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Detection and identification by real time PCR/RFLP analyses of *Cryptosporidium* species from human faeces

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ABSTRACT

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Aims: To detect a wide range of *Cryptosporidium* species from human faeces by analysis of the *Cryptosporidium* oocyst wall protein gene by PCR.

Methods and Results: The nested-assay comprised an initial amplification using a conventional thermocycler followed by real time PCR using a LightCycler with SYBR Green I for the characterization of the amplicons. The technique uses four sets of primers composed of five to six oligonucleotides with one to six base differences corresponding to the inter-species sequence differences of the gene fragment. Restriction fragment length polymorphism analysis identified *Cryptosporidium hominis* and *C. parvum*. The assay was evaluated using DNA extracted from purified material and faecal specimens containing a range of potential pathogens (including *Cryptosporidium*). The assay was specific, sensitive, reproducible and rapid.

Conclusions: This unique technique enables the rapid detection of a range of polymorphic COWP gene sequences directly from faeces using real time PCR.

Significance and Impact of the study: This study demonstrates a novel approach to identification of *Cryptosporidium* species and the identification of *C. hominis* and *C. parvum*. The technique may be especially useful for the analysis of environmental samples which are likely to contain heterogeneous mixtures of *Cryptosporidium* species.

Keywords: cryptosporidiosis, *Cryptosporidium hominis*, *Cryptosporidium parvum*, LightCycler, SYBR Green I.

INTRODUCTION

Cryptosporidium is an intestinal protozoan found both in humans and a wide range of animals (Fayer *et al.* 1997). In humans, food-borne and water-borne cryptosporidiosis are common causes of gastroenteritis and a major health concern world-wide (Fayer *et al.* 1997).

Current methods for detection of this protozoan from faeces are based on light microscopy (Casemore *et al.* 1985; Arrowood 1997). However, these methods are unable to distinguish between genetically distinct parasites (Casemore *et al.* 1985; Arrowood 1997; Pedraza-Díaz *et al.* 2000). A variety of PCR/RFLP techniques have been developed to detect and distinguish *C. hominis* (formerly *C. parvum* genotype 1) from *C. parvum* (including *C. parvum* genotype 2 and subsequently referred to as *C. parvum*) (Spano *et al.* 1997; Spano *et al.* 1998; McLauchlin *et al.* 2000). Nested-PCR procedures were shown to increase considerably the amplification sensitivity of *Cryptosporidium* DNA extracted from water (Xiao *et al.* 2000; Sturbaum *et al.* 2001), whole faeces (Balatbat *et al.* 1996; Pedraza-Díaz *et al.* 2001) and from faecal smears (Amar *et al.* 2001). A further improvement in the molecular detection of these parasites would be a reduction in the time needed for amplification and alternatives to the detection of PCR products by gel electrophoresis. The use of rapid cycling real time PCR has provided such improvements for *C. hominis* and *C. parvum* (Tanriverdi *et al.* 2002). However, as other species

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of *Cryptosporidium* such as *Cryptosporidium felis*, *C. meleagridis*, *C. muris* and *C. canis* have been associated with human infections (Pieniazek *et al.* 1999; Pedraza-Díaz *et al.* 2000; Caccio *et al.* 2002; Gatei *et al.* 2002), there is a need for the development of sensitive techniques to detect a wider range of cryptosporidial species in faeces.

We previously described methods to extract DNA from intestinal pathogens present in whole faeces (McLauchlin *et al.* 1999) and faecal smears (Amar *et al.* 2001). The aim of this study was to develop and evaluate a simple, highly specific and sensitive real time PCR method applicable to faeces and able to detect a wide range of cryptosporidial species and also to distinguish *C. hominis* from *C. parvum*.

MATERIALS AND METHODS

Faecal specimens

All faecal samples containing *C. hominis* (seven samples), *C. parvum* (10 samples) (McLauchlin *et al.* 2000), *C. felis* (one sample), *C. meleagridis* (one sample), *C. canis* (one sample), and *Cyclospora* (two samples) (McLauchlin *et al.* 1999) were collected from naturally infected humans and stored at 4°C for up to 5 years without preservatives. One faecal sample from a calf experimentally infected with *C. parvum* (Moredun reference strain) was also included.

DNA samples

DNA extracts were prepared from: purified oocysts of *C. hominis* (10 genome equivalent μl^{-1}; Laboratory of Molecular Biology, MRC, Cambridge, UK), *C. parvum* (genotype 2, Iowa strain 1372; AIDS Research Reference Reagent Program, National Institutes of Health), *C. andersoni*, *C. baileyi*, *C. meleagridis*, *C. muris* and *C. serpentis* (Patel *et al.* 1999). DNA extracted from *Giardia duodenalis* trophozoites reference strains VNB3, AMC9, and DOG1 were kindly provided by W. Homan (Laboratory for Parasitology and Mycology, Bilthoven, the Netherlands). DNA samples of *Eimeria tenella* oocysts (Patel *et al.* 1999), and from bacterial suspensions of *Escherichia coli* (strain N211; PHLS Food External Quality Assessment Scheme, London, UK) and *Clostridium perfringens* type A (NCTC 8239) from the National Collections of Type Culture, London, UK, were also used.

Microscopy

Smears were produced from 17 faecal samples where *C. hominis* (seven samples) and *C. parvum* (10 samples) had previously been identified (see above). The smears were examined by immunofluorescence microscopy as previously described (Amar *et al.* 2001). The number of oocysts was estimated by calculation of the mean for 20 microscope fields by use of a ×40 objective (Zeiss, Welwyn Garden City, UK).

DNA extraction and polyvinyl pyrrolidone (PVP) treatment

DNA extraction from whole faeces and from stained smears on glass microscope slides, including further DNA purification using PVP, was performed as described before (McLauchlin *et al.* 1999; Amar *et al.* 2001). DNA from purified oocysts and bacterial suspension was also prepared by a similar method.

Nested-real time PCR amplification (PCOWPLC-PCR)

The COWP gene sequences of *C. andersoni*, *C. baileyi*, *C. canis*, *C. felis*, *C. meleagridis*, *C. muris*, *C. hominis*, *C. parvum*, *C. serpentis* and *C. wrairi* (GenBank accession numbers: AF266262, AF266276, AF266274, AF266263, AF266266, AF266264, AF266265, Z22537, AF266275 and AF266271 respectively) were aligned using the BioEdit© program (Hall 1999). Four primers (forward [F], inner forward [IF], inner reverse [IR] and reverse [R]) were designed with the Hemi-nested Oligo Selection Program (P.H. Dear, unpublished). Because of high polymorphism in the COWP gene between *Cryptosporidium* species, each primer was made with five (IF) or six (F, IR, R) oligonucleotides having one to six different bases within each set. By doing so, there was a 100% match between one oligonucleotide and each of the COWP sequences corresponding to different *Cryptosporidium* species. HPLC-purified oligonucleotides were obtained from MWG-Biotech (Ebersberg, Germany) and their sequences and positions are shown in Table 1.

The amplification was performed as a two-step PCR. A phase I reaction was performed using a conventional thermocycler (Biometra T1, Anachem Ltd, Luton, UK). The forward and reverse primers PCOWPF and PCOWPR were designed to amplify a 430-bp fragment of the COWP gene. The reaction was performed in a 10 μl volume comprising 5 μl of DNA in 1X PCR buffer, 2 mM $MgCl_2$, 0·25 mM of each deoxynucleoside triphosphate, 0·3 μM of each primer and 0·5 U of *Taq* DNA polymerase (Invitrogen, Paisley, UK). Samples were subjected to an initial denaturation of 94°C for 1 min followed by 25 cycles of 94°C for 20 s, 50°C for 30 s and 72°C for 1 min followed by a final extension at 72°C for 5 min.

The phase II reaction was designed to amplify a 341-bp fragment of the COWP gene as a real time hot-start PCR using a LightCycler. Reactions were performed in glass capillaries (total volume of 20 μl) which comprised 10 μl of 10X diluted phase I product, 3 mM $MgCl_2$, 1 μM of each primer (PCOWPIF/IR) and 2 μl of Master Mix (FastStart

Table 1 Primers used for *Cryptosporidium* oocyst wall protein (COWP) gene fragments amplification in the real time PCOWPLC-PCR. (*C. parvum* refers to *C. parvum* genotype 2)

Primers and sequences	Target	Position and GenBank number
PCOWPF (Equal mix of six oligonucleotides)		
5′-GTGTACAGTCTGATACTGCACC-3′	*C. andersoni*,	61–82 in AF266262
	C. muris	58–79 in AF266264
5′-GTGTACAGTCTGACACTGCACC-3′	*C. serpentis*	61–82 in AF266275
5′-GTGTTCAATCAGATACTGCCCC-3′	*C. baileyi*	61–82 in AF266276
5′-GCGTACAGTCAGATACTGCTCC-3′	*C. felis*	61–82 in AF266263
5′-GTGTTCAATCAGACACAGCTCC-3′	*C. meleagridis*,	419–440 in AF266266
	C. parvum, *C. hominis*	1000–1021 in Z22537
	C. wrairi	61–82 in AF266271
5′-GTGTACAATCAGATACCGCTCC-3′	*C. canis*	77–98 in AF266274
PCOWPIF (Equal mix of five oligonucleotides)		
5′-CCTCCCAACCCTGAATGTCC-3′	*C. andersoni*,	81–100 in AF266262
	C. serpentis,	81–100 in AF266275
	C. muris	78–97 in AF266264
5′-CCACCTAACCCAGAATGTCC-3′	*C. baileyi*	81–100 in AF266276
5′-CCACCAAACCCAGAATGTCC-3′	C. *felis*	81–100 in AF266263
5′-CCTCCTAATCCAGAATGTCC-3′	*C. meleagridis*,	439–458 in AF266266
	C. parvum, *C. hominis*	1020–1039 in Z22537
	C. wrairi	81–100 in AF266271
5′-CCACCAAATCCAGAATGTCC-3′	*C. canis*	97–115 in AF266274
PCOWPIR (Equal mix of six oligonucleotides)		
5′-GGGCACTCCTTAGCTGGAGC-3′	*C. andersoni*,	402–421 in AF266262
	C. muris	399–418 in AF266264
5′-GGGCATTCCTTAGCTGGAGC-3′	*C. serpentis*	402–421 in AF266275
5′-GGACATTCTTTTGCAGGAGC-3′	*C. baileyi*	402–421 in AF266276
5′-GGACATTCTTTCGCAGGAGC-3′	*C. felis*	402–421 in AF266263
5′-GGGCATTCCTTTGCAGGAGC-3′	*C. meleagridis*	523–542 in AF266266
	C. parvum, *C. hominis*	1341–1360 in Z22537
	C. wrairi	402–421 in AF266271
5′- GGGCACTCTTTTGCAGGAGC-3′	*C. canis*	418–437 in AF266274
PCOWPR (Equal mix of six oligonucleotides)		
5′-GGACATACTGGTTGTGTTG-3′	*C. andersoni*,	472–490 in AF266262
	C. serpentis,	472–490 in AF266275
	C. muris	469–487 in AF266264
5′-GGACATATAGGTTGAGTTG-3′	*C. baileyi*	472–490 in AF266276
5′-GGACATACTGGTTGAGTTG-3′	*C. felis*	472–490 in AF266263
5′-GGGCATACAGGTTGTGTTG-3′	*C. meleagridis*	830–848 in AF266266
5′-GGGCAGACAGGTTGAGTTG-3′	*C. parvum*, *C. hominis*	1411–1429 in Z22537
	C. wrairi	472–490 in AF266271
5′-GGACAAACAGGTTGAGTTG-3′	*C. canis*	488–506 in AF266274

DNA Master SYBR Green I kit; Roche Molecular Biochemicals, Lewes, UK). Cycling conditions included 95°C for 8 min followed by 40 cycles of 95°C for 15 s, 60°C for 2 s and 72°C for 10 s. The transition rate was 20°C s^{-1}. Fluorescence readings were taken after each extension step and as a final melting analysis by a treatment of 95°C for 0 s, then 70°C for 15 s followed by a transition at 0·1°C s^{-1} to 95°C. For identification of the PCR product, melting temperature (T*m*) peaks were derived from the melting curves using the LightCycler software 3·5©.

Each batch of tests contained a maximum of 30 samples plus one positive control (Iowa strain derived DNA) and one negative control (water).

Gel electrophoresis and restriction fragment length polymorphism

To visualize DNA amplified using the LightCycler, PCR products were recovered from the glass capillaries by centrifugation into microtubes and tested by horizontal

Table 2 Restriction patterns predicted from digestion with *Rsa*I and with *Alu*I of the 341 bp *Cryptosporidium* oocyst wall protein gene fragment amplified by PCOWPLC-PCR

	Restriction fragments length (bp)	
Enzyme	*Alu*I	*Rsa*I
Cryptosporidium species		
C. andersoni	341	311–30
C. muris	341	311–30
C. serpentis	341	311–30
C. baileyi	322–19	341
C. felis	322–19	296–45
C. meleagridis	322–19	296–45
C. canis	322–19	195–71–30–27–18
C. parvum (genotype 2)	193–129–19	311–30
C. wrairi	193–129–19	296–45
C. hominis	193–129–19	284–30–27

electrophoresis in 2% agarose gels stained with SYBR Green I (Molecular Probes, Europe, Leiden, the Netherlands).

Using an alignment of the COWP gene sequences of 10 *Cryptosporidium* species, *Rsa*I and *Alu*I restrictions sites were identified to distinguish seven groups of 10 species including the unequivocal identification of *C. hominis* and *C. parvum* from the other species (Table 2). RFLP analysis was performed by digesting 5 μl of PCR product with 5 U of restriction enzyme in 1X enzyme buffer (Invitrogen) in a final volume of 30 μl for at least 4 h at 37°C. PCR products were digested with each enzyme in two separate reactions. Restriction fragments were separated in 3·2% agarose ethidium bromide gels by horizontal electrophoresis and examined by UV transillumination.

DNA sequencing

PCR products were purified using StrataPrep® PCR purification kits (Stratagene Europe, Amsterdam Zuidoost, the Netherlands). Sequencing of PCR products (sense and antisense) was performed at the Advanced Biotechnology Centre Imperial College, London, UK, using an ABI 377 Automated DNA sequencer and the appropriate IF and IR primers.

RESULTS

DNA from all faeces, oocysts, trophozoites and bacterial suspensions were subjected to PCOWPLC-PCR/RFLP. PCR products amplified from all *Cryptosporidium*-derived DNA produced T*m* values between 82°C and 84°C: it was not possible to differentiate *Cryptosporidium* species by their T*m* values only. DNA extracts recovered from other

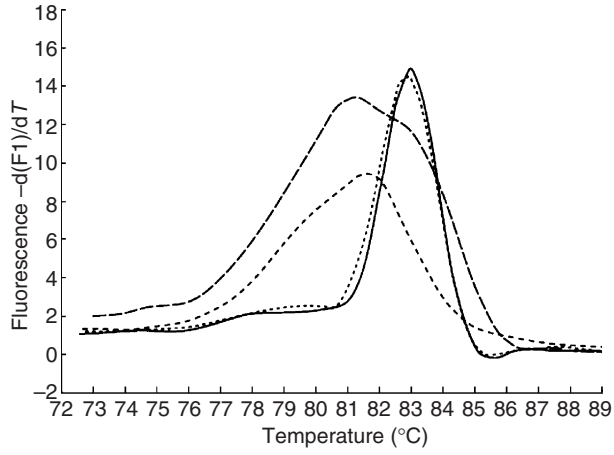

Fig. 1 Sensitivity of the real time PCOWPLC-PCR assay. The graph shows melting curves analysis for products of amplification from a DNA sample containing various genome copy number of *Cryptosporidium hominis* per reaction. Specific products have a T*m* value of approx. 83°C. ——— 20 genome copy equivalent; ..... 15 genome copy equivalent; - - - - - 10 genome copy equivalent; – – – – 0 genome copy equivalent

samples produced either no peaks or flatter peaks with a T*m* lower than 82°C (Fig. 1) representing non-specific amplification. Amplicons from the phase II reaction were also separated by SYBR Green I/agarose gel electrophoresis and the predicted 341-bp fragment was detected by electrophoresis exclusively from all DNA obtained from purified *Cryptosporidium* oocysts and from all faecal samples known to contain *Cryptosporidium* species. Results from the PCR products' digestion with *Alu*I and *Rsa*I enzymes revealed the expected RFLP patterns for each *Cryptosporidium* species tested (Table 2) and *C. hominis* and *C. parvum* could be easily distinguished from the other species. PCOWPLC-PCR products obtained from one of each *Cryptosporidium* species tested were purified and sequenced. The resulting sequences were found to be identical to the COWP gene sequences of the corresponding species obtained from the GenBank database (accession numbers cited in Table 1).

To estimate the limit of detection of the PCOWPLC-PCR, serial dilutions of a DNA extract containing 10 genome equivalents μl^{-1} of *C. hominis* were prepared and subjected to the assay. Specific melting peaks with a T*m* of 82·83–83·04°C [standard deviation (SD), 0·8–1·0] were generated from amplifications performed using ≥15 copies of the *Cryptosporidium* genome per reaction, i.e. three copies μl^{-1} (Fig. 1). The reproducibility of the COWP gene detection using DNA extracted from purified *C. hominis* oocysts subjected five times to the PCOWPLC-PCR, was 100%.

In six of the smears produced from the 17 faecal samples containing *C. hominis* or *C. parvum*, less than one oocyst per

microscope field was observed. DNA was extracted from all 17 smears and analysed in duplicate by PCOWPLC-PCR/RFLP. Amplification of the COWP gene fragment was detected (by T*m* value) from all smear extracts. Following RFLP analysis, the same genotype as previously identified from whole faeces was recovered. The reproducibility of the COWP gene detection of the duplicate results obtained from the smears was 85·3%. The reproducibility was 50% amongst samples with less than one oocyst per microscope field.

DISCUSSION

The advantage of performing PCR analyses using nested procedures (as compared with un-nested) is that the specificity and the sensitivity of reactions are greatly increased. This is especially useful when there is a need to detect small numbers of the target organism within a diverse microflora, as described here for DNA extracted directly from whole faeces and faecal smears. The nested-PCOW-PLC-PCR developed here was shown to serve this purpose. Products were amplified from DNA extracted directly from faeces or from faecal smears (without the need for oocyst purification or concentration) and the reaction was shown to be specific for COWP gene amplification and highly sensitive. The results of the assay were 100% reproducible when using DNA from purified material, however, from assays performed using DNA recovered from faecal smears, the reproducibility was 85·3%. This reduced fidelity may be caused by the co-extraction of inhibitors of the PCR or of DNA from the faecal microflora although previous analysis suggest that this is unlikely (Amar *et al.* 2001). As DNA is extracted from a smaller sample of the faecal smears than from whole faeces (15% of material), a more likely explanation is that the poorer reproducibility reflects sampling error because of the very low original template concentration. In addition, the number of parasites seen by microscopy might not reflect the level of target because some oocysts may be damaged prior to extraction and contain reduced or no intact target DNA.

The COWP gene was selected to amplify cryptosporidial DNA for its high degree of polymorphism which allowed the identification by RFLP analysis of *C. hominis* and *C. parvum* which are present in the majority of human infections (McLauchlin *et al.* 2000). However, the polymorphism within the primer binding sites necessitated the use of a mixture of five or six oligonucleotides with one to six base differences corresponding to the inter-specific sequence variation. This strategy has the additional advantage of an increased ability to detect mixtures of targets as all suitable oligonucleotide sequences are included in the reaction. This advantage has been demonstrated in a further study: mixtures of *Cryptosporidium* spp. were detected from shellfish using this method. However, it was necessary to perform DNA sequencing of the PCR fragments for identification of the *Cryptosporidium* species (Gómez-Couso *et al.* 2003). Hence, further analysis by direct sequencing is probably necessary when testing samples from food, water, the environment and from non-human hosts.

Phase I of the PCOWPLC-PCRs was performed in a conventional thermocycler and only the nested-phase was adapted to the LightCycler system. This format retained the high specificity and sensitivity provided by a nested reaction and the use of diluted phase I PCR product avoided the saturation in fluorescence because of the SYBR Green I binding to the double stranded DNA recovered from the faeces. We chose to use the double stranded DNA-binding dye SYBR Green I (rather than sequence-specific fluorescent probes) for detection of amplicons in the LightCycler for three reasons. First, the design and use of such probes is complex, especially when dealing with polymorphic sequences. Secondly, the two-phase PCR (which was necessary for adequate sensitivity) has much greater sequence specificity than a single-phase reaction and thus, confirmation of amplicon sequences (which would be afforded by a sequence-specific probe) is less important. Thirdly, our protocol allows the same oligonucleotides to be used for assays both on the LightCycler and on conventional PCR machines (results not shown). However, the real advantage of using the LightCycler method was the rapidity to perform the assay. With the exception of the RFLP analysis, which is common to both systems, the assay took approx. 1 h and 50 min by PCOWPLC-PCR and $3\frac{1}{2}$ h using conventional thermocycler and the gel based system. The gain of time was mainly because of the use of glass capillaries that allow faster thermocycling, and to the replacement of agarose gel electrophoresis by the use of melting point analyses. However, one disadvantage of using the LightCycler was that each batch was limited to 30 assays plus one positive and one negative control.

In summary, a highly sensitive real time PCR method for the detection of a wide range of *Cryptosporidium* species was developed and evaluated. It was applicable to DNA extracted directly from whole faeces and from faecal smears. The assay was specific, highly sensitive and provided reproducible results. New sequence variants of *Cryptosporidium* will inevitably be reported and added to the genetic database. The protocol described here is likely to allow the incorporation of additional primers to the assay to enable the detection of these new sequences as they become available. We have already shown that this assay can be multiplexed together with primers designed to detect *G. duodenalis* (Amar, unpublished). The use of LightCycler hybridization probes is currently being evaluated which will reduce the time currently needed for

RFLP analysis. The authors believe that this simple and rapid method is applicable to routine laboratory diagnosis and will increase the understanding of the epidemiology of cryptosporidiosis.

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