Detection and differentiation of *Cryptosporidium* by real-time polymerase chain reaction in stool samples from patients in Rio de Janeiro, Brazil

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This study reports the first genetic characterisation of Cryptosporidium *isolates in Brazil using real-time polymerase chain reaction (RT-PCR). A total of 1,197 faecal specimens from children and 10 specimens from human immunodeficiency virus-infected patients were collected between 1999-2010 and screened using microscopy. Forty-eight* Cryptosporidium *oocyst-positive isolates were identified and analysed using a generic TaqMan assay targeting the 18S rRNA to detect* Cryptosporidium *species and two other TaqMan assays to identify* Cryptosporidium hominis *and* Cryptosporidium parvum. *The 18S rRNA assay detected* Cryptosporidium *species in all 48 of the stool specimens. The* C. parvum *TaqMan assay correctly identified five/48 stool samples, while 37/48 stool specimens were correctly amplified in the* C. hominis *TaqMan assay. The results obtained in this study support previous findings showing that* C. hominis *infections are more prevalent than* C. parvum *infections in Brazil and they demonstrate that the TaqMan RT-PCR procedure is a simple, fast and valuable tool for the detection and differentiation of* Cryptosporidium *species.*

Key words: *Cryptosporidium* - RT-PCR - molecular epidemiology

Species of the genus *Cryptosporidium* are recognised as important enteropathogens of immunocompetent and immunocompromised vertebrate hosts worldwide (Xiao & Fayer 2008). To date, more than 20 species of *Cryptosporidium* have been recognised and at least eight species (*Cryptosporidium hominis*, *Cryptosporidium parvum*, *Cryptosporidium meleagridis*, *Cryptosporidium felis, Cryptosporidium canis*, *Cryptosporidium suis*, *Cryptosporidium muris* and *Cryptosporidium andersoni*) have been detected in humans (Chalmers & Davies 2010).

Because the conventional methods for detecting *Cryptosporidium* oocysts cannot identify *Cryptosporidium* at the species level, molecular tools have been developed to detect and differentiate *Cryptosporidium* at the species/genotype and subtype levels (Xiao & Ryan 2004, Fayer 2010). Since the description of the first polymerase chain reaction (PCR)-based tool for differentiating between *C. hominis* and *C. parvum* (Morgan et al. 1995), other genotyping tools, including real-time PCR (RT-PCR), restriction fragment length polymorphism, microarray, melting curve analysis, single-strand conformation polymorphism analysis, random amplified polymorphic DNA PCR and DNA sequencing have been used in the characterisation of *Cryptosporidium* epidemiology (Xiao & Ryan 2008, Pangasa et al. 2009). Although tools based on the SSU rRNA region are the most widely used, other genetic targets (e.g., *Cryptosporidium* oocyst wall protein, 70-kDa heat shock protein HSP70, thrombospondin-related adhesive protein of *Cryptosporidium*-1, dihydrofolate reductase and actin) can also detect and differentiate between *Cryptosporidium* species (Spano et al. 1998, Sulaiman et al. 2002, Xiao et al. 2002, Jiang & Xiao 2003, Xiao 2010). Some recent studies have described a RT-PCR approach to detect and identify *Cryptosporidium* species (Higgins et al. 2001, Fontaine & Guillot 2002, Limor et al. 2002, McDonald et al. 2002, Guy et al. 2003, Keegan et al. 2003, Jothikumar et al. 2008, Alonso et al. 2011).

In Brazil, there are several studies focusing on the occurrence of *Cryptosporidium*, but the molecular characterisation of the isolates is found in only few of these studies (Gonçalves et al. 2006, Bushen et al. 2007, Huber et al. 2007, Araújo et al. 2008).

In this study, we used a RT-PCR TaqMan procedure to detect *Cryptosporidium* species and to differentiate between *C. hominis* and *C. parvum* in stool specimens from children and human immunodeficiency virus (HIV)-infected patients from the state of Rio de Janeiro, Brazil.

SUBJECTS, MATERIALS AND METHODS

Faecal sample collection - A total of 1,197 faecal specimens from children and 10 specimens from HIV-infected patients (total n = 1,207) were screened for intestinal parasite infections using a centrifuge-sedimentation technique. Briefly, 3 mL of diethyl ether was added to the washed faecal samples (10 mL) and the samples were

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vortexed for 30 s and then centrifuged at 2,500 rpm for 2 min (CELM, LS-3 plus). Both the fat layer and the supernatant were discarded and the pellet was suspended in 50 mL of grade water and centrifuged at 2,500 rpm for 2 min. This washing procedure was repeated twice and the pellet was suspended in high purity grade water to a final volume of 5 mL.

These stool samples were collected between 1999-2010 from a public day care and two different public hospitals in the city of Rio de Janeiro. For *Cryptosporidium* oocyst identification, stool samples were subjected to a modified Kinyoun acid-fast staining technique (Ma & Soave 1983). After identification, all samples were stored at 4ºC until molecular characterisation. This study was conducted with the approval of the Ethical Review Committee for Research, Faculty of Medicine, Rio de Janeiro State University.

DNA extraction - The extraction was based on a previously described protocol (Huber et al. 2007). Briefly, 200 µL of faecal sample, 500 µL of DNAzol 1 (Life Technologies, Carlsbad, CA), 0.5% (final concentration) of polyvinylpyrrolidone (Sigma-Aldrich Corp, St. Louis, MO) and approximately 0.2 g of 425-600 mm diameter glass beads (Sigma) were added to a 1.5-µL Eppendorf microtube. The microtubes were vortexed three times and incubated at 96ºC for 60 min. After centrifugation, the supernatant was transferred to another microtube, precipitated with 1 mL of pure ethanol and centrifuged again. The supernatant was discarded and the pellet was washed twice with 500 µL of ethanol (95%). After precipitation, the DNA was purified using a QIAamp® DNA Stool Kit (QIAGEN, Hilden, Germany) according to the manufacturer's protocol and was stored at -20ºC until further PCR reactions could be performed.

TaqMan PCR assays - primers and probes - The RT-PCR procedure combined a duplex reaction for the detection of *Cryptosporidium* species and *C. parvum* and a simple reaction for the detection of *C. hominis*, as described previously (Jothikumar et al. 2008). To detect *Cryptosporidium* species, we used a TaqMan probe (JVAP18S), 5'-Cy5-CGC-GCCTGCTGCCTTCCTTAGATG-BHQ-3', targeting the 18S rRNA. The sequence of the forward primer (JVAF) was 5'-AT-GACGGGTAACGGGGAAT-3' and the sequence of the reverse primer (JVAR) was 5'-CCAATTACAAAACCAAAAAGTCC-3'. The oligonucleotide sequences for the *C. parvum* TaqMan assay were 5'-ACTTTTTGTTTGTTTTACGCCG-3' (JVAGF forward primer), 5'-AATGTGG-TAGTTGCGGTTGAA-3' (JVAGR reverse primer) and 5'-FAM-ATTTATCTCT-TCGTAGCGGCG-BHQ-3' (JVAGP2 probe). The oligonucleotide sequences for the *C. hominis* TaqMan assay were 5'-ACTTTTTGT-TTGTTTTACGCCG-3'- (JVAGF forward primer), 5'-ATGTGGTAGTTGCGGTTGA-A-3' (JVAGR reverse primer) and 5'-FAM-ATTTATTAATT-TATCTCTT-ACTTCGT-BHQ-3' (JVAGP1 probe).

TaqMan PCR assays - conditions - The PCR assays were performed with a 7500 System thermocycler (Life Technologies, Carlsbad, CA). Each 20 µL duplex reaction (to identify *Cryptosporidium* species and *C. parvum*) contained 10 µL 2X Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 100 nM of each probe (JVAP 18S and JVAGP2), 250 nM of each primer (JVAF, JVAR, JVAGF and JVAGR) and 5 µL of DNA. For the *C. hominis* assay, each 20 µL reaction contained 10 µL 2X Platinum Quantitative PCR SuperMix-UDG (Invitrogen), 250 nM of each primer (JVAF, JVAR, JVAGF and JVAGR), 5 mM $MgCl_2$, twice the probe concentration used for the duplex assay (200 nM) and 5 µL of DNA. The *Cryptosporidium* PCR cycling conditions consisted of denaturation at 95ºC for 2 min followed by 45 cycles of denaturation at 94ºC for 10 s, annealing at 55ºC for 30 s and extension at 72ºC for 20 s. All assays included positive controls (*C. hominis* and *C. parvum*) and negative controls (DNA extracted from faecal samples negative for any parasites).

RESULTS

A total of 48 faecal samples (38 from children and 10 from HIV-infected patients) were positive for *Cryptosporidium* oocysts using the modified Kinyoun acid-fast staining technique. These samples were subjected to RT-PCR assays. The results of the dual TaqMan PCR procedure for the stool specimens from the children and the HIV-infected patients are shown in Table. For the 48 positive faecal sample isolates, the 18S rRNA TaqMan assay detected *Cryptosporidium* species in all 48 specimens. Five samples (1 from the HIV-infected group and 4 from children) were amplified in the *C. parvum*

TABLE

Differentiation of *Cryptosporidium* species by using polymerase chain reaction TaqMan procedure applied to faecal samples from children and human immunodeficiency virus (HIV) patients at the city of Rio de Janeiro, Brazil

	Cryptosporidium species			
Group ofstool samples	*Cryptosporidium* sp.	*Cryptosporidium hominis*	*Cryptosporidium parvum*	Total
Children	6[a]	28	4	38
HIV-infected patients	-	9	1	10
Total	6[a]	37	5	48

a: positive only in the 18S rRNA assay.

assay. The other nine samples from HIV-infected patients and 28 samples from children were amplified in the *C. hominis* assay. Six specimens previously determined to be *Cryptosporidium*-positive were reported as *Cryptosporidium*-negative using PCR and 18S sequencing (data not shown), but were determined to be positive using the 18S rRNA TaqMan assay. Neither *C. hominis* nor *C. parvum* were identified as being present in these six specimens. The *C. hominis* TaqMan probe did not cross-react with any of the *C. parvum* controls and the *C. parvum* TaqMan probe did not cross-react with the *C. hominis* controls or the negative controls.

DISCUSSION

This study reports a dual TaqMan assay procedure for the epidemiological investigation of cryptosporidiosis and is the first to report on the application of a RT-PCR method for detecting and differentiating between *Cryptosporidium* species in Brazil.

A total of 48 specimens were analysed. The 18S rRNA TaqMan PCR assay was able to detect the presence of *Cryptosporidium* DNA in all 48 specimens. *C. hominis* and *C. parvum* were detected in 37 and five DNA specimens, respectively, indicating the absence of mixed infections (samples containing both species). The same 18S rRNA TaqMan PCR assay protocol also detected the presence of *Cryptosporidium* DNA in 67 of 103 specimens (65%) in an epidemiological investigation of cryptosporidiosis in USA and Botswana (Jothikumar et al. 2008). Our results showed that the TaqMan PCR assay can detect *Cryptosporidium* DNA efficiently and allowed for the differentiation of *C. hominis* and *C. parvum* at the species level. However, six samples were amplified only by the 18S rRNA TaqMan assay, indicating that other *Cryptosporidium* species involved in human cryptosporidiosis could be present in these samples. In this case, other molecular tools can be used to detect different *Cryptosporidium* species that are less prevalent in human infections.

Recent studies using RT-PCR procedures have reported molecular characterisations of *Cryptosporidium* spp and have differentiated between *C. hominis* and *C. parvum* (Higgins et al. 2001, McDonald et al. 2002, Guy et al. 2003). These authors described a RT-PCR assay for the quantification of *Cryptosporidium*, but the primer-probe set they developed was not able to differentiate between *Cryptosporidium* species. Alonso et al. (2011) evaluated the performance of a locked nucleic acid (LNA) TaqMan probe in a RT-PCR assay to quantify *Cryptosporidium* and *Giardia*. This RT-PCR assay was able to detect *C. hominis*, *C. parvum*, *C. meleagridis* and *Cryptosporidium wrairi,* but was not able to discriminate among these species. Tanriverdi et al. (2002) described a fluorescence resonance energy transfer (FRET) probe assay for differentiating between *C. hominis* and *C. parvum*, but the sensitivity of the test was not reported. Limor et al. (2002) also reported a FRET probe PCR assay, but the probe-primer set they used did not efficiently differentiate between *C. hominis* and *C. parvum*.

The results of our study show that *C. hominis* infections are more common than *C. parvum* infections in the studied region. With some exceptions, *C. hominis* is the predominant species in humans across the world (Gatei et al. 2006, 2007, 2008, Cama et al. 2007, Hung et al. 2007). In Brazil, the predominance of *C. hominis* over *C. parvum* is also known. Araújo et al. (2008) identified eight *C. hominis*, four *C. parvum* and two *C. meleagridis* species in isolates obtained from stool samples from HIV-infected patients and immunocompetent children in the state of São Paulo (SP). Another study conducted in SP revealed the presence of *C. hominis* in all 29 stool samples analysed during an outbreak at a day care centre (Gonçalves et al. 2006). A third study performed in the municipality of São Paulo (Lucca et al. 2009) reported that among 27 isolates from HIV infected patients, 17 were *C. hominis* (63%), four were *C. parvum* (14,8%), five were *C. felis* (18,5%) and one was *C. canis* (3,7%). Additionally, in Northeast Brazil, Bushen et al. (2007) reported the predominance of *C. hominis* (57.1%) over *C. parvum* (42.9%) among 42 isolates from children.

Recent molecular epidemiological studies have improved the knowledge of human cryptosporidiosis. *C. parvum* and *C. hominis* are associated with most food-borne, waterborne and direct contact-associated (i.e., person-to-person and animal-to-person) outbreaks of cryptosporidiosis (Xiao & Ryan 2008). Some studies in Brazil have demonstrated that the anthroponotic cycle of cryptosporidiosis appears to be of more relevance than the zoonotic cycle (Gonçalves et al. 2006, Sevá et al. 2010). Our findings also suggest the predominance of the anthroponotic cycle in the studied area, but new genotyping studies are necessary to understand the transmission dynamics of human cryptosporidiosis in our region.

RT quantitative PCR methodology has been used to develop high-throughput screening assays for a variety of applications. The results of the dual TaqMan assays for the stool specimens from Brazil reported in this study demonstrated that the RT-PCR procedure targeting the 18S rRNA (a multicopy gene) was able to detect the presence of *Cryptosporidium* species and is a valuable tool for rapid differentiation between *C. hominis* and *C. parvum* in stool specimens collected for epidemiological investigations.

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