Cryptosporidium is a parasite that causes the majority of waterborne protozoan outbreaks of gastrointestinal disease worldwide. 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. Post infection sequelae have also been reported. 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. Molecular detection of human infective Cryptosporidium species is usually based on conserved housekeeping genes, as is the case for other microorganisms. 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. 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. 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. 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.

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. 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) and C. parvum Iowa (gp60 subtype IIaA15G2R1) 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. Comparative analysis of sequence data was achieved by aligning subtelomeric regions spanning 30000 bp from the 5′- and 3′-ends of
the 8 Cryptosporidium hominis and C. parvum chromosomes using the Artemis Comparison Tool (ACT) 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 IlaA19G1R2, UKP3 gp60 IlaA18G2R1, UKP7 gp60 IlaA17G1R1, UKP8 gp60 IldA22G1) 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 subtelomeric regions were independently validated through The European Molecular Biology Open Software Suite (EMBOSS) Stretcher pairwise sequence alignment of orthologous coding sequences were independently validated through The European Molecular Biology Open Software Suite (EMBOSS) Stretcher pairwise sequence alignment of nucleotide ORFs.

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'-AACCT-3’) on chromosome 5 (Fig. 1). In C. parvum, a 419 bp stretch of genomic sequence was missing at the 3’ 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.05057. 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 insulinate-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 IlaA15G2R1 (UKP6) and C. hominis subtype IaA14R3 (UKH4). Sequences were aligned and compared using the Artemis Comparison Tool (ACT). Orthologous coding sequences were independently validated through The European Molecular Biology Open Software Suite (EMBOSS) Stretcher pairwise sequence alignment of nucleotide ORFs.
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 Ct 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 Ct 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 Ct difference between singleplex and duplex formats was negligible, showing that no competition was occurring when a duplex format is used.

| Assay name | Primer - Probe | Sequence (5’-3’) | nt | Tm | % GC | SIZE |
|------------|----------------|-----------------|----|----|------|------|
| Chos-1     | Chos-1F        | AAATTCCAGATTAGACGACAG | 23 | 59.2 | 43   | 140 bp |
|            | Chos-1R        | TCAATCGGAATATCCGACAAGACT | 25 | 59.1 | 40   |      |
|            | Chos-1T        | CTCCTTGAGCTTGCACC | 17 | 69 | 59   |      |
| Chos-1R2   | Chos-1F        | AAATTCCAGATTAGACGACAG | 23 | 59.2 | 43   | 127 bp |
|            | Chos-1R2       | GTAGTGAATATCTCTTTGGAAGTGATGC | 27 | 56.8 | 37   |      |
|            | Chos-1T        | CTCCTTGAGCTTGCACC | 17 | 69 | 59   |      |
| Chos-2     | Chos-2F        | GGTCTCAATATTTTTGTCCTGGTT | 23 | 58.8 | 39   | 139 bp |
|            | Chos-2R        | TTCCTGTCAATATTGGCATAAGCA | 23 | 58.4 | 35   |      |
|            | Chos-2T        | CGTCCACGCTTGCATGC | 18 | 69 | 50   |      |
| Chos-2F2   | Chos-2F2       | CGCATATGCAATATTGCTTCA | 23 | 58.9 | 39   | 167 bp |
|            | Chos-2R        | TTCCTGTCAATATTGGCATAAGCA | 23 | 58.4 | 35   |      |
|            | Chos-2T        | CGTCCACGCTTGCATGC | 18 | 69 | 50   |      |
| Cops-2     | Cops-2F        | AAGTCCGGAAGCCATAATGTCC | 22 | 58.1 | 45   | 148 bp |
|            | Cops-2R        | GTAAAGACACTGTGTTGTTTCCAAATG | 26 | 58.5 | 35   |      |
|            | Cops-2T        | TAGCACAACCTACTAAGCA | 19 | 70 | 42   |      |
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.

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

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

| Species and isolate | gp60 subtype | Chos-1 | Chos-2 | Chos-2F2 | Chos-1R2 | Cops-2 |
|---------------------|--------------|--------|--------|---------|---------|--------|
| C. hominis TU502    | Ia25R3       | 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    | Ia14R3       | 26.35  | 34.42  | 26.66   | 26.67   | neg    |
| C. hominis UKH5     | 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   | Ila17G1R1    | neg    | neg    | neg     | neg     | 27.72  |
| C. parvum UKP12     | Ila17G3      | neg    | neg    | neg     | neg     | neg    |
| C. parvum UKP13     | Ila17G5      | neg    | neg    | neg     | neg     | 28.86  |
| C. parvum UKP35     | Ila15G2R1    | neg    | neg    | neg     | 32.97   | neg    |
| C. parvum UKP36     | Ila17G1R1    | neg    | neg    | neg     | neg     | 28.40  |
| C. parvum UKP37     | Ilda24G1     | neg    | neg    | neg     | neg     | 29.34  |
| Natural co-infection C. hominis and C. parvum UKHP1 | Ilda15G1 | 30.02 | 33.27 | 30.68 | 31.86 | 33.19 |
| C. felis UKFEL3     | nk           | neg    | neg    | neg     | 27.72   | 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. meleagris UKMEL7 | nk           | neg    | neg    | neg     | neg     | neg    |
| C. meleagris UKMEL11| 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.

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

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

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