. A signal of >500 LU was considered positive, as indicated by the manufacturer.

Plasmids. Five different positive-control plasmids were constructed by cloning the PCR product, using primers FP and RP, of the respective microsporidial species into the pGem-T Easy vector (Promega, Leiden, The Netherlands). DNA for the PCRs was obtained from the respective cultures of *E. intestinalis*, *E. hellem*, *E. cuniculi*, and *V. corneae*, while for *E. bieneusi*, feces from a patient, diagnosed by microscopy, was used. DNA inserts were sequenced to confirm the identity, and plasmid concentration was measured by spectrophotometry at 260 and 280 nm. Plasmids were serially diluted in 10-fold steps, ranging from 10^6 copies to 1 copy per PCR.

Feces samples. Feces samples from four groups of subjects were tested.

Group 1. The microsporidium-positive group consisted of 24 feces samples from 22 adult patients; 23 samples contained *E. bieneusi* spores as detected by Uvitex 2B stain during routine patient care examination, and one sample contained spores of *E. intestinalis* confirmed by electron microscopy. All patients were infected with or suspected of having HIV. The feces samples, taken between 1994 and 2001, had been stored at 4°C until this study.

In addition, a negatively staining stool sample from a different HIV-positive patient diagnosed with sinusitis by *E. intestinalis* and with positive stains of urine and nasal excretion samples was also tested.

Group 2. The microsporidium-negative group of at-risk patients consisted of 31 feces samples obtained from 31 HIV-infected or otherwise immunocompromised patients (four of whom were children aged between 8 and 15 years) with diarrhea, clinically suspected of microsporidial infection but which were negative by Uvitex 2B stain for microsporidium spores in routine clinical workup. Samples were taken between July 2001 and October 2002 and were stored at 4°C until retrospective testing. Twenty-three patients were known to be HIV positive, three children were agammaglobulinemic, and one patient had traveled to the tropics.

Group 3. The microsporidium-negative group of healthy controls consisted of 14 feces samples from healthy laboratory workers and their household members, including five children.

Group 4. The microsporidium-negative group of patients with other pathogens consisted of 16 feces samples from patients, including 10 children aged between 2 and 16 years, with other gastrointestinal pathogens. Microorganisms found in the feces samples of the three microsporidium-negative groups were *Aeromonas hydrophila*, *Blastocystis hominis*, *Campylobacter jejuni*, *Clostridium difficile*, *Cryptosporidium parvum*, *Dientamoeba fragilis*, *Endolimax nana*, *Entamoeba coli*, *Entamoeba dispar*, *Entamoeba hartmanni*, *Giardia lamblia*, *Shigella sonei*, *Strongyloides stercoralis*, *Taenia* species, *Trichuris trichiuria*, and yeast.

RESULTS

To test the analytic sensitivity of the PCR procedure, each plasmid with an insert of the different microsporidium species was serially diluted and tested. Plasmids containing inserts of *E. intestinalis* and *E. cuniculi* were positive from 10 copies/PCR, and plasmids of *E. bieneusi*, *E. hellem*, and *V. corneae* were positive from 10^2 copies/PCR (Table 1). The input copy number ($\log_{10}$ transformed) correlated with the LU values from the general probe and the positive respective species-specific probe, with squared Pearson's correlation coefficients between 0.86 and 0.97 (Table 1).

TABLE 1. Serial dilutions of plasmids with inserts of five different microsporidial species after PCR and hybridization with the general microsporidium probe and the specific probe for the respective species

Species	Probe	ECL signal (LU) with plasmid input in copies/PCR[a]							R^{2b}
		1	10	10^2	10^3	10^4	10^5	10^6	
E. bieneusi	General	ND	360	2,499	20,471	13,599	25,094	35,454	0.87
	Specific	ND	397	3,078	16,185	20,047	33,565	46,897	0.96
E. intestinalis	General	207	597	2,604	8,929	25,744	24,823	34,808	0.91
	Specific	243	707	3,026	12,964	31,264	22,031	40,824	0.86
E. cuniculi	General	ND	3,347	5,860	26,559	38,664	33,716	47,189	0.89
	Specific	ND	3,006	7,245	26,007	33,094	29,034	45,180	0.89
E. hellem	General	ND	383	2,436	8,217	29,660	37,503	37,407	0.91
	Specific	ND	475	4,015	15,412	31,662	44,121	47,330	0.97
V. corneae	General	ND	261	1,207	5,886	18,264	20,383	25,804	0.94

[a] Values represent ECL signal expressed in luminosity units (LU). ND, not done.
[b] Correlation (squared Pearson's correlation coefficient) between $\log_{10}$-transformed input copy number and ECL signal.

Negative PCR controls (water) from 33 experiments gave a mean value in the ECL of 252 LU (standard deviation [SD], 37 LU) with the general microsporidium probe and means between 247 and 291 LU (SD, 36 to 60 LU) for the different species-specific probes, justifying the manufacturers cutoff of 500 LU. No cross-reactivity was observed with probes directed to microsporidial species other than the one used (data not shown).

Next, feces samples from uninfected subjects were spiked with cultured spores of the different species, and the PCR results were compared with results obtained with detection by light microscopy after Uvitex 2B staining. *E. cuniculi* and *E. hellem* could each be detected by PCR at concentrations of 3.5×10^2 spores/g of feces (17 gene copies per PCR), and *E. intestinalis* and *V. corneae* were detected at 3.5×10^3 spores/g of feces (1.7×10^2 gene copies per PCR). With light microscopy, *E. intestinalis* and *E. cuniculi* could be detected at 3.5×10^4 spores/g of feces, and *E. hellem* and *V. corneae* could be detected at 3.5×10^5 spores/g of feces (Table 2). The input copy number ($\log_{10}$ transformed) correlated with the LU values from the general probe and the respective positive species-specific probe, with squared Pearson's correlation coefficients between 0.71 and 0.99 (Table 2).

TABLE 2. Spiked feces with serial dilutions of cultured spores from four different microsporidial species after PCR and hybridization with the general microsporidium probe and the specific probe for the respective species compared with Uvitex 2B stain and light microscopy

Species[a]	Probe	ECL signal (LU) with spore input per g of feces (gene copies per PCR) of[b]:				R^{2c}
		3.5×10^2 (17)	3.5×10^3 (1.7×10^2)	3.5×10^4 (1.7×10^3)	3.5×10^5 (1.7×10^4)	
E. intestinalis	General	215	3,032	13,527	22,266	0.96
	Specific	234	4,996	17,642	35,372	0.94
	Uvitex	Neg.	Neg.	Sporadic	Some	
E. cuniculi	General	562	4,277	26,987	27,967	0.87
	Specific	564	4,305	35,538	35,786	0.84
	Uvitex	Neg.	Neg.	Sporadic	Few	
E. hellem	General	568	4,146	4,972	30,171	0.72
	Specific	650	4,951	6,492	45,498	0.71
	Uvitex	Neg.	Neg.	Neg.	Sporadic	
V. corneae	General	280	3,780	8,077	13,523	0.99
	Uvitex	Neg.	Neg.	Neg.	Sporadic	

[a] *E. bieneusi* spores were not available, as they cannot be maintained in long-term culture.
[b] Values represent ECL signal expressed in LU.
[c] Correlation (squared Pearson's correlation coefficient) between $\log_{10}$-transformed input copy number and ECL signal.

The spiked urine samples were positive with the general and the corresponding specific probes, with ECL signals comparable to those from the spiked PBS samples. The noncorresponding probes gave negative ECL values, and the unspiked samples were negative (Table 3).

Of the 24 stool samples from the microsporidium-positive group, 23 samples tested positive with the general microsporidium probe, with a mean ECL signal of the positive samples of 27,528 (SD, 9,574) LU. All positive samples reacted with one species-specific probe, corresponding to the microscopically diagnosed species (22 *E. bieneusi* and 1 *E. intestinalis*) and were negative for the other probes. One sample, microscopically found to contain structures assumed to be *E. bieneusi* spores, was negative with all probes, also upon repeat PCR.

The microscopically negative feces sample from the patient with *E. intestinalis* sinusitis gave a low positive ECL signal of 575 LU with the general probe and 728 LU with the specific *E. intestinalis* probe. Upon repeating the PCR and hybridization, signals for the sample were 585 and 774 LU, respectively.

All 31 feces samples from the microsporidium-negative group of at-risk patients tested negative in the PCR with all probes. The 14 samples from the microsporidium-negative group of healthy controls and the 16 samples from microsporidium-negative group of patients with other pathogens were also all PCR negative.

Using microscopy as the "gold standard " and omitting repeated samples from the same individuals and the microscopically negative sample from the patient with *E. intestinalis* sinusitis, our PCR has a sensitivity of at least 95% and a specificity of 100%.

DISCUSSION

We describe a single PCR with differential hybridization to detect and identify the four clinically most relevant microsporidial species in stool specimens. Several studies on the diagnosis of intestinal microsporidiosis by PCR-based methods have been published. However, most studies do not include differentiation of *E. bieneusi* and all three *Encephalitozoon*

TABLE 3. Two urine samples and PBS after PCR and hybridization with the general microsporidium probe and the specific probes for the respective species

Sample[a]	Probe	ECL Signal (LU)								
		E. intestinalis (DNA copies/PCR)			*E. cuniculi* (DNA copies/PCR)			*E. hellem* (DNA copies/PCR)		
		2.5×10^6	2.5×10^4	2.5×10^2	5.0×10^6	5.0×10^4	5.0×10^2	1.9×10^6	1.9×10^4	1.9×10^2
Urine I	General	23,590	18,142	3,972	29,368	18,913	461	25,558	15,805	872
	Specific	28,465	25,477	5,183	34,359	19,990	478	30,089	19,733	626
Urine II	General	29,912	7,539	11,916	36,322	28,507	2,095	30,847	27,737	2,884
	Specific	34,117	11,929	14,124	35,581	29,362	2,755	33,581	31,292	3,971
PBS	General	23,458	15,467	864	28,507	2,082	1,809	24,136	15,957	235
	Specific	33,459	18,557	1,182	29,362	19,568	2,484	33,906	17,567	277

[a] Samples were spiked with three serial dilutions of cultured spores from each of the three *Encephalitozoon* species.

species (25, 32, 33), including three studies applying real-time technology that detected either only *E. bieneusi* (27) or only the *Encephalitozoon* species (21, 42).

In comparison to microscopy with Uvitex 2B fluorescent stain, the spiking experiments showed a 10- to 1,000-fold-higher sensitivity of the PCR compared to microscopy, comparable to data from other studies (27, 32, 42). A further advantage of PCR over microscopy, although not detected in our study, could be the detection of infections by two (or more) different species in one patient (31, 32). One clinical sample was positive by PCR, while it was negative by microscopy. As this sample came from a patient with *E. intestinalis* sinusitis with positive stains of urine and nasal excretion samples, the positive PCR more likely reflects a higher sensitivity rather than a lower specificity of our PCR compared to microscopy after Uvitex 2B staining.

No PCR-positive samples were found among the microscopy-negative stool samples of other patients at risk for microsporidial infection. This result could in part be related to the strong decrease in incidence of microsporidial diarrhea among HIV-infected patients since the introduction of potent antiretroviral therapy, as these stool samples were obtained several years later than the positive samples (7, 15).

One sample was negative by the PCR while it was originally reported as positive for *E. bieneusi* spores by microscopy. As the sample was stored for 6 years at 4°C before the PCR was performed, degradation of the spores and microsporidial DNA is possible, although the other positive samples were stored under identical conditions for the same duration. Spiking of the sample with a known amount of *E. intestinalis* showed no sign of inhibitors of DNA extraction or the PCR (data not shown). Alternatively, the discrepancy may be due to false-positive microscopy. A reexamination of the stool sample, both the original slide from 1996 and a newly stained slide, showed some microsporidium-like structures, not highly suspect for *E. bieneusi* spores. As the patient was microscopically negative for microsporidia in six previous and four subsequent stool samples, false-positive microscopy appears to be the most likely explanation for these discrepant results. Therefore, the sensitivity of 95% of the PCR may be an underestimation.

The specificity of our PCR in combination with the differential hybridization was shown by the lack of cross-reactivity between the different *Encephalitozoon* species obtained from culture and the lack of cross-reactivity of the species-specific probes with *V. corneae*, while the general microsporidium probe gave a clearly positive signal. *V. corneae* has more similarity with *E. bieneusi* in the small subunit rRNA gene sequence than with the *Encephalitozoon* species, but *V. corneae* has not been isolated from stool specimens, making it a very suitable control to test specificity (12, 19). Also, none of the samples from healthy subjects or feces samples containing other enteric pathogens showed a positive PCR.

Isolation of DNA from spores in fecal specimens can be difficult, and feces is known to contain PCR inhibitors (13, 16). The nucleic acid isolation method of Boom et al. (3) has been evaluated for use with feces with different microorganisms (5, 37). To enhance DNA extraction efficiency from the spores, the samples were heated in lysis buffer for 10 min at 80°C. The addition of α-casein and bovine serum albumin to the PCR mixture has previously been shown to relieve PCR inhibition of feces samples (1, 5).

The method of Boom et al. used for nucleic acid isolation, with the addition of α-casein, has also been evaluated for urine, another clinical specimen known to contain PCR inhibitors (2). We tested two spiked urine samples and found results comparable to those found with spiked PBS, suggesting that our methods can be used to test urine from patients suspected of having renal microsporidiosis. A substantial proportion of the case reports on disseminated microsporidiosis in non-HIV-infected patients concerns renal transplant patients, indicating that a more systematic evaluation in this patient group should be performed (6, 18, 24, 26, 29).

The correlations between input in the reconstruction experiments and the ECL signal show that our PCR system could be used in a quantitative manner. Furthermore, the PCR could be used for detection of other microsporidial species, as positive PCR products that reacted with the general probe but not with any of the four specific probes could be sequenced.

In conclusion, we demonstrate a sensitive and specific PCR with differential hybridization to detect and identify the four medically most important microsporidial species, allowing rapid differentiation between *E. bieneusi* and the three *Encephalitozoon* species.

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