

Development of a Multiplex Real-Time PCR Assay for Quantitative Detection of Microsporidia in Clinical Samples

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ABSTRACT

Background: While fourteen microsporidial species from seven genera are recognized worldwide as etiological agents of disease in humans, the overwhelming majority of microsporidiosis cases are attributed to two species, *E. bienewsi* and *E. intestinalis*. Infections caused by these spore-forming, protozoan parasites have been predominantly identified within immunologically compromised populations such as transplant patients and those with AIDS. Routine identification of microsporidia within clinical samples relies on light microscopic analysis of samples treated with non-selective histochemical stains such as the modified trichrome stain and fluorescent brighteners. Due to the insensitivity of these microscopic techniques, many clinical parasitologists believe there is a gross underreporting of microsporidiosis. Over the last few years, molecular-based techniques such as real-time PCR assays have shown great applicability for the specific and sensitive detection of human-pathogenic microsporidia in clinical samples. **Methods:** The real-time PCR assay presented here utilizes probes and primer sets that specifically target the multi-copy 16S rRNA gene sequence of *E. bienewsi*, *E. intestinalis*, *E. hellem*, *E. cuniculi*, *V. corneae*, *T. hominis* and *N. algerae*. Efficient nucleic acid extraction methods were developed so that microsporidial DNA can be detected in a wide range of clinical matrices. **Results:** When *E. intestinalis* spores were seeded into clinical matrices, the limit of detection for the assay was determined to be 100 spores/g of feces and 10 spores/mL in urine, respiratory secretions and cerebral spinal fluid. As compared to the detection limits reported for microscopy (1,000,000 spores/mL of liquid feces), this PCR assay is considerably more sensitive. The specificity of the assay was verified with DNA isolated from numerous microsporidial species and a host of clinically relevant bacteria and parasites. **Conclusions:** This multiplex real-time PCR assay offers a superior alternative to the less sensitive and time-consuming microscopy-based techniques that are currently in use within clinical laboratories.

INTRODUCTION

Microsporidia are recognized worldwide as an emerging opportunistic human pathogen. These spore-forming, protozoan parasites have predominantly been recognized within the immunologically compromised population, including transplant patients and those with acquired immune deficiency syndrome (AIDS). However, recently these pathogens have also been identified in association with immunocompetent populations and travelers. While the most common clinical manifestation of microsporidiosis is either diarrhea, encephalitis, ocular infection, sinusitis and myositis, disseminated infections have been reported. The overwhelming majority of microsporidiosis in humans is attributed to two species, *Enterocytozoon bienewsi*, and *Encephalitozoon intestinalis*. However, other species such as *Encephalitozoon hellem*, *Encephalitozoon cuniculi*, *Vittaforma corneae*, *Trachipleistophora hominis* and *Nosema algerae* have been infrequently identified with human infections.

The diagnosis of microsporidiosis in clinical samples at present is difficult due to the small size and non-distinct shape of these infectious spores. Routine detection of microsporidia relies on light microscopic analysis of samples treated with non-selective histochemical stains such as the modified trichrome stain and fluorescent brighteners. Molecular-based techniques such as real-time PCR assays have been shown in numerous reports to have detection limits as low as 100 spores per mL of liquid feces while current staining techniques are far less sensitive with detection limits greater than 1,000,000 per mL of liquid feces. Due to the insensitivity of these staining techniques, many clinical parasitologists believe there is a gross underreporting of microsporidiosis.

The multiplex real-time PCR assay developed in this study has shown great applicability for the detection of human-pathogenic microsporidial species in clinical samples. This PCR assay utilizes probes and primer sets that specifically target the multi-copy 16S rRNA gene sequence of the clinically relevant human-pathogenic microsporidia *E. bienewsi*, *E. intestinalis*, *E. hellem*, *E. cuniculi*, *T. hominis*, *N. algerae* and *V. corneae*.

METHODS

Extraction and Isolation of Microsporidial DNA

Hemocytometrically enumerated microsporidial spores were seeded into either reagent water or clinical matrix (e.g. feces, urine, mucous or CSF). Microsporidial DNA was extracted from these seeded samples using the following kits: MoBio UltraClean Fecal DNA Kit, Qiagen QIAamp DNA Mini Kit, Qiagen QIAamp Stool Mini Kit, Qiagen QIAamp Viral RNA Mini Kit and Roche High Pure PCR Template Preparation Kit. When mentioned, microsporidial spores were disrupted by agitation using glass beads and a Mini-Bead Beater.

Real-Time PCR Assays

Each 25 μ l PCR reaction contained 0.1 μ M of both EbieP1 and MicroP1 Taqman probes, 0.5 μ M of the each forward and reverse primers, 1.8 μ g/ μ l of bovine serum albumin Fraction V (Sigma-Aldrich Co., St. Louis, MO), 1 x Taqman Universal PCR Master Mix [which consists of a mixture of AmpliTaq Gold DNA polymerase, Amperase UNG, dNTPs (dCTP, dGTP, dATP and dUTP) (Applied Biosystems), passive reference 6-carboxy-X-rhodamine (ROX), MgCl₂ and buffer components] and 5 μ l of extracted DNA template. Five No Template Control (NTC) PCR samples were run as negative controls in each round of PCR to ensure that reagents were free from contamination. PCR was performed with an ABI-Prism 5700 Sequence Detector using PCR cycling conditions consisting of 2 min at 50°C, 10 min at 95°C, followed by 40 cycles of 95°C for 0.25 min and 60°C for 1 min.

RESULTS

This study describes a multiplex real-time PCR assay that is suitable for rapid, sensitive, high-throughput detection of the clinically relevant, human-pathogenic microsporidia *E. bienewsi*, *E. intestinalis*, *E. hellem*, *E. cuniculi*, *V. corneae*, *N. algerae* and *T. hominis*. This assay utilizes species-specific primer sets and two dual fluorescently-labeled probes with differing specificities (Table 1). Genetic diversity between the 16S rRNA of *E. bienewsi* and the other human-pathogenic microsporidia required that two different Taqman probes be utilized in this multiplex assay. The probe, EbieP1 specifically targets a region within the 16S rRNA of *E. bienewsi*, while the probe, MicroP1 targets a conserved region within 16S rRNA of *E. intestinalis*, *E. hellem*, *E. cuniculi*, *V. corneae*, *N. algerae* and *T. hominis*.

Since human-pathogenic microsporidia can disseminate throughout the human host, bodily fluids such as feces, urine, respiratory secretions and CSF are commonly examined for the presence of infectious spores. Due to the varying complexity of each type of clinical sample, each requires a slightly different nucleic acid extraction procedure. As shown in Table 2, when *E. intestinalis* spores were seeded into control feces, the Qiagen QIAamp DNA Stool Mini Kit coupled with a bead beating procedure was found to provide the highest sensitivity. When DNA from 10 fecal samples seeded with *E. intestinalis* spores were isolated and the reproducibility determined with the multiplex real-time PCR assay, the coefficient of variation was determined to be only 1.3%. For respiratory secretions and CSF, the Qiagen QIAamp DNA Mini Kit coupled with a bead beating procedure provided high sensitivity and reproducibility. Because urine contains numerous PCR inhibitors, the Qiagen QIAamp Viral RNA Mini Kit coupled with a bead beating procedure was consistently shown to provide both high sensitivity and reproducibility.

The sensitivity of the multiplex real-time PCR assay was determined in feces, urine mucous and CSF as shown in Table 3. When *E. intestinalis* spores were seeded into the various clinical matrices, the limit of detection for the assay was determined to be 100 spores/g of feces and 10 spores/mL in urine, respiratory secretions and CSF. A typical real-time PCR calibration curve/amplification curve is shown in Figure 1 where DNA was extracted from control feces seeded with 100,000, 10,000, 1,000, 100 and 10 *E. intestinalis* spores, respectively.

The specificity of the assay was confirmed with DNA isolated from the various microsporidial isolates located in Table 4 as well as DNA extracted from numerous other protozoa, algae and bacteria.

Table 2. Evaluation of commercially available DNA extraction kits

Fecal DNA Extraction Kits	W/O Feces (C _T)	Feces (C _T)
MoBio UltraClean Fecal DNA Kit	<i>n.d.</i>	32.3 (n=4)
Qiagen DNA Mini Kit (+Bead Beating)	22.5 (n=2)	26.0 (n=3)
Qiagen DNA Stool Mini Kit	33.5 (n=3)	33.9 (n=3)
Qiagen DNA Stool Mini Kit (+Bead Beating)	26.7 (n=3)	26.1 (n=3)
Roche High Pure PCR Kit	24.8 (n=3)	29.4 (n=3)

^a4x10⁴ *E. intestinalis* spores/gram feces

Table 3. Sensitivity of the multiplex real-time PCR assay

<i>E. intestinalis</i> spores	Feces (C _T)	Urine (C _T)	Mucous (C _T)	CSF (C _T)
100,000	25.66 ± 0.08			
10,000	29.32 ± 0.19			
1,000	33.33 ± 0.23	29.43 ± 0.13	30.30 ± 0.15	
100	37.39 ± 0.90	32.87 ± 0.06	33.56 ± 0.10	31.09 ± 0.14
10	39.59 ± 0.49	38.00 ± 0.62	35.66 ± 0.81	38.06 ± 0.39

E. intestinalis spore counts are per gram of feces or mL of urine, mucous and CSF, respectively
C_T values correspond to the mean of nine replicates total, three replicates from each of three individual DNA extraction procedures
Shaded cells indicate the limit of detection for each clinical matrix

Figure 1. Calibration curve/amplification plot generated with feces seeded with *E. intestinalis* spores

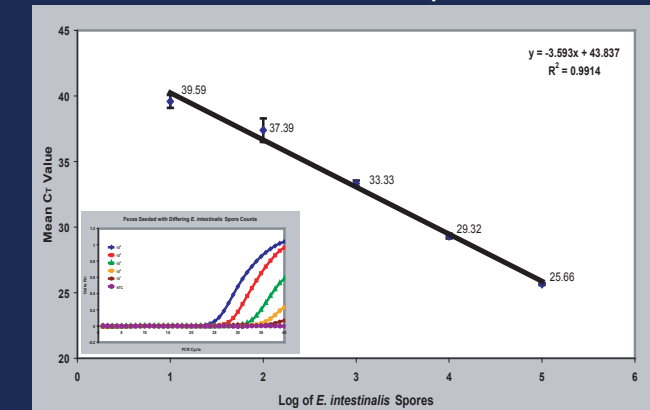


Table 4. Specificity testing

Specificity Testing
<i>Encephalitozoon cuniculi</i> (ATCC#50505)
<i>Encephalitozoon hellem</i> (CDC:0291:V213)
<i>Encephalitozoon intestinalis</i> (ATCC#50603)
<i>Vittaforma corneae</i> (ATCC#50505)
<i>Enterocytozoon bienewsi</i>
CDC Sample 6560
CDC Sample 6562
CDC Sample 6564
CDC Sample 6566
CDC Sample 6568
<i>Cyclospora cayetanensis</i>

DISCUSSION

The goal of this present study was to validate a method that overcomes the shortcomings of the microscopy-based techniques that are routinely used in clinical reference laboratories. Current literature would suggest that the sensitivity of this multiplex real-time PCR assay is a 1000-fold higher than the microscopy-based techniques. Furthermore, this assay has the potential to provide clinicians with species-level identification which is rarely possible with the microscopy-based techniques. This is extremely important since the efficacy of a particular therapeutic regimen has been shown to be dependent on the microsporidial species.

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Table 1. Oligonucleotides used in the multiplex real-time PCR assay

Probes/Primers	Sequence (5'-3')	Genome Localization	Length (nucleotides)	T _m (°C)
MicroP1	6FAM-ACA CAC CGC CCG TC-BHQ1	1170-1183 ^a 1190-1203 ^b 1171-1184 ^c 1256-1269 ^d 1236-1249 ^e 1135-1148 ^f	14	67.4
EbieP1	6FAM-CAG CTG AAG GAT CAT TT-BHQ1	90-106 ^g	17	69.7
EbieF1	CGT AAC AAG GTT TCA GTT GGA GAA	65-88 ^g	24	58.4
EbieR1	CGA TAC CCA CAC CCC AAA AA	130-111 ^g	20	59.1
EintF1IBT	TTG AAT GGG TCC CTG TCC TTT	1148-1168 ^c	21	59.3
EintR1IBT	TCC ACT GCG TCA TCT TAG ATA GC	1207-1185 ^c	23	57.3
EcunF1IBT	CTT TGA ATG TGT CCC TGT CCT TT	1145-1167 ^g	23	58.2
EcunR1IBT	AGG ACT CAG ACC TTC CGA TCT TC	1230-1208 ^g	23	58.9
EhelF1IBT	TGA ATG TGT CCC TGT CCT TTG T	1168-1189 ^g	22	58.0
EhelR1IBT	GTC CAC TGC GTC ATC TTA GAT AGC	1227-1204 ^b	24	58.1
EhelR2IBT	TGC CAC TGC GTC ATC TTA GAT AGC	1227-1204 ^b	24	61.5
VcorF1	AAG GCA CAT ACG AAA GAG GAA TTC	1061-1084 ^f	24	58.7
VcorR1	CTT CAT CCT GAA AAA CAT CTG AGA TAG T	1176-1149 ^f	28	58.4
NalgF1	ATG AAT GAG TCC CTG TTC TTT GC	1233-1255 ^f	23	58.3
NalgR1	CTT CAT AAA AAC ATC CAT CTC AGA TAG C	1297-1270 ^f	28	58.8
ThomF1	CTC ATT AAG ACG CGA TGA ATA CGT	1199-1222 ^g	24	58.4
ThomR1	CGC CTG ACT CCA TCT TCG AT	1272-1253 ^g	20	58.8

^a Nucleotide positions are based on the *E. cuniculi* 16S rRNA gene sequence (GenBank accession no. X98469).
^b Nucleotide positions are based on the *E. hellem* 16S rRNA gene sequence (GenBank accession no. L39108).
^c Nucleotide positions are based on the *E. intestinalis* 16S rRNA gene sequence (GenBank accession no. U09929).
^d Nucleotide positions are based on the *N. algerae* 16S rRNA gene sequence (GenBank accession no. AF069063).
^e Nucleotide positions are based on the *T. hominis* 16S rRNA gene sequence (GenBank accession no. AJ002605).
^f Nucleotide positions are based on the *V. corneae* 16S rRNA gene sequence (GenBank accession no. 954830).
^g Nucleotide positions are based on the *E. bienewsi* 16S rRNA gene sequence (GenBank accession no. 3983258).
T_m calculated using Primer Express software (Applied Biosystems)
6FAM - 6-carboxyfluorescein
BHQ-1 - Black Hole Quencher-1