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LETTER TO THE EDITOR

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Novel real-time PCR assays for the specific detection of human infective *Cryptosporidium* species

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Cryptosporidium is a parasite that causes the majority of waterborne protozoan outbreaks of gastrointestinal disease worldwide.[1] Cryptosporidiosis usually presents as self-limiting, although often prolonged, diarrhea with abdominal pain, nausea, vomiting and low-grade fever. Infection can be life threatening in some severely immunocompromised groups.[2] Post infection sequelae have also been reported.[3] On a global level, *Cryptosporidium hominis* and *Cryptosporidium parvum* cause the majority of human infections and drinking waterborne outbreaks. Recently, *Cryptosporidium cuniculus* has also caused a sizeable waterborne outbreak.[4] Molecular detection of human infective *Cryptosporidium* species is usually based on conserved housekeeping genes, as is the case for other microorganisms.[5] However, novel detection targets have been investigated for *Cryptosporidium*, but previous investigations aiming to identify *C. hominis* and *C. parvum* specific genes had limited success because the majority of putative species-specific genes were subsequently found to be present in the other species.[6,7] This was attributed to the limited numbers of genome sequences available and their low quality that hindered the accurate prediction of genes' presence. Nevertheless, evidence was found for one *C. hominis* specific gene (Chos-1), which was further validated independently using comparative genomics of newly sequenced clinical isolates.[8] Chos-1 has a telomeric location and the predicted secreted protein of 50 kDa has interesting features, (highly glycosylated, serine rich and several internal repeats), suggestive of a role in host pathogen interaction.[6] It is plausible that other *Cryptosporidium* species-specific genes and virulence factors are also located at the telomeres. Subtelomeres are hotspots for genetic recombination in other parasitic protozoa, resulting in significant chromosome length and sequence composition polymorphisms and an over-representation of highly-diverged loci which have been utilized for discriminatory diagnostic purposes.[9-12]

Thanks to decreased costs and technological advances, an increasing number of full genome sequences are becoming available for *Cryptosporidium* spp. and are contributing to improved comparative genomics analyses. While many investigators used these genomes to identify genes and proteins based on their presence in other parasites and bacteria, we have continued to look for species-specific genes that are likely to be involved in host parasite interaction and virulence. In addition, these genes provide a source of highly specific and stable detection markers. In this study, we used an increased number of genome sequences to identify species-specific genes, focusing on genes close to the chromosome ends. Subsequently, primers and probes targeting these loci were designed and evaluated as novel real-time PCR assays for the specific detection of the major human infective waterborne parasites *C. hominis* and *C. parvum*.

Previous investigation of putative species-specific genes led to the characterization of the telomeric Chos-1 gene.[6] This finding suggested that further genes are likely to be analogously positioned and warranted further investigation of subtelomeric regions of *C. hominis* and *C. parvum* genome sequences. In the first instance, the reference genome sequences of *C. hominis* TU502 (gp60 subtype IaA25R3) [13] and *C. parvum* Iowa (gp60 subtype IIaA15G2R1) [14] were retrieved from CryptoDB (http://cryptodb.org) and used for analysis. Subsequently, genomic data of newly sequenced genomes from *C. hominis* and *C. parvum* clinical isolates (UKH4 gp60 subtype IaA14R3 and UKP6 gp60 IIaA15G2R1, respectively) were used to verify our findings.[15] Comparative analysis of sequence data was achieved by aligning subtelomeric regions spanning 30000 bp from the 5′- and 3′-ends of

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the 8 *C. hominis* and *C. parvum* chromosomes using the Artemis Comparison Tool (ACT) [16] http://www.webact.org/WebACT/home. The subtelomeric regions were scanned in order to identify species-specific coding sequences. The specificity of these sequences was further validated against other *Cryptosporidium* isolates with different gp60 subtypes: 2 *C. hominis* (UKH5 gp60 IbA10G2, UKH3 gp60 IbA10G2) and 4 *C. parvum* (UKP2 gp60 IIaA19G1R2, UKP3 gp60 IIaA18G2R1, UKP7 gp60 IIaA17G1R1, UKP8 gp60 IIdA22G1) using a standalone nucleotide blast test generated using BioEdit (version 7.2.5) and against all non-*Cryptosporidium* isolates using blastn software tool provided by NCBI (https://blast.ncbi.nlm.nih.gov/Blast.cgi?PAGE_TYPE=BlastSearch).

The analysis showed that a 14000 bp stretch of genomic sequence was putatively specific to *C. parvum* and missing from *C. hominis* along the subtelomeric arm extending from the 5′ telomeric repeat (5′-AAACCT-3′) on chromosome 5 (Fig. 1). In *C. parvum*, a 419 bp stretch of genomic sequence was missing at the 3′ telomeric end of chromosome 5 and therefore putatively *C. hominis* specific. Four open reading frames (ORFs) were encoded within the *C. parvum* specific sequence and the *C. hominis* specific sequence partially spanned a single ORF. Two of the *C. parvum* specific ORFs are the result of a *C. parvum* specific gene duplication event of gene cgd5_4580, which is located at the subtelomeric 3′-end of chromosome 6 in *C. parvum*, and which contains an ortholog in *C. hominis* called Chro.50507. The third open reading frame, identified as gene cgd6_5500, also contains an ortholog in *C. hominis*, which has non syntenic localization compared to its *C. parvum* counterpart as it is located on a different chromosome. The fourth coding sequence, Cgd6_5510 (Cops-2) encoding an insulinase-like peptidase, was wholly specific to *C. parvum*, and hence selected for inclusion in this study. The genomic sequence identified in *C. hominis*, Chro.00007 (Chos-2), was not entirely species-specific, with an orthologous stretch of 100 bp sequence identified at the 3′ end of chromosome 7 in *C. parvum*. However, the degree of divergence for this orthologous segment (88% nucleotide similarity) and the specificity of the remaining 319 bp stretch warranted inclusion of this marker for discriminatory purposes. Chos-1, Chos-2 and Cops-2 were the 3 species-specific loci used to develop and evaluate real-time PCR assays for the specific detection of *C. hominis* and *C. parvum*.

Sequence data for each locus (Chos-1, Chos-2 and Cops-2) from all available *C. hominis* and *C. parvum* isolates (3 *C. hominis* UKH3, UKH4, UKH5 and 5 C. *parvum* UKP2, UKP3, UKP6, UKP7, UKP8) were retrieved and aligned separately (for each species) using BioEdit version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). Primer sequences were selected to amplify fragments of interest and appropriate length (70–150 bp), while avoiding undesirable molecular interactions such as cross reactivity or self-annealing. Primer length, annealing temperature, %GC content and the potential for undesirable molecular interactions were assessed using the Primer Express software program (Applied Biosystems). The predicted PCR products were analyzed *in silico* and no orthologs were found in other *Cryptosporidium* spp. MGB hydrolysis probes were designed according to the optimum criteria: probe having an annealing temperature 10°C higher than the primers and avoiding runs of identical nucleotides and nucleotides likely to quench the reporter fluorescence.

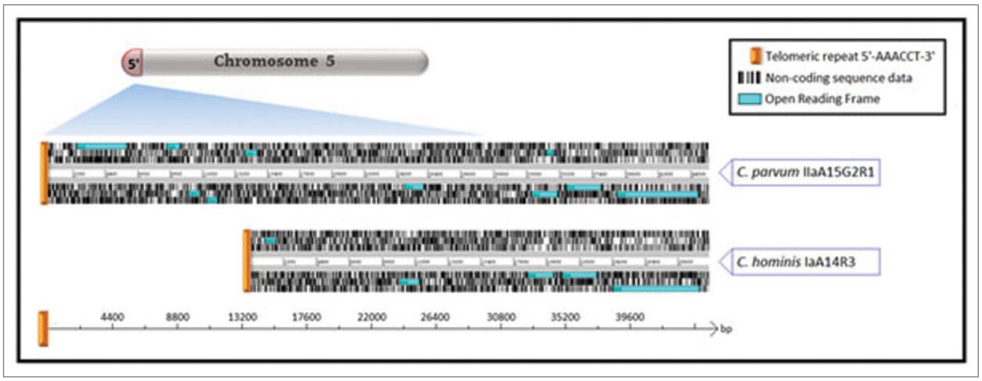

Figure 1. Subtelomeric sequence data alignment at the 5′-end of chromosome 5 reveals significant sequence incongruence between *C. hominis* and *C. parvum*. Subtelomeric sequence data extending <30,000bp inwards from the 5′-telomeric end of chromosome 5 was extracted from newly-sequenced whole genome sequences for *C. parvum* subtype IIaA15G2R1 (UKP6) and *C. hominis* subtype IaA14R3 (UKH4). Sequences were aligned and compared using the Artemis Comparison Tool (ACT).[16] Orthologous coding sequences were independently validated through The European Molecular Biology Open Software Suite (EMBOSS) Stretcher pairwise sequence alignment of nucleotide ORFs.[18]

Primers and probes sequences and characteristics are presented in Table 1. For Chos-1, 2 different reverse primers with a common forward primer and probe were tested. For Chos-2, 2 different forward primers with a common reverse primer and probe were tested and for Cops-2, a single forward and reverse primer and probe were used. Primer and probe sequences were checked for cross-reactions with non-target sequences on the GenBank database using the Basic Local Alignment Search Tool (BLAST; http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). No complete (both primers and probe) cross reactions with non-*Cryptosporidium* sequences were detected. Primers and probes (all MGB-non-fluorescent quencher, NFQ probes) were ordered from Life Technologies.

The primer and probe sets were tested in a singleplex real-time PCR format. PCR conditions were TaqMan Environmental Master Mix 2.0 (ABI, Life Technologies), 600 nM of each primer and 100 nM probe. Cycling conditions were 95°C for 10 min, 95°C for 15 seconds and 60°C for 1 min, 50 cycles. PCRs were run on a Rotorgene thermocycler (Qiagen). Additionally, 2 primer and probe sets were evaluated in a duplex format for the detection of single and dual *C. parvum* and *C. hominis* infections.

Each real time assay was evaluated using target and non-target genomic DNA from *Cryptosporidium* species and genotypes isolated from human and animal infections, which included human infective species (such as *C. cuniculus* and *C. viatorum*) as well as non-human infective species (such as *C. andersoni, C. baileyi* and *C. bovis*). All samples were from anonymised clinical human and animal sources and provided by the *Cryptosporidium* Reference Unit (Table 2).

While optimising PCR conditions, a small number of samples were tested using the primer sets in a conventional PCR format. This resulted in DNA amplification from species other than *C. hominis* and *C. parvum* (data not shown). The amplified bands were very faint and did not allow sequence analysis of PCR products. However, the use of probe-based real-time PCR format substantially increased the specificity of the assay. Real-time PCR results presented as C_t values are summarised in Table 2. Ct values ranged from 22.30 for TU502 isolate detection using Chos-1 assay to 38.44 for UKH15 detection using Chos-2 assay. Overall, only *C. hominis* and *C. cuniculus* were detected using Chos-1 and Chos-2 primer-probe sets. This result is not surprising because *C. cuniculus* is closely related to *C. hominis* and has been documented to amplify using *C. hominis* assays.[17] For Cops-2, only *C. parvum* DNA was amplified. However, one of the 7 *C. parvum* isolates tested (UKP12) did not amplify using the Cops-2 assay. Our results support the species specificity of these detection assays within the genus *Cryptosporidium*, demonstrated by the testing of a comprehensive panel of human and non-human infective *Cryptosporidium* species and genotypes. This specificity is conferred by the use of primers and probes in a real time PCR format.

The performance of a duplex assay for the simultaneous detection of both *C. hominis* and *C. parvum* was assessed using Chos2-F2 and Cops-2 primers and probes. This is particularly important for clinical settings as the duplex PCR would allow detection of both human pathogens in a single test at a decreased cost and time compared to individual singleplex PCRs. Individual *C. hominis* and *C. parvum* DNA as well as an artificially mixed DNA sample were tested using singleplex and duplex formats. The samples were correctly identified by both assay formats, but the duplex format was associated with some increase in C_t values compared with those from the simplex PCR (Table 3). This is a well-known trade off of the multiplex PCR. However, for the Cops-2 assay the C_t difference between singleplex and duplex formats was negligible, showing that no competition was occurring when a duplex format is used.

Table 1. Sequences of forward (F) and reverse (R) primers and probes (T) designed to investigate the potential of Chos-1 and Chos-2 for the specific detection of *C. hominis* and Cops-2 for the specific detection of *C. parvum*.

Assay name	Primer - Probe	Sequence (5′-3′)	nt	Tm	% GC	SIZE
Chos-1	Chos-1F	AAATTCACGATTAGCACGACCAG	23	59.2	43	140 bp
	Chos-1R	TCAATCGGAAATCCACAGAACTACT	25	59.1	40	
	Chos-1T	CCTCTTGAGCTTGCACC	17	69	59	
Chos-1R2	Chos-1F	AAATTCACGATTAGCACGACCAG	23	59.2	43	127 bp
	Chos-1R2	GTAGTGAAATATCCTTTTGGAGTATGC	27	56.8	37	
	Chos-1T	CCTCTTGAGCTTGCACC	17	69	59	
Chos-2	Chos-2F	GTTCTCAAATTTTGGTTCGCTTG	23	58.8	39	139 bp
	Chos-2R	TTCTTGTTCAATTGCCATAAGCA	23	58.4	35	
	Chos-2T	CGTTTCAAGTCGTGCATC	18	69	50	
Chos-2F2	Chos-2F2	CGCATATGCAAATCAGTTCTCAA	23	58.9	39	167 bp
	Chos-2R	TTCTTGTTCAATTGCCATAAGCA	23	58.4	35	
	Chos-2T	CGTTTCAAGTCGTGCATC	18	69	50	
Cops-2	Cops-2F	AAGTCGGAAGCCATAATGATCC	22	58.1	45	148 bp
	Cops-2R	GTAAAAGCACTTGTTTCTCCAAAATG	26	58.5	35	
	Cops-2T	TAGCACACCTACTAAAGCA	19	70	42	

Table 2. Real time PCR results using a comprehensive *Cryptosporidium* DNA panel from clinical and animal sources. Results are presented as C_t values.

Species and isolate	gp60 subtype	Chos-1	Chos-2	Chos-2F2	Chos-1R2	Cops-2
C. hominis TU502	IaA25R3	22.30	27.72	25.27	26.33	neg
C. hominis UKH15	IbA10G2	31.71	38.44	32.25	30.53	neg
C. hominis UKH14	IaA14R3	26.35	34.42	26.66	26.67	neg
C. hominis UKH6	IdA30	27.33	32.28	27.11	27.22	neg
C. cuniculus UKCU13	Va	26.10	30.68	27.64	26.78	neg
C. cuniculus UKCU14	Vb	30.12	35.04	32.27	31.64	neg
C. parvum Moredun	IIaA17G1R1	neg	neg	neg	neg	27.72
C. parvum UKP12	IIcA5G3	neg	neg	neg	neg	neg
C. parvum UKP13	IIcA5G3	neg	neg	neg	neg	28.86
C. parvum UKP35	IIaA15G2R1	neg	neg	neg	neg	32.97
C. parvum UKP36	IIaA17G1R1	neg	neg	neg	neg	28.40
C. parvum UKP37	IIdA24G1	neg	neg	neg	neg	29.34
Natural co-infection C. hominis and C. parvum UKHP1	IIdA15G1	30.02	33.27	30.68	31.86	33.19
		neg	neg	neg	neg	neg
C. felis UKFEL3	nk	neg	neg	neg	neg	neg
C. canis UKCAN1	nk	neg	neg	neg	neg	neg
C. andersoni UKAND1	nk	neg	neg	neg	neg	neg
C. baileyi UKBLY1	nk	neg	neg	neg	neg	neg
C. viatorum UKVIA2	nk	neg	neg	neg	neg	neg
C. bovis UKBOV1	nk	neg	neg	neg	neg	neg
Skunk gt UKSK1	nk	neg	neg	neg	neg	neg
C.meleagridis UKMEL7	nk	neg	neg	neg	neg	neg
C.meleagridis UKMEL11	nk	neg	neg	neg	neg	neg
C. ubiquitum UKUB7	nk	neg	neg	neg	neg	neg
C. ubiquitum UKUB7	nk	neg	neg	neg	neg	neg
Horse gt UKHOR1	nk	neg	neg	neg	neg	neg

Note. gt: genotype, nk=not known, or not applicable. Neg: negative PCR result

Comparative genomics are an important aspect of the post-genomic era. Analysis of genome sequences is continuously improving our understanding of pathogen biology, pathogenicity, evolution and host adaptation. Comparative genomics also allow identification of conserved and specific genes. We exploited the increasing numbers of published *Cryptosporidium* genome sequences to identify putative unique genes that are likely to be involved in virulence and host adaptation. Subsequently, we developed assays based on these genes as specific detection tools. Our previous attempt had a limited success as only one *C. hominis* specific gene was found.[6] This was mainly attributed to the low quality of the TU502 genome sequence available at the time. Technological advances have enabled the generation of an ever increasing number of genome sequences of improved quality.[8,15] These improvements are likely to contribute to more accurately predicted species-specific genes.

Table 3. Real time PCR results (C_t values) comparing single and duplex Chos-2F2 and Cops-2 assays.

	Singleplex assays		Duplex assays	
Cryptosporidium species and isolate	Chos-2F2	Cops-2	Chos-2F2	Cops-2
C. hominis UKH15	32.25	neg	35.14	neg
C. parvum UKP36	neg	28.4	neg	28.96
Artificial mix C. hominis and C. parvum	nk	nk	36.57	29.64
No template control	neg	neg	neg	neg

Note. nk=not known. Neg: negative PCR result Figure legend.

Indeed, assays targeting these genes were confirmed experimentally as species-specific as shown by our results. The use of probe-based real-time PCR assay dramatically increased the specificity of the assays.

These novel real-time PCR assays for the detection of *C. hominis* and *C. parvum* were evaluated using a comprehensive panel of human and non-human infective *Cryptosporidium* species and genotypes. Only DNA from *C. parvum* for real time PCR Cops-2 assay and *C. hominis* and *C. cuniculus* for Chos-1 and Chos-2 real time PCR assays were amplified. However, further specificity assessment needs to be performed using DNA from other human-infective parasites likely to be present in human stool samples such as *Giardia duodenalis, Cyclospora cayetanensis, Entamoeba histolytica/dispar, Toxoplasma gondii, Blastocystis hominis* and *Dientamoeba fragilis*. Further validation work needs to be undertaken, including assessment of analytical and diagnostic sensitivity and specificity, repeatability and reproducibility, as well as consideration of clinical and epidemiological application prior to use these assays in a clinical setting.

For the Cops-2 assay, 1 out the 7 *C. parvum* isolates tested (UKP12) did not amplify. The reason for this is unknown. It could be due to sequence variation, DNA quality or quantity or presence of inhibitors, although the latter is unlikely as the sample amplified using other detection targets. Therefore, it would be useful to include an internal positive control in any diagnostic PCR based assay. The positive control could target a

Cryptosporidium conserved gene and the performance of the modified assay will need to be assessed.

While extensively characterized genes continue to be the targets of choice for the detection of human infective *Cryptosporidium* species, identification and validation of species-specific genes are sometimes preferable, especially when investigating host adaptation and evolution. Additionally, the use of these loci is likely to add robustness to the assay especially in comparison to detection and typing assays relying on small sequence variation and even on a single nucleotide polymorphism for successful amplification and sequence analysis. More genes continue to be discovered in the post genomic era and are likely to contribute to major breakthroughs in the fight against infectious diseases.

Disclosure of potential conflicts of interest

No potential conflicts of interest were disclosed.

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