Detection and differentiation of *Cryptosporidium hominis* and *Cryptosporidium parvum* by dual TaqMan assays

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Rapid identification of the two major species of *Cryptosporidium* associated with human infections, *Cryptosporidium hominis* and *Cryptosporidium parvum*, is important for investigating outbreaks of cryptosporidiosis. This study reports the development and validation of a real-time PCR TaqMan procedure for detection of *Cryptosporidium* species and identification of *C. hominis* and *C. parvum* in stool specimens. This procedure comprised a generic TaqMan assay targeting the 18S rRNA for sensitive detection of *Cryptosporidium* species, as well as two other TaqMan assays for identification of *C. hominis* and *C. parvum*. The generic *Cryptosporidium* species assay can be duplexed with the *C. parvum*-specific assay. The generic *Cryptosporidium* species assay was able to detect ten *Cryptosporidium* species and did not cross-react with a panel of ten other protozoan parasites. The generic *Cryptosporidium* species assay could detect 1–10 oocysts in a 300 µl stool specimen, whilst each of the species-specific TaqMan assays had detection sensitivities that were approximately tenfold higher. The 18S rRNA assay was found to detect *Cryptosporidium* species in 49/55 DNA extracts from stool specimens containing either *C. hominis* or *C. parvum*. The *C. hominis* TaqMan assay correctly identified *C. hominis* in 24/31 validation panel specimens containing this species. The *C. parvum*-specific assay correctly identified *C. parvum* in 21/24 validation panel specimens containing this species. This real-time PCR procedure was used to detect and identify *C. hominis* and *C. parvum* in stool specimens from outbreak investigations in the USA and Botswana, resulting in identification of *C. hominis* and/or *C. parvum* in 66/67 stool specimens shown to be positive for these species using other techniques. From the outbreak specimens tested, the TaqMan procedure was found to have a specificity of 94 %. This TaqMan PCR procedure should be a valuable tool for the laboratory diagnosis of cryptosporidiosis caused by *C. hominis* and *C. parvum* during outbreak investigations.

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INTRODUCTION

Cryptosporidium is an important diarrhoea-causing parasitic protozoan found in both humans and animals (Fayer, 2004). The genus *Cryptosporidium* consists of at least 16 valid species, with two species, *Cryptosporidium hominis* and *Cryptosporidium parvum*, responsible for most cases of cryptosporidiosis in humans (Xiao *et al.*, 2004). *C. parvum* is a zoonotic species, whereas *C. hominis* is anthroponotic. Other *Cryptosporidium* species that have been shown to cause illness in humans include *Cryptosporidium meleagridis*, *Cryptosporidium felis* and *Cryptosporidium canis* (Caccio & Pozio, 2001; Gatei *et al.*, 2002; Morgan *et al.*, 2000; Pedraza-Diaz *et al.*, 2000; Pieniazek *et al.*, 1999; Xiao

et al., 2001). Species discrimination is important for molecular epidemiological purposes to evaluate potential sources of infections. Conventional methods for detecting *Cryptosporidium* oocysts in faecal specimens involve microscopic detection of oocysts using either a direct fluorescent antibody (DFA) assay with broadly reactive *Cryptosporidium* species antibodies or a modified acid-fast staining technique. However, neither of these methods can identify *Cryptosporidium* at the species level, and their diagnostic strength depends on the skills of the examiner (Casemore *et al.*, 1985; Pedraza-Diaz *et al.*, 2000). An ELISA using mAbs against *Cryptosporidium* antigens has been developed and successfully used; however, this method cannot identify *Cryptosporidium* at the species level, despite being practical as a screening method (Graczyk *et al.*, 1996).

Abbreviations: COWP, *Cryptosporidium* oocyst wall protein; DFA, direct fluorescent antibody.

Molecular and biological differences were the basis for the classification of the *C. parvum* anthroponotic strain into a distinct species, *C. hominis*. The creation of this new taxon made it necessary to define the standards for specific molecular-based laboratory identification of the two most common *Cryptosporidium* species associated with diarrhoeal outbreaks worldwide. Various PCR formats have been employed to distinguish species of *Cryptosporidium*. PCR-based detection has been shown to be sensitive and specific for the detection of *C. parvum* in clinical specimens and environmental samples (Amar *et al.*, 2001; Morgan *et al.*, 1998; Sturbaum *et al.*, 2001; Webster *et al.*, 1996; Xiao *et al.*, 2000). PCR-RFLP and PCR followed by DNA sequencing analysis have been described as reliable approaches for the distinction of *C. hominis* from *C. parvum* (formerly known as *C. parvum* genotypes 1 and 2, respectively) (McLauchlin *et al.*, 2000; Pieniazek *et al.*, 1999; Spano *et al.*, 1997, 1998). Nevertheless, they are time-consuming and labour-intensive, making them inadequate for a rapid diagnostic response during outbreak investigations.

Real-time PCR with specific primers and probes represents an alternative to conventional PCR for increasing the speed of sample analysis while decreasing the potential risks for contamination of the laboratory environment with amplicons. In the present study, we developed and validated a dual real-time PCR protocol in which a duplex TaqMan assay was used to detect *Cryptosporidium* species and *C. parvum*. A second TaqMan assay was used to detect *C. hominis*.

METHODS

Protozoan parasite and faecal specimens. *C. hominis* and *C. parvum* oocysts were obtained from human and bovine faecal specimens, respectively, by the method of Arrowood & Donaldson (1996) and stored in 2.5% PBS at 4 °C. The purified *C. hominis* and *C. parvum* stocks were determined to have concentrations of 2.2×10^7 and 33×10^7 oocysts ml^{-1} , respectively, by counting oocysts on a haemocytometer. These stocks were used for specificity testing, as well as seeding of *Cryptosporidium*-negative stools to investigate the detection sensitivity of the molecular assays. The *Cryptosporidium* species identified in Table 1 were confirmed using a genotyping procedure based on the 18S rRNA gene. In addition, analysis of the *Cryptosporidium* oocyst wall protein (COWP) gene (i.e. C- and N-terminal portions of the gene) was performed in the case of the *C. parvum*-like specimen obtained from lemurs (da Silva *et al.*, 2003; Pieniazek *et al.*, 1999; Spano *et al.*, 1997; Xiao *et al.*, 2001). All of the protozoan parasites identified in Table 1 as DNA specificity controls were analysed previously by the CDC Division of Parasitic Diseases reference diagnostics laboratory as part of different studies. They were confirmed by PCR using methods relying on sequencing analysis of a fragment of the 18S rRNA gene. The TaqMan assays developed for this study were validated using the panel of ten identified *Cryptosporidium* specimens reported in Table 1, as well as a blind panel consisting of 69 DNA extracts from human and animal stools, mussels and flies. This panel comprised 24 specimens known to be positive for *C. parvum*, 31 specimens known to be positive for *C. hominis* and 14 specimens known to contain non-*Cryptosporidium* protozoan parasites. The presence of *C. hominis* and *C. parvum* in

Table 1. Specificity testing results for genus- and species-level TaqMan assays

Species tested	18S rRNA assay	<i>C. hominis</i> assay	<i>C. parvum</i> assay
<i>C. hominis</i>	+	+	–
<i>C. parvum</i>	+	–	+
<i>C. canis</i>	+	–	–
<i>C. felis</i>	+	–	–
<i>C. parvum</i> -like (from lemur)*	+	–	–
<i>C. muris</i>	+	–	–
<i>C. andersoni</i>	+	–	–
<i>C. baileyi</i>	+	–	–
<i>C. serpentis</i>	+	–	–
<i>C. wrairi</i>	+	–	+

*da Silva *et al.* (2003).

these samples was confirmed by PCR amplification of the COWP and 18S rRNA genes and of the microsatellite loci ML-1 and ML-2, followed by DNA sequencing analysis; the PCR primers and conditions used have been described previously (Caccio *et al.*, 2000; Johnson *et al.*, 1995; Spano *et al.*, 1997, 1998; Xiao *et al.*, 2001). DNA sequencing reactions were performed by cycle sequencing using BigDye version 3.1 chemistry (ABI). Sequencing data were obtained using an ABI Prism 3100 sequence analyser equipped with data collection software version 2.0 and DNA Sequence Analysis Software version 5.1. Sequences were assembled, edited and aligned in DNASTAR SeqMan, as well as in the GeneStudio suite. The validation panel was used to evaluate the ability of the real-time PCR protocol to correctly detect *Cryptosporidium* species and *C. parvum* or *C. hominis* (i.e. using the duplex TaqMan assay for *Cryptosporidium* species and *C. parvum*, with a separate TaqMan assay for the detection of *C. hominis*). The validation panel was prepared by CDC staff not involved with the analysis of the specimens and was provided to CDC analytical staff as blind panels (i.e. with no descriptive identifiers).

Unpreserved stool specimens collected for epidemiological investigations of cryptosporidiosis in the USA and Botswana were shipped in chilled coolers from the study sites to CDC in Atlanta, USA. These stool specimens were refrigerated and tested using the DFA test and molecular assays [the real-time PCR assays of the present study, the ML-2 assay of Caccio *et al.* (2001) and the COWP assay of Spano *et al.* (1997)] within 1 week by the CDC laboratory staff.

DNA extraction. Total genomic DNA was extracted from 300–500 μl of stool sample spiked with *Cryptosporidium* oocysts and clinical samples using a modification of the FastDNA method (MP Biomedicals), as described previously (da Silva *et al.*, 1999). Samples were disrupted in an FP120 cell disruptor (MP Biomedicals) at a speed of 5.5 for 10 s. Potential inhibitors were removed by further purification using a QIAquick PCR purification kit (Qiagen) following the manufacturer's instructions. Purified DNA was stored at 4 °C until used in PCRs.

DFA assay. Microscopic examination of faecal specimens using the DFA assay was performed as a reference diagnostic technique that is commonly used in clinical laboratory practice. For microscopic examination, stool specimens were processed by ethyl acetate sedimentation (Ash & Orihel, 1987). Direct wet smears prepared from each of the specimens in quadruplicate were air-dried and then processed separately with an acid-fast stain (Ash & Orihel, 1987) and

the DFA assay from the MeriFluor test kit (Meridian Diagnostic) (Nizeyi *et al.*, 1999). Slides were examined for *Cryptosporidium* oocysts by light microscopy as described previously (Nizeyi *et al.*, 1999).

Primers and TaqMan probe design

Cryptosporidium species TaqMan assay. The 18S rRNA sequences for various species of *Cryptosporidium* were retrieved from GenBank. The alignments were performed using CLUSTAL_X (<http://bips.u-strasbg.fr/en/Documentation/ClustalX/>, version 1.81) and BioEdit sequence alignment software version 5.09 (<http://www.mbio.ncsu.edu/BioEdit/bioedit.html>). Consensus primers were designed after comparing several partial 18S rRNA gene sequences for each *Cryptosporidium* species. The sequence of the forward primer (JVAF) was 5'-ATGACGGGTAACGGGGAAT-3', designed on nt 100–118 of GenBank accession number AY458612. The sequence of the reverse primer (JVAR) was 5'-CCAATTACAAAACCAAAA-GTCC-3', designed on nt 258–236 of GenBank accession number AY458612. The *Cryptosporidium* species TaqMan probe (JVAP18S), 5'-Cy5-CGCGCCTGCTGCCTTCCTTAGATG-BHQ-3', was designed based on nt 184–161 of GenBank accession number AY458612. This assay was designed to amplify all *Cryptosporidium* spp. for which 18S DNA data were available. A BLAST analysis of GenBank indicated that the reverse primer would bind only when DNA from *Cryptosporidium* species was available as a template. GeneRunner version 3.05 (Hastings Software) was used to analyse the oligonucleotide primers and probes for the presence of secondary structure and to determine their thermal profiles. The final targeted sequences for the primers and probe were subjected to BLAST searches to ascertain their specificity for *Cryptosporidium* species sequences.

Species-specific TaqMan assays. A similar approach was used to design the primers and probes for the *C. hominis*- and *C. parvum*-specific TaqMan real-time PCR assays. These oligonucleotides were designed on the basis of GenBank accession number AF190627, which has been identified as being polymorphic, but with an undefined function (Widmer *et al.*, 2000). The oligonucleotide sequences for the *C. hominis* TaqMan assay were: 5'-ACTTTTGTGTTGTTTACG-CCG-3' (JVAGF forward primer), 5'-AATGTGGTAGTTGCGGTT-GAA-3' (JVAGR reverse primer) and 5'-FAM-ATTATTAATTTAT-CTCTTACTTCGT-BHQ-3' (JVAGP1 probe). The oligonucleotide sequences for the *C. parvum* TaqMan assay were: 5'-ACTTTTGTGTTGTTTACGCCG-3' (JVAGF forward primer), 5'-AATGTGGTAGTTGCGGTTGAA-3' (JVAGR reverse primer) and 5'-FAM-ATTATCTCTTCGTAGCGGCG-BHQ-3' (JVAGP2 probe). The JVAGP2 probe, which was designed for specific detection of *C. parvum*, had a GC content of 48 mol% and a T_m value of 63 °C. For the *C. parvum*-specific probe (JVAGP2), a non-conventional design approach was needed in order to obtain a sufficient T_m for this GC-poor template. The 3' end of the probe sequence (5'-CGGCG-3') was made complementary to the 3' end of the forward primer (5'-CGCCG-3') to raise its GC content. Because the forward primer had positive polarity and the probe was designed to have negative polarity, they should not compete for the same strand. The JVAGP1 probe had a GC content of 19 mol% and a T_m value of 53 °C based on the nearest-neighbour method, but it was demonstrated in thermal gradient real-time PCR tests that the probe could hybridize effectively over a range of 45–60 °C. All primers and probes reported in the present study were synthesized at the CDC Biotechnology Core Facility.

Real-time PCR conditions. Amplification of *Cryptosporidium* DNA was performed using an iCycler iQ4 Real-Time PCR Detection System (Bio-Rad) and an Mx 3000p system (Stratagene). For the duplex reaction (consisting of the 18S rRNA assay and *C. parvum* assay), both TaqMan probes (JVAP18S and JVAGP2) were used at a

final concentration of 100 nM each and the four primers were used at a final concentration of 250 nM each. For the separate *C. hominis* assay, the primers were used at the same concentration as in the duplex assay (250 nM), but the concentration of the TaqMan probe (JVAGP1) was 200 nM (twice the probe concentration used for the duplex assay). Additional $MgCl_2$ was added to a final concentration of 5 mM in the *C. hominis* assay reactions, to compensate for the low T_m of the probe. Real-time PCR amplifications were performed using the following conditions: denaturation at 95 °C for 2 min, followed by 45 cycles of denaturation at 94 °C for 10 s, annealing at 55 °C for 30 s and extension at 72 °C for 20 s. Each 20 µl reaction contained 10 µl 2× Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 5 µl DNA, and primers, probe(s) and $MgCl_2$ as described above.

Quantification and sensitivity analysis. The sensitivity of each TaqMan assay (18S rRNA, *C. hominis*-specific and *C. parvum*-specific) was determined using *Cryptosporidium*-negative stool specimens seeded with tenfold dilutions of either *C. parvum* or *C. hominis* from stocks quantified as described above. Quantitative PCR results were expressed as the number of *Cryptosporidium* oocysts per reaction. Standard curves were generated using DNA extracted from samples spiked with the equivalent of approximately 10^5 – 10^{-1} oocysts of each species. For the generation of standard curves, threshold cycle values were plotted proportionally to the logarithm of the input copy numbers. To assess the log-linear relationship of the assays, the linear regression coefficient (R^2) was calculated by the iCycler iQ4 software for each assay. In addition, the sensitivity of the dual TaqMan assay protocol for detecting mixed infections was investigated by adding tenfold dilutions of DNA from the stock of *C. parvum* oocysts to DNA extracts from two clinical samples known to contain *C. hominis*. The effective range of *C. parvum* in these mixed DNA samples was from one to 10^7 oocysts.

RESULTS AND DISCUSSION

Specificity of the real-time PCR assays

This study reports the development of a dual TaqMan assay procedure, consisting of a duplex assay targeting the 18S rRNA gene for sensitive detection of *Cryptosporidium* species and a chromosomal gene of unknown function to identify *C. parvum*, combined with a singleplex TaqMan assay to identify *C. hominis* based on the same gene used for identification of *C. parvum*. The species-specific assays employed the same primers, but two distinct TaqMan probes were used. A panel of ten stool DNA extracts, each representing a different *Cryptosporidium* species, was tested to evaluate the detection scope of the generic 18S rRNA TaqMan assay and the specificity of the *C. hominis* and *C. parvum* TaqMan assays (evaluated as singleplex assays). The 18S rRNA assay was able to detect all ten *Cryptosporidium* species tested (Table 1). The *C. parvum* TaqMan assay was found to amplify only *C. parvum* and *Cryptosporidium wrairi*, which is rarely detected in the environment and is not known to infect humans (Xiao *et al.*, 2004). The *C. hominis* TaqMan probe did not cross-react with any other species on the panel, including *C. wrairi*. The 18S rRNA and species-specific TaqMan assays were found not to cross-react with ten DNA samples that contained protozoan parasites other than *C. hominis* and *C. parvum* (Table 2).

Table 2. Validation testing results for *Cryptosporidium* species, *C. hominis* and *C. parvum* TaqMan PCR assays using a blind validation panel

Specimen	No. of positive specimens/total no. of specimens tested (%)				
	DFA assay	ML-2 assay	18S rRNA assay	<i>C. parvum</i> assay	<i>C. hominis</i> assay
<i>C. hominis</i>	26/31 (84)*	25/25 (100)	28/31 (90)*	0/31	24/31 (77)
<i>C. parvum</i>	21/23 (91)*	19/20 (95)	21/24 (88)*	21/24 (88)	0/24
Negative†	NA	NA	0/14	0/14	0/14

NA, Not analysed.

*For *Cryptosporidium* species.†Negative for *Cryptosporidium* species. Stool specimens contained parasites other than *Cryptosporidium* as follows: *Cyclospora cayetanensis* (n=2 isolates), *Entamoeba histolytica* (n=1), *Toxoplasma gondii* (n=1), *Enterocytozoon bienersi* (n=3), *Entamoeba dispar* (n=1), *Encephalitozoon cuniculi* (n=1), *Giardia intestinalis* (n=1), *Encephalitozoon hellem* (n=1), *Entamoeba invadens* (n=1) and *Encephalitozoon intestinalis* (n=2).

This is the first study to report the development of real-time PCR assays that can efficiently distinguish between *C. hominis* and *C. parvum* using TaqMan probes. Tanriverdi *et al.* (2002) reported a fluorescence resonance energy transfer (FRET) probe assay to distinguish between *C. hominis* and *C. parvum*, but the authors did not report sensitivity data and also indicated that the FRET probes did not perform well in resolving mixtures of *C. hominis* and *C. parvum*. Limor *et al.* (2002) also reported a FRET probe PCR assay that could discriminate between *C. hominis* and *C. parvum*, but the amplification efficiency of the assay was not sufficient to ensure sensitive detection of these two species. Tanriverdi *et al.* (2002, 2003) reported two different SYBR Green assays to distinguish between *C. hominis* and *C. parvum* based on melting curve analysis. Although effective in amplifying *C. hominis* and *C. parvum* DNA, these assays resulted in differentials of less than 0.5 °C in the melting peaks of the two species, which could limit their usefulness for definitive identification of *C. hominis* or *C. parvum* in clinical specimens. Nevertheless, comparative studies could be useful to ascertain the strength and weaknesses of the techniques mentioned above.

Sensitivity of the TaqMan assays

Standard curves developed using seeded stool specimens are shown in Fig. 1(a) for the 18S rRNA and *C. hominis* TaqMan assays and Fig. 1(b) for the 18S rRNA and *C. parvum* assays. The R^2 values for the standard curves were at least 0.99 for both assays over a quantification range of six orders of magnitude. The 18S rRNA assay could detect 1–10 *C. hominis* and *C. parvum* oocysts seeded into 300 µl stool specimens. Thus the lower limit of detection was 0.5 oocysts per reaction, which was a tenfold lower detection limit than the species-specific assays. This generic TaqMan assay targets the 18S rRNA gene, a multicopy gene (20 copies per oocyst) that offers a high degree of sensitivity. This level of sensitivity is similar to the detection limits reported by other researchers for detection of *Cryptosporidium* species, *C. parvum* or *C. hominis*

(Fontaine & Guillot, 2002; Guy *et al.*, 2003). As also shown in Fig. 1, the relative difference in the speed of detectable PCR product generation was ~6 threshold cycle values between the 18S rRNA and the species-specific assays. This difference in detection limits highlights an important design aspect of this TaqMan PCR procedure: a probe-based real-time PCR assay is used to target a gene present at high copy numbers for genus-level detection of *Cryptosporidium* species, whilst *C. hominis* and *C. parvum* identification is accomplished simultaneously by targeting

Fig. 1. Standard quantification curves for *Cryptosporidium* 18S rRNA and species-specific TaqMan assays for *C. hominis* (a) and *C. parvum* (b).

a different gene, present in lower copy numbers but having sufficient polymorphisms for species-level identification.

The sensitivity of the *C. hominis* and *C. parvum* assays for identifying a sample containing both species (as in an example of mixed infection) was also evaluated. Two *C. hominis* specimens containing approximately 10^3 oocysts per reaction were mixed with tenfold dilutions of *C. parvum* oocysts. When the *C. parvum* oocyst addition was in the order of 10^2 – 10^5 oocysts per reaction, the dual TaqMan assay protocol detected both *C. hominis* and *C. parvum*. When the *C. parvum* oocyst addition was in the order of 1–10 oocysts or 10^6 – 10^7 oocysts per reaction, the dual TaqMan assay protocol only detected the species that was more abundant in the mixed sample.

Validation of the TaqMan assays versus reference molecular and microscopy methods

Using a blind panel of 69 DNA extracts (containing 31 known *C. hominis* specimens, 24 known *C. parvum* specimens and 14 negative specimens), the 18S rRNA and species-specific TaqMan assays were found to produce false-negative (FN) rates that were similar to, or lower than, DFA microscopy and slightly higher than ML-2 PCR (Table 2). For the 31 known *C. hominis* specimens, the DFA and 18S rRNA TaqMan assays detected *Cryptosporidium* species in 26 (16 % FN) and 28 (10 % FN) specimens, respectively. The *C. hominis* TaqMan assay correctly identified the presence of *C. hominis* in 24 of the 31 known *C. hominis*-positive specimens (23 % FN). The *C. parvum* TaqMan probe did not cross-react with any of the *C. hominis* specimens. For the known *C. parvum* specimens, the DFA and 18S rRNA TaqMan assays detected *Cryptosporidium* species in 21/23 (9 % FN) and 21/24 (12 % FN) specimens, respectively. The *C. parvum* TaqMan assay correctly identified the presence of *C. parvum* in 21 of the 24 known *C. parvum* specimens (12 % FN). The *C. hominis* TaqMan probe did not cross-react with any of the *C. parvum* specimens.

Use of dual TaqMan assay protocol for epidemiological investigation of cryptosporidiosis

A total of 103 stool specimens from epidemiological investigations of cryptosporidiosis in the USA and Botswana were analysed by DFA microscopy, ML-2 PCR and our dual TaqMan PCR procedure to diagnose the presence of *Cryptosporidium* species. *Cryptosporidium* oocysts were observed in 51 (49 %) of the 103 specimens using DFA microscopy (Table 3). The 18S rRNA TaqMan PCR assay detected the presence of *Cryptosporidium* species DNA in 67 (65 %) of the 103 specimens, whilst the ML-2 assay was able to detect *Cryptosporidium* in 61 (59 %) of the specimens. *Cryptosporidium* was detected in 71 of the 103 specimens using either the DFA or ML-2 assay, indicating that the TaqMan procedure had a specificity of 94 % (67/71) for detecting *Cryptosporidium* in clinical specimens.

Table 3. Sensitivity of *Cryptosporidium* species detection using the DFA, ML-2 PCR and dual TaqMan PCR protocols for epidemiological investigations of cryptosporidiosis in the USA and Botswana

Specimen	Analytical results for assay method			
	DFA	ML-2	18S rRNA assay	All assays
<i>Cryptosporidium</i> species	51	61	67*	71
Negative†	52	42	36	32

**C. hominis* ($n=35$), *C. parvum* ($n=29$) or a mixture of *C. hominis* and *C. parvum* ($n=2$) was identified in 66/67 specimens using the species-specific TaqMan assays.

†Negative for *Cryptosporidium* species.

Three DFA-positive specimens were reported as *Cryptosporidium*-negative using the 18S rRNA TaqMan and ML-2 assays, but were positive by other PCR and 18S sequencing methods (data not shown). Nevertheless, the 18S rRNA TaqMan assay was able to detect *Cryptosporidium* in 16 specimens that were reported as negative by DFA assay and in six specimens that were reported as negative using the ML-2 assay. Of the 67 specimens for which the TaqMan procedure detected *Cryptosporidium* species, *C. hominis* and/or *C. parvum* were detected in 66 specimens: 35 contained *C. hominis*, 29 contained *C. parvum* and two contained both *C. hominis* and *C. parvum*.

A total of 24 stool specimens from the Botswana set were subjected to DNA sequencing analysis after amplification with COWP N-terminal PCR primers CRY9 and CRY15 (Spano *et al.*, 1997) to confirm the results obtained by the specific TaqMan assays. COWP-based sequencing analysis identified 12 specimens as containing *C. hominis*, 11 specimens as containing *C. parvum* and one specimen as coming from a case of mixed infection of *C. hominis* and *C. parvum*. The mixed infection was determined by sequencing analysis of cloned COWP N-terminal amplicons, as reported by Bandyopadhyay *et al.* (2007). The same identification results were achieved for the 24 stool specimens using our TaqMan procedure.

The results of the dual TaqMan assays for the stool specimens from the USA and Botswana showed that the real-time PCR procedure of the present study can provide accurate detection and identification of *C. hominis* and *C. parvum* in stool specimens collected for epidemiological studies. In addition to identifying *C. hominis* and *C. parvum* as the sole aetiological agents of cryptosporidiosis cases, the species-specific TaqMan assays also identified two cases of mixed infection, of which one was confirmed by COWP-based DNA sequencing analysis. The blind-panel validation of the dual TaqMan assay method showed that it has a detection sensitivity that is better than conventional microscopy and comparable to other

molecular methods used for confirmatory identification of *Cryptosporidium* species. These data and capabilities indicate that this method could be a simple and valuable tool for fast and reliable laboratory diagnosis of *C. hominis* and *C. parvum* cryptosporidiosis.

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