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

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Diagnosis of microsporidial infections is routinely performed by light microscopy, with unequivocal non-molecular species identification achievable only through electron microscopy. This study describes a single SYBR Green real-time PCR assay for the simultaneous detection and species identification of such infections. This assay was highly sensitive, routinely detecting infections containing 400 parasites (g stool sample)⁻¹, whilst species identification was achieved by differential melt curves on a Corbett Life Science Rotor-Gene 3000. A modification of the QIAamp DNA tissue extraction protocol allowed the semi-automated extraction of DNA from stools for the routine diagnosis of microsporidial infection by real-time PCR. Of 168 stool samples routinely analysed for microsporidian spores, only five were positive by microscopy. By comparison, 17 were positive for microsporidian DNA by real-time analysis, comprising 14 *Enterocytozoon bienersi*, one *Encephalitozoon cuniculi* and two separate *Pleistophora* species infections.

Received 6 October 2010
Accepted 17 December 2010

INTRODUCTION

The phylum Microspora contains a diverse set of unicellular spore-forming parasites, evolutionarily related to fungi. None are obligate parasite of humans; however, several can give rise to opportunistic infections. Whilst these infections are found most commonly in immunocompromised patients, *Enterocytozoon bienersi* has been reported in the stool samples of immunocompetent individuals (Gumbo *et al.*, 2000), and serological data suggest that infections due to *Enterocytozoon bienersi* and *Encephalitozoon* species could be common (Sak *et al.*, 2010; van Gool *et al.*, 1997). Along with *Enterocytozoon bienersi*, 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 bienersi* 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 *Enterocytozoon bienersi* and *Encephalitozoon* species (Verweij *et al.*, 2007). Discrimination among multiple human pathogenic

Abbreviations: CI, confidence limit; C_t, cycle threshold; SSU, small subunit; TEM, transmission electron microscopy.

The GenBank/EMBL/DDBJ accession numbers for the SSU-rRNA sequences of samples #084, #085, #086, #100 and #105 are FR729097, FR729098, FR729100, FR729096 and FR729099, respectively.

microsporidian species has been achieved using both oligo-nucleotide 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, b; De Groote *et al.*, 1995) on archived samples and on stool specimens submitted for routine diagnosis requiring testing for microsporidial 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 *Encephalitozoon 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, 2 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 kit 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 & Skaletsky, 2000) against the *Enterocytozoon bienersi* SSU-rRNA sequence. Primers MsRTf1 (5'-CAGGTGATCTCTGCCTGACG-3') and MsRTr1 (5'-CCATCTCT-CAGGCTCCCTCT) were chosen to ensure amplification of *Encephalitozoon intestinalis*, *Encephalitozoon cuniculi* and *Encephalitozoon hellem* SSU-rRNA sequences in addition to the *Enterocytozoon bienersi* sequence. Amplification was performed in a Rotor-Gene 3000 (Corbett Life Sciences) using a QuantiTect SYBR Green PCR kit (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 bienersi*, *Encephalitozoon intestinalis* and *Encephalitozoon cuniculi* was performed using the EBIEF1/EBIER1 (da Silva *et al.*, 1997b), SINTF1/SINTR (da Silva *et al.*, 1997a; Visvesvara *et al.*, 1995) and ECUNF/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 MsRTr1 primers. A single contig was compiled for each product using the Seqman 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 MsRTf1/MsRTr1 real-time PCR, except that the MsRTf1/MsRTr1 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_t) of PhHV-1 (mean C_t + 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/dpdx/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 *Encephalitozoon cuniculi* spore samples. The amplicons produced from *Enterocytozoon bieneusi*, *Encephalitozoon intestinalis*, *Encephalitozoon hellem* and *Encephalitozoon 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

The results of the amplifications are shown as + (positive) or – (negative). Where multiple amplifications were carried out with a given sample to examine the limit of detection, the number of positive results is shown in square brackets.

Samples (no.)	Source	Previous species diagnosis	MsRTf1/MsRTr1 real-time pan-phylum	EBIEF1/EBIER1 standard <i>Ent. bieneusi</i>	SINTF1/SINTR standard <i>Enceph. intestinalis</i>
<i>Encephalitozoon intestinalis</i> (1)	Patient	TEM	+	–	+
<i>Enterocytozoon bieneusi</i> (3)	Patient	TEM	+	+	–
Uncharacterized positives (14)	Patient	None	+	+	–
<i>Encephalitozoon hellem</i>	Culture	PCR	+	–	–
<i>Encephalitozoon cuniculi</i>	Culture	PCR	+	–	–
<i>Encephalitozoon intestinalis</i> (20 spores)	Culture	PCR	[24/24]	–	[10/10]
<i>Encephalitozoon intestinalis</i> (2 spores)	Culture	PCR	[23/24]	–	[0/10]
Negative control stools (3)	Patient	None	–	–	–
Negative control H ₂ O	Patient	None	–	–	–
Non-microsporidian 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).

Fig. 1. Melting curves of *Encephalitozoon cuniculi* (■), *Encephalitozoon hellem* (□), *Encephalitozoon intestinalis* (○) and *Enterocytozoon bieneusi* (●) real-time PCR amplicons. 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*.

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 C_t 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 C_t 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 C_t 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.*,

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 bienersi* 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 bienersi* and *Encephalitozoon intestinalis* PCR was performed with the EBIEF1/EBIER1 and SINTF1/SINTR primer pairs, respectively. ND, Not done; NA, not applicable.

Sample no.	MsRTf1/MsRTr1		<i>Ent. bienersi</i> PCR	<i>Enceph. intestinalis</i> PCR	Microscopy	SSU-rRNA sequence
	Positive	Melting temp. (<i>Eb</i> / <i>Ei</i>) (°C)				
013	+	81.1 (81.1/82.8)	+	–	–	ND
018	+	81.2 (81.1/82.8)	+	–	–	ND
019	+	81.3 (81.1/82.8)	+	–	–	ND
021	+	81.1 (81.1/82.8)	+	–	–	ND
030	+	81.2 (81.1/82.8)	+	–	–	ND
054	–*	81.8 (81.8/83.4)	+	–	–	ND
084	+	81.7 (81.5/83.0)	+	–	–	<i>Ent. bienersi</i>
085	+	81.9 (81.5/83.0)	+	–	–	<i>Ent. bienersi</i>
086	+	81.3 (81.5/83.0)	–	–	–	Unknown†
091	+	81.3 (81.5/83.0)	+	–	+	ND
100	+	83.5 (81.5/83.0)	–	–	–	<i>Enceph. cuniculi</i>
105	+	80.3 (81.4/83.0)	–	–	–	Unknown†
160	+	81.4 (81.6/82.8)	+	–	+	ND
168	+	81.4 (81.3/82.7)	+	–	+	ND
215	+	81.9 (82.1/83.5)	+	–	+	ND
267	+	81.1 (81.3/82.7)	+	–	–	ND
276	+	81.7 (81.6/83.0)	+	–	–	ND
324	+	81.4 (81.3/82.7)	+	–	+	ND
Remaining 150 samples	–	NA	–	–	–	NA

*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 PCRs; 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 bienersi*, *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 bienersi*. 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 bienersi* infections, as this is the only species to show resistance to albendazole (De Groote *et al.*, 1995; Didier *et al.*, 1996; Ditrich *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 benchmark of 10^3 , 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.

ACKNOWLEDGEMENTS

S.D.P. is supported by the Foundation for Innovative and New Diagnostics (FIND). P.L.C. is supported by UCL Hospitals Comprehensive Biomedical Research Centre Infection Theme. Archived stool samples were retrieved and checked for microsporidial infection by Hilary Cotterill (Health Protection Agency North West, Manchester, UK). Cultured *Encephalitozoon cuniculi*, *Encephalitozoon hellem* and *Encephalitozoon intestinalis* spores were supplied by Dr Tom van Gool (Academic Medical Center, Amsterdam, The

Netherlands). PhHV-1 DNA was a kind gift from Adele Lee-Wriede (Barts and the London NHS Trust, London, UK).

REFERENCES

- Abe, N., Kimata, I. & Iseki, M. (2003). Identification of genotypes of *Giardia intestinalis* isolates from dogs in Japan by direct sequencing of the PCR amplified glutamate dehydrogenase gene. *J Vet Med Sci* **65**, 29–33.
- Aviles, H., Belli, A., Armijos, R., Monroy, F. P. & Harris, E. (1999). PCR detection and identification of *Leishmania* parasites in clinical specimens in Ecuador: a comparison with classical diagnostic methods. *J Parasitol* **85**, 181–187.
- Berry, A., Fabre, R., Benoit-Vical, F., Cassaing, S. & Magnaval, J. F. (2005). Contribution of PCR-based methods to diagnosis and management of imported malaria. *Med Trop (Mars)* **65**, 176–183.
- Bertrand, I., Gantzer, C., Chesnot, T. & Schwartzbrod, J. (2004). Improved specificity for *Giardia lamblia* cyst quantification in wastewater by development of a real-time PCR method. *J Microbiol Methods* **57**, 41–53.
- Chabchoub, N., Abdelmalek, R., Mellouli, F., Kanoun, F., Thellier, M., Bouratbine, A. & Aoun, K. (2009). Genetic identification of intestinal microsporidia species in immunocompromised patients in Tunisia. *Am J Trop Med Hyg* **80**, 24–27.
- Chupp, G. L., Alroy, J., Adelman, L. S., Breen, J. C. & Skolnik, P. R. (1993). Myositis due to *Pleistophora* (microsporidia) in a patient with AIDS. *Clin Infect Dis* **16**, 15–21.
- da Silva, A. J., Slemenda, S. B., Visvesvara, G. S., Schwartz, D. A., Wilcox, C. M., Wallace, S. & Pieniazek, N. J. (1997a). Detection of *Septata intestinalis* (microsporidia) Cali et al. 1993 using polymerase chain reaction primers targeting the small subunit ribosomal RNA coding region. *Mol Diagn* **2**, 47–52.
- da Silva, A. J., Bornay-Llinares, F. J., del Aguila de la Puente, Cdel. A., Moura, H., Peralta, J. M., Sobottka, I., Schwartz, D. A., Visvesvara, G. S., Slemenda, S. B. & Pieniazek, N. J. (1997b). Diagnosis of *Enterocytozoon bienzei* (microsporidia) infections by polymerase chain reaction in stool samples using primers based on the region coding for small-subunit ribosomal RNA. *Arch Pathol Lab Med* **121**, 874–879.
- De Groote, M. A., Visvesvara, G., Wilson, M. L., Pieniazek, N. J., Slemenda, S. B., daSilva, A. J., Leitch, G. J., Bryan, R. T. & Reves, R. (1995). Polymerase chain reaction and culture confirmation of disseminated *Encephalitozoon cuniculi* in a patient with AIDS: successful therapy with albendazole. *J Infect Dis* **171**, 1375–1378.
- Didier, E. S., Rogers, L. B., Brush, A. D., Wong, S., Traina-Dorge, V. & Bertucci, D. (1996). Diagnosis of disseminated microsporidian *Encephalitozoon hellem* infection by PCR-Southern analysis and successful treatment with albendazole and fumagillin. *J Clin Microbiol* **34**, 947–952.
- Dieterich, D. T., Lew, E. A., Kotler, D. P., Poles, M. A. & Orenstein, J. M. (1994). Treatment with albendazole for intestinal disease due to *Enterocytozoon bienzei* in patients with AIDS. *J Infect Dis* **169**, 178–183.
- Ditrich, O., Kucerová, Z. & Koudela, B. (1994). *In vitro* sensitivity of *Encephalitozoon cuniculi* and *E. hellem* to albendazole. *J Eukaryot Microbiol* **41**, 37S.
- Fabre, R., Berry, A., Morassin, B. & Magnaval, J. F. (2004). Comparative assessment of conventional PCR with multiplex real-time PCR using SYBR Green I detection for the molecular diagnosis of imported malaria. *Parasitology* **128**, 15–21.
- Field, A. S., Marriott, D. J., Milliken, S. T., Brew, B. J., Canning, E. U., Kench, J. G., Darveniza, P. & Harkness, J. L. (1996). Myositis associated with a newly described microsporidian, *Trachipleistophora hominis*, in a patient with AIDS. *J Clin Microbiol* **34**, 2803–2811.
- Franzen, C., Schwartz, D. A., Visvesvara, G. S., Müller, A., Schwenk, A., Salzberger, B., Fätkenheuer, G., Hartmann, P., Mahrle, G. & other authors (1995). Immunologically confirmed disseminated, asymptomatic *Encephalitozoon cuniculi* infection of the gastrointestinal tract in a patient with AIDS. *Clin Infect Dis* **21**, 1480–1484.
- Gama, B. E., Silva-Pires, F. E., Lopes, M. N., Cardoso, M. A., Britto, C., Torres, K. L., de Mendonça Lima, L., de Souza, J. M., Daniel-Ribeiro, C. T. & Ferreira-da-Cruz, M. F. (2007). Real-time PCR versus conventional PCR for malaria parasite detection in low-grade parasitemia. *Exp Parasitol* **116**, 427–432.
- Garcia, L. S. (2002). Laboratory identification of the microsporidia. *J Clin Microbiol* **40**, 1892–1901.
- Ghosh, K. & Weiss, L. M. (2009). Molecular diagnostic tests for microsporidia. *Interdiscip Perspect Infect Dis* **2009**, 926521.
- Grau, A., Valls, M. E., Williams, J. E., Ellis, D. S., Muntané, M. J. & Nadal, C. (1996). Myositis caused by *Pleistophora* in a patient with AIDS. *Med Clin (Barc)* **107**, 779–781.
- Gumbo, T., Gangaidzo, I. T., Sarbah, S., Carville, A., Tzipori, S. & Wiest, P. M. (2000). *Enterocytozoon bienzei* infection in patients without evidence of immunosuppression: two cases from Zimbabwe found to have positive stools by PCR. *Ann Trop Med Parasitol* **94**, 699–702.
- Guy, R. A., Xiao, C. & Horgen, P. A. (2004). Real-time PCR assay for detection and genotype differentiation of *Giardia lamblia* in stool specimens. *J Clin Microbiol* **42**, 3317–3320.
- Helmy, M. M., Abdel-Fattah, H. S. & Rashed, L. (2009). Real-time PCR/RFLP assay to detect *Giardia intestinalis* genotypes in human isolates with diarrhea in Egypt. *J Parasitol* **95**, 1000–1004.
- Joseph, J., Sharma, S., Murthy, S. I., Krishna, P. V., Garg, P., Nutheti, R., Kenneth, J. & Balasubramanian, D. (2006). Microsporidial keratitis in India: 16S rRNA gene-based PCR assay for diagnosis and species identification of microsporidia in clinical samples. *Invest Ophthalmol Vis Sci* **47**, 4468–4473.
- Katakai, Y., Chiabchalard, R., Komaki-Yasuda, K., Kawazu, S., Singhasivanon, P., Krudsood, S., Looreesuwun, S. & Kano, S. (2004). Application of real-time polymerase chain reaction (PCR) analysis for detection and discrimination of malaria parasite species in Thai patients. *Southeast Asian J Trop Med Public Health* **35**, S10–S14.
- Katakai, Y., Kawazu, S., Naya, T., Nagakura, K., Ito, M., Aikawa, M., Qu, J. Q., Guan, L. R., Zuo, X. P. & other authors (1998). Diagnosis of kala-azar by nested PCR based on amplification of the *Leishmania* mini-exon gene. *J Clin Microbiol* **36**, 2173–2177.
- Koudela, B., Lom, J., Vitovec, J., Kucerová, Z., Ditrich, O. & Trávníček, J. (1994). *In vivo* efficacy of albendazole against *Encephalitozoon cuniculi* in SCID mice. *J Eukaryot Microbiol* **41**, 49S–50S.
- Kumar, S. S., Ananthan, S. & Joyee, A. G. (2005). Detection of *Enterocytozoon bienzei* (Microsporidia) by polymerase chain reaction (PCR) using species-specific primer in stool samples of HIV patients. *Indian J Med Res* **121**, 215–219.
- Lubell, Y., Reyburn, H., Mbakiliwa, H., Mwangi, R., Chonya, K., Whitty, C. J. & Mills, A. (2007). The cost-effectiveness of parasitologic diagnosis for malaria-suspected patients in an era of combination therapy. *Am J Trop Med Hyg* **77**, S128–S132.
- Mangold, K. A., Manson, R. U., Koay, E. S., Stephens, L., Regner, M., Thomson, R. B., Jr, Peterson, L. R. & Kaul, K. L. (2005). Real-time PCR for detection and identification of *Plasmodium* spp. *J Clin Microbiol* **43**, 2435–2440.

- Mathis, A. & Deplazes, P. (1995). PCR and in vitro cultivation for detection of *Leishmania* spp. in diagnostic samples from humans and dogs. *J Clin Microbiol* **33**, 1145–1149.
- McGlade, T. R., Robertson, I. D., Elliot, A. D. & Thompson, R. C. (2003). High prevalence of *Giardia* detected in cats by PCR. *Vet Parasitol* **110**, 197–205.
- Menotti, J., Cassinat, B., Porcher, R., Sarfati, C., Derouin, F. & Molina, J. M. (2003a). Development of a real-time polymerase-chain-reaction assay for quantitative detection of *Enterocytozoon bieneusi* DNA in stool specimens from immunocompromised patients with intestinal microsporidiosis. *J Infect Dis* **187**, 1469–1474.
- Menotti, J., Cassinat, B., Sarfati, C., Liguory, O., Derouin, F. & Molina, J. M. (2003b). Development of a real-time PCR assay for quantitative detection of *Encephalitozoon intestinalis* DNA. *J Clin Microbiol* **41**, 1410–1413.
- Müller, A., Stellermann, K., Hartmann, P., Schrappe, M., Fätkenheuer, G., Salzberger, B., Diehl, V. & Franzen, C. (1999). A powerful DNA extraction method and PCR for detection of microsporidia in clinical stool specimens. *Clin Diagn Lab Immunol* **6**, 243–246.
- Niesters, H. G. (2002). Clinical virology in real time. *J Clin Virol* **25**, S3–S12.
- Niesters, H. G. (2004). Molecular and diagnostic clinical virology in real time. *Clin Microbiol Infect* **10**, 5–11.
- Notermans, D. W., Peek, R., de Jong, M. D., Wentink-Bonnema, E. M., Boom, R. & van Gool, T. (2005). Detection and identification of *Enterocytozoon bieneusi* and *Encephalitozoon* species in stool and urine specimens by PCR and differential hybridization. *J Clin Microbiol* **43**, 610–614.
- Perandin, F., Manca, N., Calderaro, A., Piccolo, G., Galati, L., Ricci, L., Medici, M. C., Arcangeletti, M. C., Snounou, G. & other authors (2004). Development of a real-time PCR assay for detection of *Plasmodium falciparum*, *Plasmodium vivax*, and *Plasmodium ovale* for routine clinical diagnosis. *J Clin Microbiol* **42**, 1214–1219.
- Ramos, F., Zurabian, R., Morán, P., Ramiro, M., Gómez, A., Clark, C. G., Melendro, E. I., García, G. & Ximénez, C. (1999). The effect of formalin fixation on the polymerase chain reaction characterization of *Entamoeba histolytica*. *Trans R Soc Trop Med Hyg* **93**, 335–336.
- Rozen, S. & Skaletsky, H. (2000). Primer3 on the WWW for general users and for biologist programmers. *Methods Mol Biol* **132**, 365–386.
- Sak, B., Kucerova, Z., Kvac, M., Kvetonova, D., Rost, M. & Secor, E. W. (2010). Seropositivity for *Enterocytozoon bieneusi*, Czech Republic. *Emerg Infect Dis* **16**, 335–337.
- Tosoni, A., Nebuloni, M., Ferri, A., Bonetto, S., Antinori, S., Scaglia, M., Xiao, L., Moura, H., Visvesvara, G. S. & other authors (2002). Disseminated microsporidiosis caused by *Encephalitozoon cuniculi* III (dog type) in an Italian AIDS patient: a retrospective study. *Mod Pathol* **15**, 577–583.
- Troll, H., Marti, H. & Weiss, N. (1997). Simple differential detection of *Entamoeba histolytica* and *Entamoeba dispar* in fresh stool specimens by sodium acetate-acetic acid-formalin concentration and PCR. *J Clin Microbiol* **35**, 1701–1705.
- van Gool, T., Vetter, J. C., Weinmayr, B., Van Dam, A., Derouin, F. & Dankert, J. (1997). High seroprevalence of *Encephalitozoon* species in immunocompetent subjects. *J Infect Dis* **175**, 1020–1024.
- Verweij, J. J., Schinkel, J., Laeijendecker, D., van Rooyen, M. A., van Lieshout, L. & Polderman, A. M. (2003). Real-time PCR for the detection of *Giardia lamblia*. *Mol Cell Probes* **17**, 223–225.
- Verweij, J. J., Blangé, R. A., Templeton, K., Schinkel, J., Brienens, E. A., van Rooyen, M. A., van Lieshout, L. & Polderman, A. M. (2004). Simultaneous detection of *Entamoeba histolytica*, *Giardia lamblia*, and *Cryptosporidium parvum* in fecal samples by using multiplex real-time PCR. *J Clin Microbiol* **42**, 1220–1223.
- Verweij, J. J., Ten Hove, R., Brienens, E. A. & van Lieshout, L. (2007). Multiplex detection of *Enterocytozoon bieneusi* and *Encephalitozoon* spp. in fecal samples using real-time PCR. *Diagn Microbiol Infect Dis* **57**, 163–167.
- Visvesvara, G. S., da Silva, A. J., Croppo, G. P., Pieniazek, N. J., Leitch, G. J., Ferguson, D., de Moura, H., Wallace, S., Slemenda, S. B. & other authors (1995). In vitro culture and serologic and molecular identification of *Septata intestinalis* isolated from urine of a patient with AIDS. *J Clin Microbiol* **33**, 930–936.
- Wang, Z., Orlandi, P. A. & Stenger, D. A. (2005). Simultaneous detection of four human pathogenic microsporidian species from clinical samples by oligonucleotide microarray. *J Clin Microbiol* **43**, 4121–4128.
- Weber, R., Deplazes, P., Flepp, M., Mathis, A., Baumann, R., Sauer, B., Kuster, H. & Lüthy, R. (1997). Cerebral microsporidiosis due to *Encephalitozoon cuniculi* in a patient with human immunodeficiency virus infection. *N Engl J Med* **336**, 474–478.
- Weiss, L. M., Michalakakis, E., Coyle, C. M., Tanowitz, H. B. & Wittner, M. (1994). The in vitro activity of albendazole against *Encephalitozoon cuniculi*. *J Eukaryot Microbiol* **41**, 65S.
- Wolk, D. M., Schneider, S. K., Wengenack, N. L., Sloan, L. M. & Rosenblatt, J. E. (2002). Real-time PCR method for detection of *Encephalitozoon intestinalis* from stool specimens. *J Clin Microbiol* **40**, 3922–3928.