Detection and species identification of microsporidial infections using SYBR Green real-time PCR

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INTRODUCTION

The phylum Microspora contains a diverse set of unicellular spore-forming parasites, evolutionarily related to fungi. None are obligate parasites of humans; however, several can give rise to opportunistic infections. Whilst these infections are found most commonly in immunocompromised patients, Enterocytozoon bieneusi has been reported in the stool samples of immunocompetent individuals (Gumbo et al., 2000), and serological data suggest that infections due to Enterocytozoon bieneusi and Encephalitozoon species could be common (Sak et al., 2010; van Gool et al., 1997). Along with Enterocytozoon bieneusi, the most common intestinal microsporidian parasite in humans is Encephalitozoon intestinalis, which may cause diarrhoea in infected individuals. In total, 14 different microsporidian species have been reported to infect humans (Garcia, 2002), producing infections in muscular (Chupp et al., 1993; Grau et al., 1996), ocular, genito-urinary or disseminated sites (Chabchoub et al., 2009; Didier et al., 1996; Franzen et al., 1995; Tosoni et al., 2002). The spores of these parasites can be identified as small (1–4 μm) oval bodies that exhibit fluorescence when stained with calcofluor or Fluorescent Brightener 28 (Oxoid). An experienced microscopist may be able to use the size and shape of the spores to differentiate Enterocytozoon bieneusi from other microsporidia; however, transmission electron microscopy (TEM) is still the gold standard and is necessary for the definitive identification of the microsporidian species. This is achieved by examining the morphology of the polar filaments characteristic of microsporidia. Because TEM is expensive, time-consuming and not feasible for routine diagnosis, molecular methods are increasingly being developed and employed for diagnosis and surveillance of these organisms (Chabchoub et al., 2009; Ghosh & Weiss, 2009; Joseph et al., 2006; Kumar et al., 2005).

A number of conventional species-specific PCRs and probe-based real-time PCRs exist for the detection of different microsporidian species within a sample (Menotti et al., 2003a, b; Wolk et al., 2002), whilst a multiplex real-time PCR has been developed for the detection of Encephalitozoon bieneusi and Encephalitozoon species (Verweij et al., 2007). Discrimination among multiple human pathogenic...
microsporidian species has been achieved using both oligonucleotide microarrays (Wang et al., 2005) and PCR followed by differential hybridization (Notermans et al., 2005). However, the requirement to cover the full spectrum of potential human parasites could represent significant financial and technical barriers for routine diagnosis and/or surveillance. Here, we investigated a novel set of primers to a conserved region of the small-subunit (SSU) rRNA gene to develop a sensitive pan-phylum real-time PCR assay for the detection and differentiation of enteric microsporidial infections.

**METHODS**

**Samples.** The new assay was developed by comparing its performance and that of routine microscopy against a set of Centers for Disease Control and Prevention (CDC)-validated PCRs (da Silva et al., 1997a; de Groote et al., 1995) on archived samples and on stool specimens submitted for routine diagnosis requiring testing for microsporidal infection. No additional samples were taken for the purpose of assay development.

Initial work on the assay used 18 archived stool samples positive for microsporidia by microscopy, which had been stored at 4 °C for between 3 and 7 years. Sixteen negative-control stool samples were either donations from uninfected immunocompetent individuals or archived patient samples containing Blastocystis sp., Cyclospora cayetanensis, Cryptosporidium species, Ascaris lumbricoides, Giardia intestinalis and Entamoeba histolytica. Cultured spores of Enterocytozoon intestinalis, Encephalitozoon cuniculi and Encephalitozoon hellem used in the assay development were a kind gift from Dr Tom van Gool. For the sample of *Encephalitozoon intestinalis* spores, an accurate spore count was made using a haemocytometer and a dilution series prepared in PBS. DNA was extracted from 200 μl aliquots containing 20,000, 2000, 200, 20 and 2 spores into 100 μl elution buffer. Five microlitres of the resulting eluates were calculated to yield DNA equivalent to that from 2000, 200, 20 and 0.2 spores of *Encephalitozoon intestinalis*, respectively (assuming complete recovery of DNA).

Subsequent assay development used 168 consecutive human stool samples from immunocompromised patients, sent for routine diagnosis to the Department of Clinical Parasitology at the Hospital for Tropical Diseases, and to the Diagnostic Parasitology Laboratory at the London School of Hygiene and Tropical Medicine. These patients included human immunodeficiency virus-positive and post-transplantation patients, as well as patients on immunosuppressive therapy.

**DNA extraction.** DNA was extracted from 100 mg stool or 200 μl purified spore suspension using a modified QIAamp DNA Mini kit protocol for tissue extraction (Qiagen). For stool samples, 100 mg solid material was washed twice in 1 ml PBS (pH 7.2) before being spun at 14,000 g for 5 min. The supernatant was removed and 400 μl ATL buffer and 40 μl proteinase K (both supplied in kit) were added to the resultant pellet. The sample was vortexed for 1 min and incubated at 56 °C for 2 h with pulse vortexing every 30 min. The sample was spun at 6500 g for 1 min and 240 μl supernatant was transferred to Safe-Lock 2 ml tubes (Qiagen) for processing in the QIAcube DNA extraction system using a modified version of the DNA mini tissue programme supplied by Qiagen in which DNA was eluted with 2 × 50 μl aliquots of AE buffer (in kit).

To evaluate the effects of formalin treatment (a common procedure in diagnostic laboratories) on the yield of amplifiable DNA, four replicates of the same sample were incubated for 7 days at 4 °C with an equal volume of 10% formalin or PBS before extraction with ATL buffer. The level of DNA amplification achieved with each sample was tested using the real-time PCR listed below and the mean DNA titre and 95% confidence interval (CI) was calculated for each extraction condition. Alongside these extractions, four replicate samples stored in PBS were also extracted using a standard Qiagen Stool protocol to evaluate its performance against our modified tissue protocol.

**Real-time and conventional PCR amplification and sequencing.** Two new primers for real-time SYBR Green amplification were designed using Primer3 software (Rozen & Skaltsky, 2000) against the *Enterocytozoon bieneusi* SSU-rRNA sequence. Primers MsRTf1 (5'-CACGGTGGATTCTGGCAGGAG-3') and MsRTf1 (5'-CCATCTCCTCAGGCTCCCTCT-3') were chosen to ensure amplification of *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi* and *Encephalitozoon hellem* SSU-rRNA sequences in addition to the *Enterocytozoon bieneusi* sequences. Amplification was performed using the TIGR MSRT primers (Qiagen) Forward and reverse primers (10 pmol each) were used in 20 μl reactions containing 5 μl template DNA. The optimum cycling conditions were 95 °C for 10 s, 60 °C for 20 s and 72 °C for 20 s. The assay was set up in duplicate for each sample and run for 35 cycles. A sample was recorded as positive for microsporidial DNA when the normalized fluorescence in both tubes was greater than 0.1 (as determined by Rotor-Gene 6 software, version 6.1; Corbett Research). DNA from an *Encephalitozoon intestinalis* spore dilution series was also used to determine the sensitivity threshold for the assay by setting up multiple replicate reactions containing DNA equivalent to that of 20 or 2 spores.

Conventional PCR for *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis* and *Encephalitozoon cuniculi* was performed using the EBI Elf1/EBIER1 (da Silva et al., 1997b), SINTF1/SINTR (da Silva et al., 1997a; Visvesvara et al., 1995) and ECUF6/ECUNR (De Groote et al., 1995) primer pairs, respectively, with 45 rounds of amplification, according to published conditions.

Where amplification was achieved using real-time analysis but not the conventional species-specific PCR, the real-time product was purified using a QIAquick PCR cleanup kit (Qiagen). The purified product was then sequenced using BigDye version 3.1 chemistry on an ABI 3730 automated sequencer (Applied Biosystems) using the MsRTf1 and MsRTf1 primers. A single contig was compiled for each product using the Sequen program (DNASTAR). The resultant sequences were identified using a BLAST search against the EMBL database on the EBI website (www.ebi.ac.uk).

Before the assay could be deployed as a routine diagnostic assay, acceptance criteria for an internal control (to monitor the inhibition of PCR) had to be established. A SYBR Green-based real-time PCR using phocine herpesvirus type 1 (PhHV-1) DNA was chosen. The conditions for this reaction were identical to those of the *Enterocytozoon intestinalis* real-time PCR, except that the MsRTf1/MsRTf1 primers were replaced by 15 pmol each of the PhHV-267s and PhHV-337 primers (Niesters, 2002) and PhHV-1 control DNA was spiked into the reaction mix. Thirty-three DNA samples positive for microsporidial DNA were reanalysed in order to set an amplification cycle threshold (*C*<sub>β</sub>) of PhHV-1 (mean *C*<sub>β</sub> + 1.96 SD) to be used in identifying samples needing to be retested during the routine deployment of this assay.

**Microscopy.** A small volume (~20 μl) of stool sample was smeared onto a slide, air dried and fixed with methanol. The sample was then stained with Fluorescent Brightener 28 (Oxoid) before examination under a Zeiss UV microscope at 360–370 nm. All Fluorescent Brightener 28-positive samples were examined by Chromotrope 2R (BDH) staining to confirm the presence of microsporidian spores. All procedures were carried out as listed on the CDC website detailing staining procedures for the diagnostic examination of stool samples (http://www.dpd.cdc.gov/dpx/HTML/DiagnosticProcedures.htm), except for the replacement of Fast Green with 0.5 g Aniline Blue.
RESULTS

Real-time analysis with pan-phylum primers produced positive amplification with all 18 DNAs extracted from archived microsporidia-positive stool samples (Table 1). Conventional PCR on these samples using species-specific primers identified 17 Enterocytozoon bieneusi infections (three confirmed by TEM) and one Encephalitozoon intestinalis infection (confirmed by TEM). The MsRTf1/MsRTr1 primer set was also capable of amplifying DNA from cultured Encephalitozoon intestinalis, Encephalitozoon hellem and Enterocytozoon cuniculi spore samples. The amplicons produced from Enterocytozoon bieneusi, Encephalitozoon intestinalis, Encephalitozoon hellem and Enterocytozoon cuniculi DNA (262, 282, 291 and 281 bp, respectively) were investigated using melting-curve analysis. A clear difference in the melting temperatures of amplicons from all four species was evident, suggesting that this assay could be used on patient samples to identify the infecting species (Fig. 1).

Real-time amplification of DNA extracted from purified Encephalitozoon intestinalis spores produced robust amplification with DNA amounts equivalent to that of both 20 and 2 spores per reaction (24/24 and 23/24 reactions, respectively). Amplification of DNA equivalent to 0.2 spores was achievable when 40 cycles of amplification were used, although not routinely (three out of eight reactions performed). With conventional PCR, it required a minimum DNA level equivalent to 20 spores of Encephalitozoon intestinalis to achieve consistent amplification using the EBIEF1/EBIER1 primer set.

Employing a standard curve produced using DNA extracted from the dilution series of Encephalitozoon intestinalis spores (efficiency=0.89, $r^2=0.99$), the effects of different stool treatments and DNA extraction regimes on the real-time analysis could be examined. Using replicate aliquots of a patient stool sample containing Enterocytozoon bieneusi, an estimated spore density of 4962 ml$^{-1}$ (95% CI 4294–5558) was observed when using the modified tissue extraction protocol on aliquots stored in PBS at 4°C for 7 days. Storage of identical aliquots in formalin resulted in a 100-fold reduction in DNA amplification (estimated spore density: 52 ml$^{-1}$; 95% CI 47–58). Extraction of aliquots stored in PBS using a standard QIAamp Stool kit instead of the modified tissue protocol produced an estimated spore density of 848 ml$^{-1}$ (95% CI 733–962).

Real-time amplification with the MsRTf1/MsRTr1 primer set and conventional PCR with the EBIEF1/EBIER1 and SINTF1/SINTR primer sets were performed prospectively on 168 consecutive non-formalin-treated stool samples sent for routine diagnosis to the Hospital for Tropical Diseases. Of the 168 samples, only five were found to be positive for microsporidial infection by microscopic analysis. Using the real-time assay, positive amplification was achieved with 17 of the samples. Melting temperature analysis suggested 15 Enterocytozoon bieneusi infections, an unknown microsporidial infection and an Encephalitozoon

Table 1. Amplification of microsporidial DNA from archived microscopy-positive stool samples, negative-control stool samples and purified spores using different primer sets

<table>
<thead>
<tr>
<th>Samples (no.)</th>
<th>Source</th>
<th>Previous species diagnosis</th>
<th>MsRTf1/MsRTr1 real-time pan-phylum</th>
<th>EBIEF1/EBIER1 standard Ent. bieneusi</th>
<th>SINTF1/SINTR standard Enceph. intestinalis</th>
</tr>
</thead>
</table>
| Encephalitozoon intestinalis (1) | Patient | TEM | + | - | + |+
| Enterocytozoon bieneusi (3) | Patient | TEM | + | + | - |-
| Uncharacterized positives (14) | Patient | None | + | + | - |-
| Encephalitozoon hellem | Culture | PCR | + | - | - |-
| Encephalitozoon cuniculi | Culture | PCR | + | - | - |-
| Encephalitozoon intestinalis (20 spores) | Culture | PCR | [24/24] | - | [10/10] |
| Encephalitozoon intestinalis (2 spores) | Culture | PCR | [23/24] | - | [0/10] |
| Negative control stools (3) | Patient | None | - | - | - |-
| Negative control H$_2$O | Patient | None | - | - | - |-
| Non-microsporidial intestinal pathogens (13)† | Patient | Microscopy | - | - | - |-

*Two of these samples only amplified with the Enterocytozoon bieneusi conventional PCR after tenfold dilution of the DNA.
†The non-microsporidian pathogens were Blastocystis sp. (two samples), Cyclospora cayetanensis (one sample), Cryptosporidium species (two samples), Ascaris lumbricoides (one sample), Giardia intestinalis (four samples) and Entamoeba histolytica (three samples).
cuniculi infection (Table 2). All microscopy-positive samples were positive by real-time amplification. All 168 routine diagnostic samples were subsequently analysed by conventional PCR using the EBIEF1/EBIER1 and SINTF1/SINTR primer sets. With the EBIEF1/EBIER1 primer set, amplification was seen in 14/15 samples identified as Enterocytozoon bieneusi by the real-time assay (sample #086 failed to amplify). Neither of the putative non-Enterocytozoon bieneusi microsporidial samples (samples #100 and #105) amplified with the EBIEF1/EBIER1 primer set. Out of the remaining 151 samples deemed negative for microsporidial DNA by the real-time assay, only a single sample (#054) produced an amplification product with these primers. Reanalysis of this sample using 40 cycles of real-time amplification produced a positive result in one of the two duplicate tubes (equating to a DNA level equivalent to approximately 0.5 spores per reaction by reference to the internal standard curve). None of the 168 samples were amplified using the Encephalitozoon intestinalis SINTF1/SINTR primer set.

For the prospective study, using the EBIEF1/EBIER1 and SINTF1/SINTR PCR as the gold standard, the sensitivity of microscopy and the MsRTf1/MsRTr1 real-time assay for the diagnosis of Enterocytozoon bieneusi infection was 26.7% (95% CI 49.0–4.4%) and 93.3% (95% CI 100–80.5%), respectively. The specificity of these two assays under these criteria was 100 and 99.3% (95% CI 100–98.0%), respectively. Looking at the microscopy-positive and microscopy-negative samples, the median Ct values (and ranges) were 23.9 (21.1–33.0) and 28.02 (22.1–33.1).

Two of the samples (#084 and #085) identified as Enterocytozoon bieneusi in both the real-time and the conventional PCRs were analysed by sequencing, together with the three samples that were MsRTf1/MsRTr1-positive but EBIEF1/EBIER1-negative (#086, #100 and #105). BLAST searches confirmed that samples #084 and #085 contained Enterocytozoon bieneusi DNA. The amplicon from the potential Encephalitozoon cuniculi sample (#100, melt temperature 83.5 °C) had 99% identity to several published Encephalitozoon cuniculi sequences including GenBank accession number X98470. This sample subsequently amplified with the ECUNF/ECUNR primer pair. The other two undetermined positive samples (#086 and #105, melt temperatures 81.3 and 80.3 °C, respectively) returned closest matches to published Pleistophora species sequences when BLAST searches were performed (84% identity to GenBank accession no. AJ252953 and 96% identity to GenBank accession no. AJ252959).

Of the 33 microsporidia-positive DNAs used to examine amplification of the control PhHV-1, 30 of these samples produced positive normalized fluorescence (>0.1). The mean Ct for positive fluorescence was 32.1 cycles (range 29.3–34.97) with a SD of 1.5. The remaining three samples showed no amplification of PhHV-1 DNA, despite being positive for amplification of microsporidial DNA. The cut-off for the acceptance criteria (to be used during routine deployment of this assay) was set as positive normalized fluorescence before 35 cycles (mean of the 30 positive Ct results + 1.96 SD).

**DISCUSSION**

For routine diagnostic use, an assay should be simple to use (thus eliminating the possibility of errors during processing), affordable and, most importantly, show good sensitivity and specificity. The use of PCR and real-time PCR in routine diagnosis has led to significant increases in the ability to detect many protozoan diseases including malaria (Berry et al., 2005; Fabre et al., 2004; Gama et al., 2007; Katakai et al.,…

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**Fig. 1.** Melting curves of Encephalitozoon cuniculi (■), Encephalitozoon hellem (□), Encephalitozoon intestinalis (○) and Enterocytozoon bieneusi (●) real-time PCR ampl cons. DNA was extracted from three positive clinical samples identified as Enterocytozoon bieneusi, Encephalitozoon cuniculi and Encephalitozoon intestinalis by conventional PCR and from a cultured sample of Encephalitozoon hellem.
Table 2. Amplification and analysis of 168 patient samples with MsRTf1/MsRTr1 real-time and EBIEF1/EBIER1 and SINTF1/SINTR conventional PCR primers

Diagnosis for microsporidial infection is shown as positive (+) or negative (−) for each set of primers, along with the mean melting temperature for each pair of real-time PCR products. The comparative melting temperatures for the Enterocytozoon bieneusi and Encephalitozoon intestinalis control samples used on the same real-time run are shown to the right of each melting temperature in parentheses. The SSU-rRNA sequence was determined by analysing the MsRTf1/MsRTr1 real-time PCR products. Enterocytozoon bieneusi and Encephalitozoon intestinalis PCR was performed with the EBIEF1/EBIER1 and SINTF1/SINTR primer pairs, respectively. ND, Not done; NA, not applicable.

<table>
<thead>
<tr>
<th>Sample no.</th>
<th>MsRTf1/MsRTr1</th>
<th>Ent. bieneusi PCR</th>
<th>Enceph. intestinalis PCR</th>
<th>Microscopy</th>
<th>SSU-rRNA sequence</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Positive</td>
<td>Melting temp. (Eb/Ei) (°C)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>013</td>
<td>+</td>
<td>81.1 (81.1/82.8)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>018</td>
<td>+</td>
<td>81.2 (81.1/82.8)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>019</td>
<td>+</td>
<td>81.3 (81.1/82.8)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>021</td>
<td>+</td>
<td>81.1 (81.1/82.8)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>030</td>
<td>+</td>
<td>81.2 (81.1/82.8)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>054</td>
<td>− *</td>
<td>81.8 (81.8/83.4)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>084</td>
<td>+</td>
<td>81.7 (81.5/83.0)</td>
<td>+</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>085</td>
<td>+</td>
<td>81.9 (81.5/83.0)</td>
<td>+</td>
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<td>−</td>
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<tr>
<td>086</td>
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<td>−</td>
<td>−</td>
<td>−</td>
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<tr>
<td>091</td>
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<td>105</td>
<td>+</td>
<td>80.3 (81.4/83.0)</td>
<td>−</td>
<td>−</td>
<td>−</td>
</tr>
<tr>
<td>160</td>
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<td>81.4 (81.6/82.8)</td>
<td>+</td>
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<tr>
<td>168</td>
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<tr>
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<td>267</td>
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<td>276</td>
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<td>324</td>
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<td>81.4 (81.3/82.7)</td>
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<tr>
<td>Remaining 150 samples</td>
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<td>NA</td>
<td>−</td>
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</table>

*Sample #054 was originally deemed negative by 35 cycles of real-time amplification, but 40 cycles yielded a positive result.
†Nearest matches to the unknown sequences were both Pleistophora sp.

2004; Lubell et al., 2007; Mangold et al., 2005; Perandin et al., 2004), leishmaniasis (Aviles et al., 1999; Katakura et al., 1998; Mathis & Deplazes, 1995), giardiasis (Abe et al., 2003; Bertrand et al., 2004; Guy et al., 2004; Helmy et al., 2009; McGlade et al., 2003; Verweij et al., 2003) and cryptosporidiosis (Verweij et al., 2004). Real-time PCR analyses remove the requirement for the separate amplification and analysis of amplicons, providing a rapid, sealed-tube system. This format decreases both the turnaround time and the possibility of contamination of subsequent reactions by amplicons released into the environment during gel loading, as well as removing the need for the teratogenic substance ethidium bromide used in gel electrophoresis for visualizing DNA. Thus, real-time PCR offers considerable advantages for routine diagnosis or surveillance of disease, tempered only by the initial cost of the hardware required.

The new MsRTf1/MsRTr1 primer set described here showed sensitivity that was much greater than that achieved by light microscopy and was equivalent to (or better than) that achieved by conventional PCR. The ability to use a single primer set with SYBR Green amplification removes the requirement for multiplexed primer sets when amplifying a broad range of microsporidial infections, whilst still allowing discrimination between different microsporidian species by the use of melting curves. The resultant assay is therefore potentially cheaper and simpler to use than multiplex probe-based assays. One limitation of the current SYBR Green assay may be the inability to easily multiplex additional non-microsporidian real-time PCR for the simultaneous analysis of multiple pathogens. No obvious pan-phylum probe site exists for adaptation of the MsRTf1/MsRTr1 primers to such a format; however, binding sites for species-specific probes have been identified should multiplexing be required. Such a probe-based assay would, however, limit the flexibility of this technique to investigate less commonly observed microsporidian species such as Pleistophora species.

It is common practice in diagnostic laboratories to fix stool samples upon receipt, especially those from HIV-positive patients. Given that the use of formalin has been shown to result in significant loss of DNA amplification both here and in other diagnostic assays (Ramos et al., 1999; Troll...
et al., 1997), it is essential that such practices be altered before the implementation of molecular screening programmes for diagnosis or surveillance. This is especially relevant as our internal control DNA was added subsequent to the removal of formalin through sample processing (as with many diagnostic PCR; Niesters, 2004). It is interesting that three samples were positive for amplification of microsporidial DNA but not for amplification of PhHV-1. It could be that these samples had a sufficient number of microsporidial spores in them to overcome any inhibition. Alternatively, the microsporidial PCR may be more robust to PCR inhibition than the PhHV-1 PCR, making this a very conservative internal control. A robust real-time reaction would explain why two of the DNA samples used in the assay development amplified with the real-time assay when used neat but would only amplify with the conventional PCR upon dilution. It would appear, therefore, that the new SYBR Green assay shows greater resistance to PCR inhibitors carried through during sample clean-up than published conventional PCRs.

A limited number of different microsporidian species have been tested with this assay at present, although the major human pathogens Enterocytozoon bieneusi, Encephalitozoon intestinalis, Encephalitozoon cuniculi and Encephalitozoon hellem all amplified successfully, together with different Pleistophora infections. There remains the possibility that additional species will have a similar melting profile to those above, confusing the ability of the assay to identify definitively the species present in a positive sample. Indeed, patient sample #86 (positive for Pleistophora species DNA) gave a melting temperature within the range often seen for Enterocytozoon bieneusi. It may therefore be necessary to perform a secondary species-specific PCR before treatment regimens are decided upon, although this may only be relevant for potential Enterocytozoon bieneusi infections, as this is the only species to show resistance to albendazole (De Groote et al., 1995; Didier et al., 1996; Dittrich et al., 1994; Koudela et al., 1994; Weiss et al., 1994). Alternatively, a high-resolution melting analysis (beyond the capability of the Rotor-Gene 3000) may further extend the ability of this assay to differentiate among different species, rendering a second PCR unnecessary. The ability to use real-time analysis to quantify parasite burden lends itself to in vivo drug sensitivity studies where the kinetics of parasite clearance needs to be examined (Dieterich et al., 1994). This primer set would allow clearance rates of any microsporidian species to be determined accurately and quickly for in situ drug sensitivity scores. Some caution should be applied in this assay, however, when determining spore concentrations in stool preparations using a standard curve of DNA prepared from cultured spores.

It was interesting to note that, for the patient presenting with the Encephalitozoon cuniculi infection, a high burden of microsporidial spores was observed in the liver biopsy but spores were not seen in the stool sample when using microscopy. The amplification of Encephalitozoon cuniculi DNA from the stool sample raises the possibility that infections in the liver or other tissues may result in the presence of DNA but not intact spores within faeces or urine. It may be possible, therefore, to extend this assay for the diagnosis of non-intestinal infections where biopsies of the affected tissue have not been taken or are unavailable to the diagnostic facility. Alternatively, it may be that spores were present at such low levels in the available stool samples that only the increased sensitivity of molecular biological methods allowed an accurate diagnosis. Indeed, Encephalitozoon cuniculi spores have been detected previously in human stools by microscopy (Weber et al., 1997).

The increasing sensitivity of molecular biological techniques raises the question of what constitutes an actual infection requiring treatment and what may comprise spores that have been ingested before passing, intact, through the intestinal tract. This may be of particular interest for the two samples that were positive for Pleistophora species DNA, a parasite known to produce myositis rather than intestinal infections. Previously reported Pleistophora species infections (producing localized myositis) have been negative for microsporidial spores in stool samples examined by microscopy (Field et al., 1996). The two instances of positivity for Pleistophora species DNA observed in this phase of the work may therefore be solely indicative of the ingestion of infected fish muscle rather than the presence of a human enteric infection. It is notable that, during the 10-month period in which this assay has been implemented at the Hospital for Tropical Diseases for the routine diagnosis of microsporidial infections, a single instance of high-burden infection with Pleistophora species has been observed, with a spore burden of 73 800 spores (g stool sample)$^{-1}$, suggesting that Pleistophora species are capable of establishing true enteric infections (these would be missed by existing probe-based real-time assays). It is the ability of real-time amplification to assign a definitive spore burden to a known mass of stool that may allow the determination of what constitutes a true enteric infection, with a bench mark of $10^6$, $10^4$ or even $10^5$ spores (g stool)$^{-1}$ being established in future. The current limit for detection by light microscopy would appear to be between $10^4$ and $10^6$ spores (g stool)$^{-1}$ (Müller et al., 1999). Future analysis of the relationship between spore burden and presentation of symptoms remains to be carried out, but this primer set offers the tools required for such a study.

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