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 cornea* as a control. In addition, the assay was tested with feces spiked with cultured spores from each of the three *Encephalitozoon* species and *V. cornea*. An analytical sensitivity of 3.5 × 10³ to 3.5 × 10⁴ 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 albendazole, 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).
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 cornea were used.

To construct the microsporidia 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 × 10², 3.5 × 10³, 3.5 × 10⁴, and 3.5 × 10⁵ 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 × 10⁴, 1.7 × 10⁵, and 1.7 × 10⁶ 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 fluorescein-labeled anti-E. bieneusi antibody (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-hybridization suspension was added to 100 μl of water, and the ECL signal, expressed in luminescence 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 plasmids was obtained from the respective cultures of E. intestinalis, E. hellem, E. cuniculi, and V. cornea. For each sample, the DNA of interest was amplified by PCR with a specific probe, with squared Pearson's correlation coefficients above 0.95. A general microsporidium probe was used (positions 20 to 39 [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16). Hybridization. A general microsporidium probe was used (positions 20 to 39 of E. bieneusi [GenBank accession no. AF012324]), 5'-CAGGTTGATTCTGCCTGACG, and reverse primer RP (positions 263 to 244 of E. bieneusi subunit rRNA gene (16).
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 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 by light microscopy after Uvitex 2B staining. E. cuniculi and E. hellem could each be detected by PCR at concentrations of 3.5 \times 10^3 spores/g of feces (1.7 \times 10^5 gene copies per PCR), and E. intestinalis and V. corneae were detected at 3.5 \times 10^5 spores/g of feces (1.7 \times 10^7 gene copies per PCR). With light microscopy, E. intestinalis and E. cuniculi could be detected at 3.5 \times 10^5 spores/g of feces, and E. hellem and V. corneae could be detected at 3.5 \times 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).

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 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.

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

<table>
<thead>
<tr>
<th>Sample</th>
<th>Probe</th>
<th>E. intestinalis (DNA copies/PCR)</th>
<th>E. cuniculi (DNA copies/PCR)</th>
<th>E. hellem (DNA copies/PCR)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>2.5 × 10⁶</td>
<td>5.0 × 10⁶</td>
<td>10²</td>
</tr>
<tr>
<td>Urine I</td>
<td>General</td>
<td>23,590</td>
<td>29,368</td>
<td>2,558</td>
</tr>
<tr>
<td></td>
<td>Specific</td>
<td>28,465</td>
<td>34,359</td>
<td>30,089</td>
</tr>
<tr>
<td>Urine II</td>
<td>General</td>
<td>29,912</td>
<td>36,322</td>
<td>30,847</td>
</tr>
<tr>
<td></td>
<td>Specific</td>
<td>34,117</td>
<td>33,581</td>
<td>33,906</td>
</tr>
<tr>
<td>PBS</td>
<td>General</td>
<td>23,458</td>
<td>28,507</td>
<td>24,136</td>
</tr>
<tr>
<td></td>
<td>Specific</td>
<td>33,459</td>
<td>29,362</td>
<td>33,906</td>
</tr>
</tbody>
</table>

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

### REFERENCES


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