Real-time PCR detection and speciation of *Cryptosporidium* infection using Scorpion probes

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At least eight species of *Cryptosporidium* can cause human infection and disease. A real-time PCR (qPCR) assay based on the 18S rRNA gene and utilizing a Scorpion probe was developed to detect all human-pathogenic *Cryptosporidium* without the usual need for nested amplification. Sensitivity of detection in stool samples was highest using a glass bead-based DNA extraction method (under 10³ oocysts per stool sample). The assay was validated against 123 human stool specimens from Bangladesh and Tanzania, exhibited a sensitivity and specificity of >91 % versus microscopy, and detected an additional eight microscopy-negative infections. *Cryptosporidium parvum*-specific and *Cryptosporidium meleagridis*-specific Scorpion qPCR assays that provided 100 % accurate speciation compared with *VspI* RFLP analysis and sequencing were developed subsequently. These Scorpion probe qPCR assays are simpler to perform than existing nested PCR and RFLP methods for diagnosis and epidemiological investigation of cryptosporidiosis.

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INTRODUCTION

Since first appreciated as a protozoal pathogen in 1976, *Cryptosporidium* is now established as a major cause of diarrhoea in humans (Hlavsa *et al.*, 2005). *Cryptosporidium hominis* and *Cryptosporidium parvum* are the major species that have emerged from human studies. However, the avian species *Cryptosporidium meleagridis* has been observed frequently, e.g. it was observed at a rate of 12 % in a large study of Peruvian human immunodeficiency virus (HIV)-infected cryptosporidiosis patients (Cama *et al.*, 2003). Several additional zoonotic species have been reported infrequently, including *Cryptosporidium felis*, *Cryptosporidium canis*, *Cryptosporidium muris*, *Cryptosporidium suis* and a cervine genotype (Cama *et al.*, 2003; Gatei *et al.*, 2003; Ong *et al.*, 2002; Pedraza-Díaz *et al.*, 2001; Xiao *et al.*, 2001). Species data are important to aid outbreak investigation and provide information about

Cryptosporidium transmission cycles, and may prove clinically relevant (Houpt *et al.*, 2005; Hunter *et al.*, 2004; Okhuysen *et al.*, 1999).

We therefore sought to develop a *Cryptosporidium* assay that would be sensitive, provide species information and be simple to perform. The mainstay diagnostic modalities of microscopy or antigen detection do not discriminate species. Several PCR tests have been reported for *Cryptosporidium*; however, most require nested amplification if using faecal samples and an additional RFLP analysis step for speciation (Amar *et al.*, 2004; Coupe *et al.*, 2005; Gatei *et al.*, 2003; Higgins *et al.*, 2001). In this work, a real-time PCR (qPCR) assay is described that maintains reasonable sensitivity without nested amplification by targeting a relatively short fragment (108–125 bp) of the multicopy 18S rRNA gene. *C. parvum*- and *C. meleagridis*-specific qPCR assays were also developed; these assays were able to provide

accurate species information on clinical samples from Bangladesh and Tanzania.

METHODS

Parasites and parasite DNA. *C. parvum* oocysts and *C. hominis* strain TU502 oocysts (kindly provided by Saul Tzipori, Tufts University School of Veterinary Medicine, Grafton, USA) were stored at 4 °C in PBS, sedimented and counted in a haemocytometer before spiking into 200 mg aliquots of parasite-free stool. DNA of *C. meleagridis*, *C. felis* and *C. muris* was obtained from stool specimens from patients from Moshi, Tanzania or Vellore, India (species identity was confirmed by sequencing the amplified 18S rRNA product).

Human faecal specimens. Stool specimens were obtained from individuals with and without diarrhoea at the International Centre for Diarrhoeal Diseases and Research, Dhaka, Bangladesh ($n=70$), and the Kilimanjaro Christian Medical Centre, Moshi, Tanzania ($n=53$). Informed consent was obtained from all participants and the human experimentation guidelines of the US Department of Health and Human Services, the University of Virginia, the Centre for Health and Population Research (Bangladesh) and the Kilimanjaro Christian Medical Centre Research Ethics Committee were followed. Specimens were tested for *Cryptosporidium* infection by microscopy after modified acid-fast stain. Available specimens with disparate microscopy/PCR results were tested by antigen detection via ELISA (*Cryptosporidium* II kit; Techlab) according to the manufacturer's instructions.

DNA extraction. All DNA was extracted from experimentally spiked and human faecal samples using the QIAamp DNA Stool Mini kit (Qiagen) according to the manufacturer's instructions, except that the suspension was incubated in the kit's stool lysis buffer at 95 °C and a 3 min incubation with InhibitEx tablets was performed. On experimentally spiked specimens, four methods of stool preparation prior to Qiagen DNA extraction were tested. For the glass bead method, 200 mg acid-washed glass beads (0.5 mm; Sigma) was added to 200 mg stool in Qiagen ASL buffer and the samples were vortexed for 2–5 min. For sonication, stool samples underwent two 1 min sonication bursts on ice water. For the freeze–thaw method, stool samples were subjected to one or six freeze–thaw cycles in liquid nitrogen and a 95 °C water bath. For the proteinase K/SDS method, 200 µl of 200 µg proteinase K ml⁻¹ in 0.2% SDS in water was added to the stool sample, which was then incubated at 55 °C for 1 h. For the Tanzanian clinical samples, stool samples received six freeze–thaw cycles prior to Qiagen DNA extraction. For the Bangladeshi clinical samples, 1 g stool sample was concentrated by ether-PBS sedimentation followed by sonication and six cycles of freeze–thaw prior to Qiagen DNA extraction.

Oligonucleotides. Sequences of the 18S rRNA gene of *C. hominis* (AF093491), *C. parvum* (AF164102), *C. meleagridis* (AF112574), *C. canis* (AB210854), *C. felis* (AF112575), *C. muris* (X64343) and *C. suis* (AF108861) were obtained from the NCBI database and aligned using CLUSTAL X v1.8 (<http://www.ebi.ac.uk/clustalw/>). Initially, a PCR assay was designed to amplify all human-pathogenic *Cryptosporidium* species (forward 5'-GGTTGTATTATTAGATAA-AGAAC-3', reverse 5'-AGGCCAATACCCTACCGTCT-3' and internal probe 5'-GTGACATATCATTCAAGTTTCTGAC-3'). A *C. parvum*-specific assay was designed with a *C. parvum*/*C. hominis*-specific forward primer (5'-CTCGACTTTATGGAAGGGTTG-3'), a *C. parvum*-specific reverse primer (5'-CAGAACTTGAATGAT-ATGTCACATTTAA-3') and a *C. parvum*/*C. meleagridis*-specific internal probe (5'-GAACCAATATAATTGGTGACTC-3'). A *C. meleagridis*-specific assay was designed with a *C. meleagridis*-specific forward primer (5'-GCGAAAAAACCTGACTTAATGGAAA-3'), a *C. meleagridis*/*C. canis*-specific reverse primer (5'-GAAACTTGAAT-

GATATGTCACATA-3') and a common *Cryptosporidium* probe (5'-GGTTGTATTATTAGATAAAG-3'). Primers and probes demonstrated no deleterious secondary structures or significant identity to non-*Cryptosporidium* sequences in a BLAST search (<http://www.ncbi.nlm.nih.gov/blast/>). Three Scorpion Uni-probes (Proligo) were generated: one with the pan-*Cryptosporidium* assay's reverse primer linked to the probe; one with the *C. parvum*-specific assay's reverse primer linked to the probe; and one with the *C. meleagridis*-specific assay's forward primer linked to the probe. Scorpion Uni-probes incorporated a 5' reporter dye, a stem-loop sequence, a black-hole quencher and a hexethylene glycol reverse-extension blocker. Probe binding to the respective neosynthesized strands is indicated in Fig. 1.

PCR amplification, RFLP and sequencing. Amplification took place in a 25 µl volume containing 1 × PCR buffer (Qiagen), 8.0 mM total MgCl₂ (including that contained in the PCR buffer), 0.5 mM of each deoxynucleoside triphosphate, 1.25 U *Taq* polymerase (HotStarTaq; Qiagen), 2.5 µg BSA, 7.5 pmol Scorpion probe, 30 pmol primer and 5 µl faecally extracted DNA. Amplification was performed on an iCycler (Bio-Rad) under the following cycling conditions: 95 °C for 15 min; 40 cycles of 95 °C for 15 s, 51 °C for 15 s and 72 °C for 20 s; and then 75 °C for 10 min. RFLP analysis was performed by incubating 12 U *VspI* (Promega) per 10 µl PCR product at 37 °C for 2 h. Digested products were fractionated on 2.0% agarose gel and visualized by ethidium bromide (2 µg ml⁻¹). For sequencing, amplicons were purified using the QIAquick PCR Purification kit (Qiagen) and sequenced with the appropriate primers on an Applied Biosystems 377 Prism DNA Sequencer at the University of Virginia Biomolecular Research Facility.

Statistics. Proportions were compared by Fisher's exact test. All *P* values were two-tailed.

RESULTS AND DISCUSSION

Selection of oligonucleotides

The multicopy 18S rRNA gene was chosen for amplification given the desire to optimize detection sensitivity and based on findings by others that this gene could be successfully amplified from all human-pathogenic *Cryptosporidium* species (Jiang & Xiao, 2003). 18S rRNA sequences from the eight *Cryptosporidium* species that have been reported thus far to cause disease in humans were downloaded from GenBank. Primers and probes were designed to detect all *Cryptosporidium* species that spanned the widely used *VspI* *C. hominis* restriction site (Fig. 1, pan-*Cryptosporidium* assay). A *C. hominis*-specific Scorpion assay was developed, but exhibited suboptimal performance (see below). Therefore, *C. parvum*- and *C. meleagridis*-specific reactions were designed that maximized primer and probe polymorphisms against the other species (Fig. 1, right-hand column).

Sensitivity of the *Cryptosporidium* Scorpion probe qPCR assays

The sensitivity of the pan-*Cryptosporidium* assay was determined on parasite-free stool spiked with known quantities of purified *C. parvum* or *C. hominis* oocysts. Four methods of initial stool preparation were tested including glass bead homogenization, sonication, freeze–thaw cycles and treatment with proteinase K with SDS. DNA

pan-*Cryptosporidium* assay

Species	18S rRNA sequence	# mismatches (of primers/probe vs. sequence)
<i>C. hominis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	0
<i>C. parvum</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	0
<i>C. meleagridis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	0
<i>C. canis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	0
<i>C. felis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	1
<i>C. muris</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	1
<i>C. suis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	0
<i>C. hominis</i>	5'-GGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	
	-----TACACTGTATAGTAGTTTCAAGACTGGATAGTGAAGATCTGCCATCCCAATACCGGA	
	GTGACATATCATTCAAGTTTCTGAC	
	5'-HEX-CGCGC	
	G B C D G D C D G D BHQ	

C. parvum-specific assay

Species	18S rRNA sequence	# mismatches (of primers/probe vs. sequence)
<i>C. hominis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	3
<i>C. parvum</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	0
<i>C. meleagridis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	7
<i>C. canis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	9
<i>C. felis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	18
<i>C. muris</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	15
<i>C. suis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	10
<i>C. parvum</i>	5'-TCGACCTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTG	
	-----TCTTGGTATATATAA-----CACTGAGTATTTATGAAATGCCATGTGTAATT-----TACACTGTATAGTAGTCCAAAGAG	
	GAACCAATATAATT-----GGTGACTC	
	5'-FAM-CCCGC	
	G - C - G - G - G - BHQ	

C. meleagridis-specific assay

Species	18S rRNA sequence	# mismatches (of primers/probe vs. sequence)
<i>C. hominis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	5
<i>C. parvum</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	5
<i>C. meleagridis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	0
<i>C. canis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	2
<i>C. felis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	14
<i>C. muris</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	10
<i>C. suis</i>	GCAGAAAACTCGACTTTATGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----ATGTGACATATCATTCAAGTTTCTGACCTATCAGCTTTAGACGGTAGGGTATTGGCCT	4
<i>C. meleagridis</i>	BHQ - G - G - C - G	
	HEG	
	CCAACATAAATACTTATTTTC	
	3'-CGCTTTTTCGGAAGGGTGTATTTATTAGATAAAGAACCAATATAATT---GGTGACTCATAATACTTTACGGATCACAATTAA-----TACACTGTATAGTAGTCCAAAG	

Fig. 1. Primer and Scorpion probe sequences for pan-*Cryptosporidium*, *C. parvum*-specific and *C. meleagridis*-specific qPCR. 18S rRNA sequences for *C. hominis*, *C. parvum*, *C. meleagridis*, *C. canis*, *C. felis*, *C. muris* and *C. suis* were obtained from GenBank (nt 172–322 of AF093491, AF164102, AF112574 and AB210854, nt 173–333 of AF112575, nt 150–302 of X64343 and nt 172–325 of AF108861 are shown for the seven *Cryptosporidium* species, respectively) and aligned using CLUSTAL software. Primers (boxed) and probes (bold) were designed to detect all *Cryptosporidium* species, *C. parvum* or *C. meleagridis*. Sequence mismatches of primers or probes against other species are highlighted and shown in the column on the right. Probes were linked to forward or reverse primers via Scorpion Uni-probes as indicated. Separation of fluorophore (HEX, FAM or TEX) from the black-hole quencher (BHQ) during PCR synthesis is shown below sequences. HEG, Hexethylene glycol reverse-extension blocker. The *VspI* restriction site, AT↓TAAT (underlined in the uppermost *C. hominis* sequence), is exclusive to *C. hominis*.

Fig. 2. Sensitivity of the Scorpion probe pan-*Cryptosporidium* qPCR assay. (a) 10^5 *C. parvum* oocysts were spiked into aliquots of parasite-free stool, which were then homogenized via glass bead, freeze-thaw, sonication or proteinase K/SDS methods. DNA was extracted with the QIAamp DNA Stool Mini kit. Samples were then amplified with the pan-*Cryptosporidium* Scorpion qPCR assay. (b) Serial dilutions of *C. parvum* oocysts were spiked into parasite-free stool. DNA was prepared via the glass bead/Qiagen method and qPCR-amplified with the pan-*Cryptosporidium* assay. No fluorescence was observed with unspiked faecal samples or water controls (data not shown). The fluorescence threshold for each run was calculated by Bio-Rad iCycler software v3.0 and is shown as a horizontal line. ND, Not detected.

was then purified from all preparations using the commercial QIAamp DNA Stool Mini kit and subjected to qPCR. The lowest cycle threshold (C_T) was consistently observed on the glass bead-prepared samples or after one cycle of freeze-thaw (Fig. 2a). The lower limit of detection with this assay was 10^2 – 10^3 oocysts per 200 mg stool sample after 40 cycles of PCR (Fig. 2b). Sensitivity of the *C. parvum*- and *C. meleagridis*-specific Scorpion assays for detecting *C. parvum* and *C. meleagridis*, respectively, was similar to that of the pan-*Cryptosporidium* assay (data not shown).

Specificity of the *Cryptosporidium* Scorpion probe qPCR assays

Specificity of the assays was examined. For *C. hominis* and *C. parvum*, experimentally spiked specimens were used, but

sequence-confirmed faecal specimens were used for *C. meleagridis*, *C. felis* and *C. muris*. The pan-*Cryptosporidium* assay detected all samples (Fig. 3a), including *C. felis* and *C. muris*, despite their 1 bp sequence mismatches in the forward primer and probe, respectively (Fig. 1). In contrast, the *C. parvum*-specific assay detected only *C. parvum* (Fig. 3b) and the *C. meleagridis*-specific assay detected only *C. meleagridis* (Fig. 3c). Several *C. hominis*-specific Scorpion probes were attempted within this region; however, all exhibited poor sensitivity or false-positive detection of *C. parvum* (data not shown). Such difficulty has been reported previously due to the minimal nucleotide polymorphisms in the 18S rRNA sequence of *Cryptosporidium* species (Higgins *et al.*, 2001). Finally, multiplex PCR with all primers and probes could be performed, but this is not recommended due to diminished sensitivity versus singleplex reactions (e.g. *C. hominis* C_T changed from 32.8 to 35.1 and *C. meleagridis* C_T changed from 29.1 to 33.1).

Comparison of *Cryptosporidium* Scorpion probe qPCR with microscopy on clinical specimens

The pan-*Cryptosporidium* assay was validated on 123 banked stool DNA samples obtained during case-control studies in Bangladesh ($n=70$) and Tanzania ($n=53$) during the preceding 3 years. Sixty-three patients had diarrhoea (≥ 3 loose or watery stools over the previous 24 h), 60 did not and all 53 Tanzanian patients were HIV-infected. Fresh specimens were tested by microscopy after acid-fast staining and then stored at -70°C . DNA was then extracted via a freeze-thaw/Qiagen method (note that sufficient quantities were not available for re-extraction via the more sensitive glass bead method). The qPCR assay exhibited a sensitivity of 92% (95% confidence interval of 78–98%) and a specificity of 91% (95% confidence interval of 83–96%) versus microscopy. Specifically, of 36 microscopy-positive specimens, 33 were qPCR-positive; of 87 microscopy-negative specimens, 79 were qPCR-negative. For specimens with inconsistent qPCR/microscopy results, available samples were retested by *Cryptosporidium* ELISA; results showed that all three microscopy-positive/qPCR-negative specimens were antigen-negative and six of the eight microscopy-negative/qPCR-positive specimens were antigen-positive. Overall, therefore, the qPCR sensitivity appeared to exceed that of microscopic analysis and that reported for traditional PCR assays for *Cryptosporidium* (Kostrzynska *et al.*, 1999), perhaps due to the short (~ 125 bp) target sequence, which increases PCR efficiency. Sensitivity of PCR in faecal specimens has historically been a limitation for *Cryptosporidium* such that nested amplification has frequently been used (Bialek *et al.*, 2002; Morgan *et al.*, 1998). Whereas the sensitivity of the present assay would be predicted to improve with nested amplification, we do not recommend this because of the risks of contamination and increased reagent cost of nested PCR, but rather favour future use of the glass bead method for DNA extraction (Fig. 2).

Fig. 3. Specificity of the *C. parvum*- and *C. meleagridis*-specific Scorpion probe qPCR assays. DNA was extracted from parasite-free stool spiked with 10^6 *C. hominis* or 10^6 *C. parvum* oocysts, as well as from human faecal specimens infected with *C. meleagridis*, *C. felis* and *C. muris* (confirmed by sequencing of the 18S rRNA gene). DNA samples were qPCR-amplified using (a) the pan-*Cryptosporidium* assay, (b) the *C. parvum*-specific assay and (c) the *C. meleagridis*-specific assay. The fluorescence threshold for each run was calculated by Bio-Rad iCycler software v3.0 and is shown as a horizontal line. ND, Not detected.

Speciation using Scorpion probes

The accuracy of the *C. parvum* and *C. meleagridis* assays for species identification compared with *VspI* RFLP digestion analysis was examined. First, each of the 82 pan-*Cryptosporidium* qPCR-negative specimens was tested by the *C. parvum*- and *C. meleagridis*-specific qPCR assays and no false positives were observed (Table 1). Upon testing each of the 41 pan-*Cryptosporidium* qPCR-positive specimens with the specific qPCR assays, 26 specimens were *C. parvum* qPCR-negative/*C. meleagridis* qPCR-negative, 12 were *C. parvum* qPCR-positive/*C. meleagridis* qPCR-negative, two were *C. parvum* qPCR-positive/*C. meleagridis* qPCR-positive and one was *C. parvum* qPCR-negative/*C. meleagridis* qPCR-positive. Each amplicon from the pan-*Cryptosporidium* qPCR, *C. parvum*-specific qPCR and *C. meleagridis*-specific qPCR assays (total = 58) was then analysed by *VspI* RFLP digestion to confirm species identity and identify *C. hominis* infections (the only species with the internal *VspI* site; Fig. 1). This analysis revealed no *VspI* digestion of any *C. parvum*-specific or *C. meleagridis*-specific qPCR product. Furthermore, for the two *C. parvum*

qPCR-positive/*C. meleagridis* qPCR-positive specimens, both the *C. parvum* qPCR and *C. meleagridis* qPCR products were sequenced and the presence of both species was confirmed. Finally, two of the 26 pan-*Cryptosporidium* qPCR-positive/*C. parvum* qPCR-negative/*C. meleagridis* qPCR-negative specimens that were not digested with *VspI* were sequenced and were found to be *C. felis* infections (the first *C. felis* infections reported from Bangladesh). Overall, therefore, the *C. parvum*- and *C. meleagridis*-specific qPCR assays were 100 % accurate compared with *VspI* RFLP and sequencing analysis. By combining pan-*Cryptosporidium* and *C. parvum*-specific results, *C. hominis* infection could be reasonably inferred from a pan-*Cryptosporidium* qPCR-positive/*C. parvum* qPCR-negative result (24 of 27 such samples were confirmed as *C. hominis* by RFLP analysis).

Interestingly, six of 14 *C. parvum* infections were mixed infections compared with only four of 28 *C. hominis* infections ($P=0.06$). Mixed *C. hominis*/*C. parvum* infections have been clearly documented in up to 4–12 % of cases in diverse regions including the United States, Uganda and

Table 1. Speciation using *C. parvum*- and *C. meleagridis*-specific qPCR assays

Pan- <i>Cryptosporidium</i> qPCR result	<i>n</i>	<i>C. parvum</i> qPCR result	<i>C. meleagridis</i> qPCR result	RFLP and/or sequencing result
–	82	–	–	Not applicable
+	26	–	–	<i>C. hominis</i> (24), <i>C. felis</i> (2)*
+	12	+	–	<i>C. parvum</i> (12)†
+	2	+	+	Mixed <i>C. parvum</i> / <i>C. meleagridis</i> (2)*
+	1	–	+	<i>C. meleagridis</i> (1)

*The presence of *C. felis* and mixed *C. parvum*/*C. meleagridis* infection was confirmed by sequencing.

†Four of these 12 *C. parvum* infections were found to be mixed *C. parvum*/*C. hominis* infections, as shown by *VspI* digestion of the pan-*Cryptosporidium* qPCR amplicon without *VspI* digestion of the *C. parvum*-specific qPCR amplicon.

Peru (Cama *et al.*, 2003; Tumwine *et al.*, 2005). Some have reported that this burden may be underestimated since standard PCR-RFLP assays for *Cryptosporidium* are not very sensitive for detection of the minor subtype (Reed *et al.*, 2002). The significance of human infection with particular *Cryptosporidium* species, mixed or not, remains to be elucidated on many levels. It is hoped that these assays can serve as a tool towards that end.

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REFERENCES

- Amar, C. F., Dear, P. H. & McLauchlin, J. (2004). Detection and identification by real time PCR/RFLP analyses of *Cryptosporidium* species from human faeces. *Lett Appl Microbiol* **38**, 217–222.
- Bialek, R., Binder, N., Dietz, K., Joachim, A., Knobloch, J. & Zelck, U. E. (2002). Comparison of fluorescence, antigen and PCR assays to detect *Cryptosporidium parvum* in fecal specimens. *Diagn Microbiol Infect Dis* **43**, 283–288.
- Cama, V. A., Bern, C., Sulaiman, I. M. & 7 other authors (2003). *Cryptosporidium* species and genotypes in HIV-positive patients in Lima, Peru. *J Eukaryot Microbiol* **50** (Suppl.), 531–533.
- Coupe, S., Sarfati, C., Hamane, S. & Derouin, F. (2005). Detection of *Cryptosporidium* and identification to the species level by nested PCR and restriction fragment length polymorphism. *J Clin Microbiol* **43**, 1017–1023.
- Gatei, W., Greensill, J., Ashford, R. W., Cuevas, L. E., Parry, C. M., Cunliffe, N. A., Beeching, N. J. & Hart, C. A. (2003). Molecular analysis of the 18S rRNA gene of *Cryptosporidium* parasites from patients with or without human immunodeficiency virus infections living in Kenya, Malawi, Brazil, the United Kingdom, and Vietnam. *J Clin Microbiol* **41**, 1458–1462.
- Higgins, J. A., Fayer, R., Trout, J. M., Xiao, L., Lal, A. A., Kerby, S. & Jenkins, M. C. (2001). Real-time PCR for the detection of *Cryptosporidium parvum*. *J Microbiol Methods* **47**, 323–337.
- Hlavsa, M. C., Watson, J. C. & Beach, M. J. (2005). Cryptosporidiosis surveillance – United States 1999–2002. *MMWR Surveill Summ* **54**, 1–8.
- Houpt, E. R., Bushen, O. Y., Sam, N. E. & 8 other authors (2005). Short report: asymptomatic *Cryptosporidium hominis* infection among human immunodeficiency virus-infected patients in Tanzania. *Am J Trop Med Hyg* **73**, 520–522.
- Hunter, P. R., Hughes, S., Woodhouse, S., Raj, N., Syed, Q., Chalmers, R. M., Verlander, N. Q. & Goodacre, J. (2004). Health sequelae of human cryptosporidiosis in immunocompetent patients. *Clin Infect Dis* **39**, 504–510.
- Jiang, J. & Xiao, L. (2003). An evaluation of molecular diagnostic tools for the detection and differentiation of human-pathogenic *Cryptosporidium* spp. *J Eukaryot Microbiol* **50** (Suppl.), 542–547.
- Kostrzynska, M., Sankey, M., Haack, E. & 8 other authors (1999). Three sample preparation protocols for polymerase chain reaction based detection of *Cryptosporidium parvum* in environmental samples. *J Microbiol Methods* **35**, 65–71.
- Morgan, U. M., Pallant, L., Dwyer, B. W., Forbes, D. A., Rich, G. & Thompson, R. C. (1998). Comparison of PCR and microscopy for detection of *Cryptosporidium parvum* in human fecal specimens: clinical trial. *J Clin Microbiol* **36**, 995–998.
- Okhuysen, P. C., Chappell, C. L., Crabb, J. H., Sterling, C. R. & DuPont, H. L. (1999). Virulence of three distinct *Cryptosporidium parvum* isolates for healthy adults. *J Infect Dis* **180**, 1275–1281.
- Ong, C. S., Eisler, D. L., Alikhani, A., Fung, V. W., Tomblin, J., Bowie, W. R. & Isaac-Renton, J. L. (2002). Novel *Cryptosporidium* genotypes in sporadic cryptosporidiosis cases: first report of human infections with a cervine genotype. *Emerg Infect Dis* **8**, 263–268.
- Pedraza-Diaz, S., Amar, C., Iversen, A. M., Stanley, P. J. & McLauchlin, J. (2001). Unusual *Cryptosporidium* species recovered from human faeces: first description of *Cryptosporidium felis* and *Cryptosporidium* ‘dog type’ from patients in England. *J Med Microbiol* **50**, 293–296.
- Reed, C., Sturbaum, G. D., Hoover, P. J. & Sterling, C. R. (2002). *Cryptosporidium parvum* mixed genotypes detected by PCR-restriction fragment length polymorphism analysis. *Appl Environ Microbiol* **68**, 427–429.
- Tumwine, J. K., Kekitiinwa, A., Bakeera-Kitaka, S., Ndeezi, G., Downing, R., Feng, X., Akiyoshi, D. E. & Tzipori, S. (2005). Cryptosporidiosis and microsporidiosis in Ugandan children with persistent diarrhea with and without concurrent infection with the human immunodeficiency virus. *Am J Trop Med Hyg* **73**, 921–925.
- Xiao, L., Bern, C., Limor, J., Sulaiman, I., Roberts, J., Checkley, W., Cabrera, L., Gilman, R. H. & Lal, A. A. (2001). Identification of 5 types of *Cryptosporidium* parasites in children in Lima, Peru. *J Infect Dis* **183**, 492–497.