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 C. 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 (Goñalves 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 4°C 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 96°C 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 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 -20°C 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'-ACTTTTTGT-TTGGTACTACCGCG-3' (JVAF forward primer), 5'-ATGTTGCTAGTCCGTTGA-A-3' (JVAGR reverse primer) and 5'-FAM-ATTATCTCT-TCTACTGCGG-BHQ-3' (JVAGP2 probe). The oligonucleotide sequences for the *C. hominis* TaqMan assay were 5'-ACTTTTTGT-TTGGTACTACCGCG-3' (JVAF forward primer), 5'-ATGTTGCTAGTCCGTTGA-A-3' (JVAGR reverse primer) and 5'-FAM-ATTATCTCT-TCTACTGCGG-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 MgCl₂, 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 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. 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>
<thead>
<tr>
<th>Cryptosporidium species</th>
<th>Group of stool samples</th>
<th>Cryptosporidium sp.</th>
<th>Cryptosporidium hominis</th>
<th>Cryptosporidium parvum</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Children</td>
<td>6*</td>
<td>28</td>
<td>4</td>
<td>38</td>
<td></td>
</tr>
<tr>
<td>HIV-infected patients</td>
<td>-</td>
<td>9</td>
<td>1</td>
<td>10</td>
<td></td>
</tr>
<tr>
<td>Total</td>
<td>6*</td>
<td>37</td>
<td>5</td>
<td>48</td>
<td></td>
</tr>
</tbody>
</table>

*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. meleagris 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. Limer 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 foodborne, 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 anthropontic 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 anthropontic 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.

**REFERENCES**


