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SHORT COMMUNICATION

Detection and characterization of *Cryptosporidium cuniculus* by real-time PCR

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Abstract *Cryptosporidium cuniculus* was originally detected in rabbits and has been identified as an emerging human pathogen, but the occurrence, prevalence, and epidemiology in human and rabbit populations are poorly understood. As identification of *C. cuniculus* can be time-consuming and costly using existing molecular assays, a real-time polymerase chain reaction (PCR)-based method targeting specific markers for this species was developed. The assay is based on amplification of the *C. cuniculus*-specific 60-kDa glycoprotein (GP60) gene using two PCRs targeting subtype families Va and Vb. PCR product formation was monitored by SYBR Green I fluorescence measurement followed by post-amplification melt curve analysis; high resolution melt curve analysis was found to give increased sensitivity over standard melt curve analysis. The real-time PCR correctly identified all 41 *C. cuniculus* isolates (40 from humans, one from a rabbit) tested, with subtype family in agreement with GP60 gene sequencing. Specificity was demonstrated by lack of detection of nine other *Cryptosporidium* species and genotypes, including 88 isolates of the closely related species, *Cryptosporidium hominis*. The PCRs were performed in separate tubes to maximize the possibility of detecting mixed Va–Vb infections; however, none were detected. The potential for multiplexing the reactions was also demonstrated, furthering the utility of the assay for large-scale occurrence and prevalence studies.

Introduction

Cryptosporidium cuniculus (syn. *Cryptosporidium* rabbit genotype) has recently been redescribed as a species after publication of a full morphological, molecular, and infectivity data (Robinson et al. 2010). It was originally detected in rabbits but is now an emerging human pathogen, having been identified recently as the etiological agent in a waterborne outbreak in England (Chalmers et al. 2009a). It was the third most commonly identified *Cryptosporidium* species after *Cryptosporidium parvum* and *Cryptosporidium hominis* in sporadic cases of cryptosporidiosis in the UK in 2007 and 2008 (Chalmers et al. 2011). *C. cuniculus* is most closely related to *C. hominis*, but can be differentiated by analysis of the small subunit (SSU) rRNA, 70-kDa heat shock protein, or actin genes (Robinson et al. 2010). This involves costly and time-consuming DNA sequence analysis or restriction fragment length polymorphism (RFLP) analysis with extended gel electrophoresis times (Robinson et al. 2008). Typing assays based on other targets, such as the *Cryptosporidium* oocyst wall protein (COWP) gene and Lib13 locus, fail to differentiate *C. cuniculus* from *C. hominis* (Robinson et al. 2010).

The only known hosts for *C. cuniculus* are humans and rabbits, but only a limited number of studies of the occurrence or prevalence in these populations have been reported (Robinson et al. 2010; Chalmers et al. 2011; Nolan et al. 2010; Shi et al. 2010). These studies used RFLP or sequencing of SSU rRNA gene nested polymerase chain reaction (PCR) products to identify species and nested PCR followed by sequencing of the GP60 gene to subtype. To facilitate further studies to improve understanding of *C. cuniculus* as a zoonotic cause of human disease, there is a need for a rapid, cost-effective assay to specifically detect the species in fecal samples. The use of real-time PCR to detect pathogens reduces costs and running times mainly due to the elimination of the requirement for running products on electrophoresis gels. The technique has been used to specifically detect important *Cryptosporidium* spp. in vulnerable hosts, for example, *C. parvum* in cattle as reported by Homem et al. (2011).

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The *Cryptosporidium* 60-kDa surface glycoprotein gene (GP60) is highly variable, forming several discrete subtype families based on overall sequence identity; each family also contains multiple subtypes based on variations in a serine microsatellite region (Alves et al. 2003). Sequence analysis of the GP60 gene from *C. cuniculus* isolated from humans in UK and from 14 (nine wild, five farmed) rabbit populations in four countries has identified only two subtype families, Va and Vb, which are distinct from those found in other *Cryptosporidium* species (Chalmers et al. 2009a; Chalmers et al. 2011; Nolan et al. 2010; Shi et al. 2010). Here, we describe an assay for detection and characterization of *C. cuniculus* based on real-time PCR of these species-specific GP60 gene subtype families.

Materials and methods

Samples used for assay development and evaluation

To develop the assay and evaluate analytical specificity, an evaluation panel of 97 *Cryptosporidium* and non-*Cryptosporidium* DNA samples was tested. This included 93 archived *Cryptosporidium* DNA samples from the ongoing national collection of oocysts, and related investigations, held at the Cryptosporidium Reference Unit (CRU), originally characterized by PCR–RFLP of the COWP and SSU rRNA genes and confirmed by SSU rRNA sequencing (Chalmers et al. 2009a; Chalmers et al. 2009b). Of these, 38 were *C. hominis*, nine *C. parvum*, one each of *Cryptosporidium andersoni*, *Cryptosporidium baileyi*, *Cryptosporidium felis*, *Cryptosporidium meleagridis*, *Cryptosporidium muris*, *Cryptosporidium ubiquitum* and *Cryptosporidium* horse genotype, and 39 were *C. cuniculus*. The *C. cuniculus*, *C. parvum* and a subset (due to financial constraints) of the *C. hominis* samples were also PCR-amplified and sequenced at the GP60 gene as described by Alves et al. (2003), with nomenclature as proposed previously (Chalmers et al. 2009a; Sulaiman et al. 2005; Cama et al. 2007). DNA from four other genera including human and closely related protozoa was also analyzed.

C. cuniculus-specific real-time PCR development

To design PCR primers specific for *C. cuniculus* GP60 Va and Vb, all contemporary GP60 Va and Vb sequences available from GenBank (National Center for Biotechnology Information, http://www.ncbi.nlm.nih.gov/GenBank/) were aligned using the ClustalW algorithm (Thompson et al. 1994) of the BioEdit software, version 7.0.9.0 (http://www.mbio.ncsu.edu/BioEdit/bioedit.html). NetPrimer (Premier Biosoft, Palo Alto, CA, USA) was used to calculate primer melting temperatures and check for undesirable inter- and intramolecular binding. Primer and probe sequences were then checked for cross-reactions with nontarget sequences on the GenBank database using the basic local alignment search tool (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The primer pairs GP60Va2F (5′-AGTGGAAACACTACTCAAACTAGTGATAG-3′)-GP60Va2R (5′-ATCGAACCACATTACAAATGAAGTAC-3) and GP60Vb2F (5′-GCTGATTCCCAAACTTCTGCTC-3′)-GP60Vb2R (5′-CATAGACCATAGTATAGTCACCACA CTC-3′) were designed to amplify 135nt (nt 223–357 on GenBank accession number FJ262732) and 157nt (nt 262–418 on FJ262734) of the intra-subtype family-conserved regions downstream of the microsatellite regions within subtype families Va and Vb, respectively, without amplifying DNA from other *Cryptosporidium* species or other genera. Reactions (25 μl) contained 12.5 μl ABsolute QPCR SYBR Green Mix (Thermo Scientific, Epsom, UK), 0.4 μM each primer, and 2 μl DNA. The Va and Vb PCRs were performed in separate tubes except when multiplexing was investigated. Amplification and detection was done on a high resolution melt (HRM)-capable Rotorgene 6000 (Corbett Research, Cambridge, UK) using the following conditions: 95°C for 15 min followed by 40 cycles of 95°C for 45 s, 55°C for 45 s, and 72°C for 30 s.

Amplification products were detected by SYBR Green I fluorescence measurement. Threshold cycle (C_T) was calculated from amplification plots using a standardized threshold value. Standard melt curve analysis (MCA) was performed over the temperature range 55–90°C with 1°C increments. HRM was carried out over the range 70–90°C with 0.1°C increments. For both standard MCA and HRM, derivative melt curves were produced by plotting the negative derivative of fluorescence with respect to temperature (−dF/dT) against temperature. A sample was considered positive if a distinct peak was visible with its apex between 82 and 85°C for standard MCA and 80 and 83°C for HRM.

The potential to multiplex the reactions was investigated by amplification of a small subset of six *C. cuniculus* samples (four Va and two Vb) from the evaluation panel in reactions containing both sets of primers using the thermocycler conditions described above, followed by examination of normalized (for pre- and post-melt fluorescence) HRM plots.

Application of the assay for routine detection of *C. cuniculus*

To investigate use of the assay for analyzing human clinical samples, 52 *Cryptosporidium*-positive stool samples received between January 1 and February 28, 2009 for the CRU national genotyping service that gave *C. hominis* profiles by routine COWP PCR–RFLP were retested for *C. cuniculus* by both real-time PCR and extended gel run nested SSU rRNA PCR–RFLP which differentiates *C. cuniculus* from *C. hominis* (Chalmers et al. 2009a). GP60 sequencing was performed to confirm *C. cuniculus*-positive samples, as described above.

Results and discussion

Detection of *C. cuniculus* by the real-time PCR was initially evaluated using the panel of 97 target and nontarget samples. Amplification curves were observed not only for all *C. cuniculus*-positive samples (Va mean C_T=24.7, range 17.6–29.9; Vb mean C_T=23.2, range 19.3–26.0) but also for nontarget samples (Va mean C_T=32.6, range 26.7–36.6; Vb mean C_T=28.4, range 23.6–31.9), including negative controls, due to formation of nonspecific amplification products including primer dimers. MCA was therefore required to identify true positives. Standard MCA and HRM were compared by testing

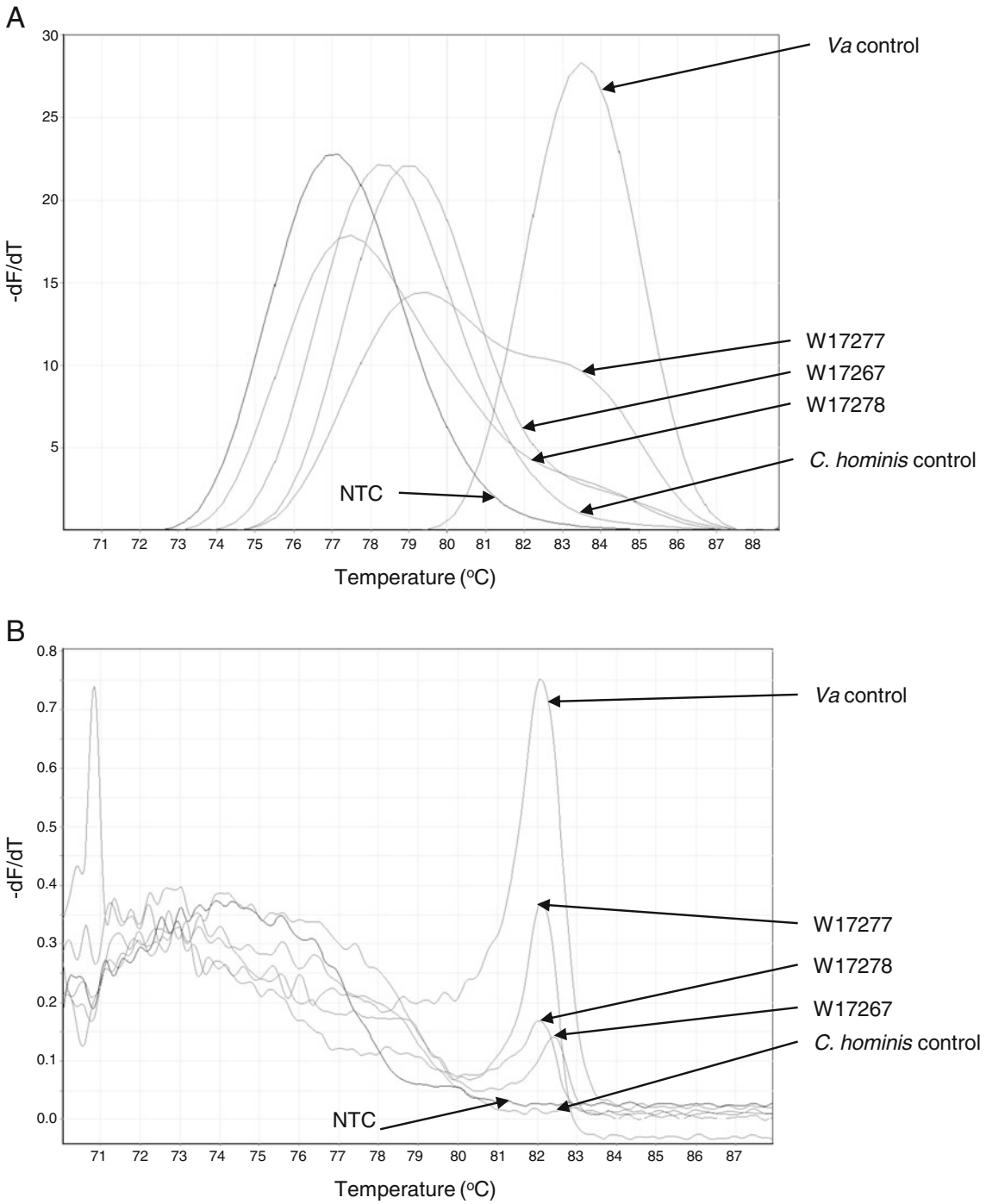

Fig. 1 Derivative melt curves for **A** standard melt curve analysis and **B** high resolution melt curve analysis (HRM) of *C. cuniculus* GP60 subtype family Va PCR products showing typical positive (*Va control*) and negative (no template control, *NTC*) reactions and improved detection of amplicons by HRM for samples *W17267*, *W17277*, and *W17278*. *T* temperature (in degree Celsius); *F* fluorescence units

a subset of 33 *C. cuniculus* samples (27 Va, 6 Vb). Of these, 30/33 (90 %) were positive by standard MCA and three gave poorly defined Va melt curves (Fig. 1). In contrast, all 33 (100 %) were positive by HRM which was therefore performed on all further samples.

Using the combination of subtype family-specific PCR and HRM, all of the *C. cuniculus* isolates and none of the *C. hominis*, *C. parvum*, *C. andersoni, C. baileyi, C. felis, C. meleagridis, C. muris, C. ubiquitum* and horse genotype or non-*Cryptosporidium* spp. in the evaluation panel were

Table 1 Analysis of 97 *Cryptosporidium* and non-*Cryptosporidium* DNA by nested GP60 gene PCR-sequence analysis and real-time GP60 Va–Vb PCR

DNA source	GP60 allele by sequencing (number of samples)	Real-time GP60 Va–Vb PCR (number of samples)
C. cuniculus	VaA18 (26)	Va (26)
	VaA19 (2)	Va (2)
	VaA22 (1)	Va (1)
	VaA23 (1)	Va (1)
	VbA20 (1)	Vb (1)
	VbA22 (1)	Vb (1)
	VbA25 (1)	Vb (1)
	VbA30 (1)	Vb (1)
	VbA32 (1)	Vb (1)
	VbA34 (2)	Vb (2)
	VbA36 (1)	Vb (1)
	Neg (1)	Va (1)
DNA of nontarget *Cryptosporidium* spp./genotypes and other genera		
C. hominis	IaA15R3 (1)	Neg (1)
	IaA18 (1)	Neg (1)
	IaA25R3 (1)	Neg (1)
	IbA9G3 (1)	Neg (1)
	IbA10G2 (2)	Neg (2)
	IdA15G1 (1)	Neg (1)
	IdA16 (1)	Neg (1)
	IeA11G3T3 (2)	Neg (2)
	IfA12G1 (2)	Neg (2)
	IgA24 (1)	Neg (1)
	Not done (25)	Neg (25)
C. parvum	IIaA15G2R1 (3)	Neg (3)
	IIaA17G1 (1)	Neg (1)
	IIaA19G2R1 (2)	Neg (2)
	IIbA15G2 (1)	Neg (1)
	IIcA5G3 (1)	Neg (1)
	IIdA17G1 (1)	Neg (1)
C. andersoni	Not done	Neg (1)
C. baileyi	Not done	Neg (1)
C. felis	Not done	Neg (1)
C. meleagridis	Not done	Neg (1)
C. muris	Not done	Neg (1)
C. ubiquitum	Not done	Neg (1)
Cryptosporidium horse genotype	VIbA13b[a]	Neg (1)
Cyclospora cayetanensis	Not done	Neg (1)
Eimeria tenella	Not done	Neg (1)
Toxoplasma gondii	Not done	Neg (1)
Homo sapiens	Not done	Neg (1)

Neg PCR negative

[a]Human isolate W6863 (Robinson et al. 2008) identified as a new GP60 subtype (GenBank accession number EU437419)

detected, indicating high analytical specificity (Table 1). HRM peaks ranged from 81.5 to 82.4°C for Va and from 81.0 to 81.6°C for Vb. The overlapping of these ranges was not problematic due to the Va and Vb reactions being carried out in separate tubes.

When the GP60 subtype family data obtained by sequencing were compared with the real-time PCR results for the 39 *C. cuniculus* samples in the evaluation panel, 38 samples amplified with the conventional PCR GP60 primers and 39 in the real-time assay. There was 100 % agreement between results for the samples amplified by both assays (Table 1). The real-time assay detected Va and Vb subtype families across a range of subtypes which included four subtypes among 30 members of the Va subtype family and seven subtypes among eight Vb isolates. No mixed Va–Vb infections were identified. One additional Va isolate was identified by real-time PCR, for which no conventional GP60 amplicon had been obtained, most likely due to low concentration of DNA. This sample gave a low HRM peak, a relatively high threshold cycle of 29.9, and a very faint SSU rRNA PCR–RFLP pattern, suggesting a similar level of sensitivity for the real-time GP60 and nested SSU rRNA PCRs. The sensitivity of the real-time assay improved on that of the nested GP60 PCR, probably due to the shorter amplicon resulting in higher reaction efficiencies.

Multiplexing of the two reactions was investigated using a small number of *C. cuniculus*-positive samples. Examination of normalized HRM plots allowed successful differentiation of four Va and two Vb samples (Fig. 2) showing the potential for using the assay in a multiplex format which would reduce consumable costs and setup time. In the present study, however, separate reactions were performed to allow identification of mixed Va–Vb isolates without the potential for competition that exists for multiplex PCR.

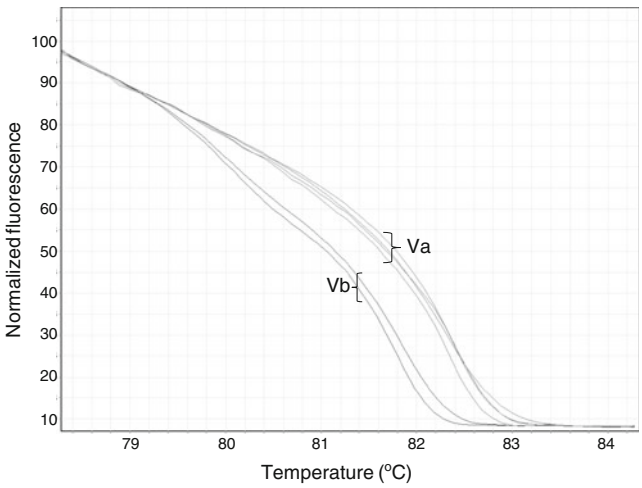

Fig. 2 Normalized high-resolution melt curve analysis plots obtained for *C. cuniculus* GP60 subtype family Va- and Vb-positive samples using multiplexed Va and Vb real-time PCRs

The results of retesting 52 human *C. hominis*-positive stools received at the CRU between January 1 and February 28, 2009 by the real-time assay and extended gel nested SSU rRNA PCR–RFLP showed 100 % agreement: the same two isolates were identified as *C. cuniculus*; the remainder were negative by real-time PCR and were *C. hominis* by SSU rRNA PCR–RFLP. The *C. cuniculus* isolates were confirmed by SSU rRNA gene sequencing and one identified as Va and one Vb by real-time PCR; the subtypes were VaA21 and VbA25 by nested GP60 PCR-sequencing.

No mixed Va–Vb infections were detected in any of the *C. cuniculus*-positive samples identified and no mixed infections have been reported previously in rabbits or humans, suggesting that mixed *C. cuniculus* GP60 subtype infections are rare. Reports of mixed GP60 subtype infections in general are rare, but most studies have been performed using broad-range GP60 PCRs followed by sequencing, making identification of mixed infections difficult due to competition. This study suggests that, as mixed infections are rare, use of the multiplexed format of the assay would be applicable.

In summary, we describe a rapid, cost-effective, sensitive, and specific assay for detection and characterization of the emerging human pathogen *C. cuniculus* based on real-time PCR of the GP60 Va and Vb subtype families. Compared with extended gel run nested SSU rRNA PCR–RFLP, turnaround times are reduced from ~9.5 h (~1.5 h hands-on) to ~3.5 h (~0.5 h hands-on) and consumable costs reduced by ~80 %. While it is possible that further subtype families of this highly variable gene may be circulating, the identification to date of only the Va and Vb subtype families in *C. cuniculus* by studies performed on three different continents (Robinson et al. 2010; Chalmers et al. 2011; Nolan et al. 2010; Shi et al. 2010) suggests the potential of these loci for the detection of the species. If other subtype families are discovered subsequently, these can be incorporated in future versions of the assay.

Although characterization to the subtype family level, as described here, lacks the resolution of full subtyping by sequence analysis, it is a more cost-effective and time-efficient screening tool for large-scale studies. Subtype family may also prove to be as (if not more) useful for epidemiological studies, as subtype family has been shown to relate to clinical symptoms in *C. hominis* and *C. parvum* infections (Cama et al. 2008; Iqbal et al. 2011). Moreover, *C. cuniculus* subtype family has been shown to be associated with patient sex and time of year (Chalmers et al. 2011). Larger studies are required to provide further insights into the epidemiology of this emerging pathogen, and the real-time PCR assay described is a useful tool for use in large-scale prevalence and epidemiological studies.

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