**Development and Evaluation of a Genus-Specific, Probe-Based, Internal-Process-Controlled Real-Time PCR Assay for Sensitive and Specific Detection of Blastocystis spp.**

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**Blastocystis** is a common intestinal parasite of unsettled clinical significance, which is not easily detected by standard parasitological methods. The genus comprises at least 13 subtypes (STs) (which likely represent separate species), 9 of which have been found in humans. Recent data indicate that at least one of the subtypes is associated with intestinal disease. A quantitative TaqMan 5′ nuclease real-time PCR (TaqMan PCR) including an internal process control (IPC) was developed for the detection of **Blastocystis** and shown to be applicable to genomic DNAs extracted directly from feces. The assay enabled successful amplification of DNAs from all relevant subtypes within the genus (ST1 to ST9). For assay evaluation, 153 samples previously tested by xenic in vitro culture (XIVC) were screened by the TaqMan assay. A total of 49/51 samples positive by XIVC and 13/102 samples negative by XIVC were positive by the TaqMan assay; samples positive by the TaqMan assay and negative by XIVC were subsequently tested by conventional PCR, and amplicons could be identified to the subtype level by sequencing in 69% of the cases. Compared to the TaqMan assay, XIVC had a sensitivity of 79%. This is the first time that a genus-specific, probe-based, internal-process-controlled real-time PCR assay for the detection **Blastocystis** has been introduced.

The incentive for the application of real-time PCR-based screening platforms in diagnostic parasitology is strong (33). Such assays are advantageous in many ways, primarily due to high specificity and sensitivity and the facts that real-time PCRs are operated in a closed-tube system with minimal risk of contamination and that a cutoff can be set to automatically distinguish positive from negative samples, thus eliminating subjective bias. Only two real-time PCR assays for **Blastocystis** have been published so far. One targeted an unknown gene and was shown to enable amplification of DNAs from ST1, ST3, and ST4 (11); it is unknown whether the assay enables the detection of **Blastocystis** strains belonging to other subtypes, and since the gene target is unknown, it is impossible theoretically to determine specificity and sensitivity based on gene copy numbers. Another assay was reported by Poirier et al. (22) and was designed as a genus-specific PCR targeting the SSU rRNA gene, enabling amplification of DNAs from **Blastocystis** strains belonging to all subtypes so far identified in humans. However, the amplicon was 339 bp long, and generally, significantly shorter amplicons are wanted in diagnostic PCRs to increase sensitivity. Moreover, the assay was based on SYBR green detection of double-stranded DNA and had only 95% specificity. Neither of these two assays included an internal amplification control; for diagnostic PCR assays, testing for potential PCR inhibition in fecal DNA samples that are PCR negative is essential.

The aim of the present study was to design and evaluate a genus-specific TaqMan assay for **Blastocystis** with an internal amplification control. (This work was carried out as part of a B.Sc. project performed by Umran Nisar Ahmed [Technical University of Denmark]).

**MATERIALS AND METHODS**

**Primer Design.** Complete SSU rDNA sequences of **Blastocystis** sp. ST1 to ST10, other **Blastocystis** species, and species of taxonomic and differential diagnostic relevance, namely, *Proteromonas lacertae*, *Candida albicans*, and *Saccharomyces cerevisiae*, were aligned (Fig. 1) using MegAlign in DNASTAR (DNASTAR, Madison, WI) and MultAlin (2), and target sequences for genus-specific primer and probes were identified and de-
Alignment of Blastocystis-specific oligonucleotides (forward, probe, and reverse) and SSU DNAs from Blastocystis sp. ST1 to ST10, other Blastocystis spp., Proteromonas lacertae, Candida albicans, and Saccharomyces cerevisiae. Polymorphic bases are highlighted in gray. Dashes indicate missing or nonexisting bases.

<table>
<thead>
<tr>
<th>Primer or probe</th>
<th>Sequence (5'-3')</th>
<th>Nucleotide position</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Primers for TaqMan assay</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Blastocystis FWD 5</td>
<td>GGTCCCGTAGAACATTTTGATT</td>
<td>1641–1663 in AY244621 sequence&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
<tr>
<td>Blastocystis R 2</td>
<td>CCTAGGGAAACCTGTACACCTCA</td>
<td>1734–1759 in AY244621 sequence&lt;sup&gt;b&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

| **Primers for construction of IPC** | | |
| Blastocystis FWD 5 IPC | GGTCCCGTAGAACATTTTGATT | 13918 in U39284 phage lambda sequence |
| Blastocystis REV 2 IPC | CCTAGGGAAACCTGTACACCTCA | 14061 in U39284 phage lambda sequence |

| **Probes** | | |
| Blastocystis probe | FAM-TCTGTTAAATCTCACCATTAGAGGA-MGBNFQ | 1705–1730 in AY244621<sup>b</sup> sequence |
| IPC probe | TAMRA-TCCCTCGGTATACGGACGGTTCCTTGAGG-BHQ2 | 14011 in phage lambda sequence |

<sup>a</sup> Boldface corresponds to the phage lambda sequence (GenBank accession number J02459). MGBFQ, minor groove binder and nonfluorescent quencher; and BHQ2, Black Hole Quencher 2 (nonfluorescent quencher).

<sup>b</sup> Sequence may exhibit polymorphism compared to oligonucleotide.
The assay was specificity tested against panel dilutions of fungal DNAs from Candida albicans (ATCC 6548), Candida glabrata (ATCC 90030), Candida parapsilosis (ATCC 22019), Candida tropicalis (UKNEQAS 0527), Candida krusei (ATCC 6258), Geotrichum candidum (UKNEQAS 1911), and Saccharomyces cerevisiae (ATCC 8258). DNAs from the following bacterial ATCC strains were also used for specificity testing: Bacillus cereus (ATCC 14579), Bacillus subtilis (ATCC 6633), Campylobacter coli (ATCC 33559), Enterobacter cloacae (ATCC 13047), Escherichia coli (ATCC 25922), and Proteus mirabilis (ATCC 12453). Tested DNAs from non-ATCC bacterial strains represented Aeromonas caviae, Bacteroides fragilis, Campylobacter jejuni, Campylobacter upsaliensis, Citrobacter freundii, Clostridium difficile, Clostridium perfringens, Clostridium sordelli, Hafnia alvei, Klebsiella pneumoniae, Listeria monocytogenes, Plesiomonas shigelloides, Pseudomonas aeruginosa, Salmonella enteritidis, Salmonella paratyphi, Serratia marcescens, Shigella dysenteriae, Shigella flexneri, Staphylococcus aureus, Staphylococcus pyogenes, Vibrio cholerae serotype Ogawa, Vibrio para-haemolyticus, and Yersinia enterocolitica.

DNAs used for diagnostic validation of the real-time PCR assay represented 51 samples positive and 102 samples negative for Blastocystis by XIVC from Danish patients submitting stools for parasitological analysis. Culture analyses had been carried out as previously described using Jones’ medium supplemented with 10% horse serum (30, 38). Of the 102 XIVC-negative samples, 42 were positive for Dientamoeba fragilis, 1 for Cryptosporidium, 1 for Entamoeba dispar, and 1 for Entamoeba histolytica. Tested DNAs from Giardia lamblia, Cryptosporidium, Entamoeba histolytica, E. dispar. and D. fragilis (33), and the prevalence of intestinal parasites among the XIVC-positive samples was comparable.

Genomic DNAs from aliquots of fecal samples tested by XIVC were extracted from fresh fecal samples using the NucliSENS easyMAG protocol (bioMérieux, Herlev, Denmark) according to the recommendation of the manufacturer.

In order to validate results obtained by real-time PCR, samples positive by the TaqMan assay and negative by XIVC were subjected to conventional PCR assays using primers described by Scicluna et al. (25). All products were sequenced unidirectionally.

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The obtained nucleotide sequences were assigned to subtypes by using BLAST against the Blastocystis database available at www.pubmlst.org/blastocystis (10, 28).

Cohen’s kappa index and comparison of means. Means and medians of cycle threshold (C<sub>T</sub>) values were calculated and a two-tailed Student’s t test for comparison of means carried out using software available at http://qudata.com/online/statcalc/ and http://studentstest.com/. Cohen’s kappa index for intertest agreement was calculated (http://olmosantonio.com/diagnostics/kappa/online/calculator.html).

RESULTS

The TaqMan assay allowed amplification of all subtypes included in the study, and no amplification of fungal or bacterial DNA was detected. DNA from 25,000 parasites per reaction was detected at a C<sub>T</sub> value of 21.89/21.90 in duplicate determinations, and reproducible C<sub>T</sub> values were obtained down to a 10<sup>-4</sup> dilution, which is equivalent to template DNA from 2.5 parasites per reaction (Table 2). DNA from a lower number of parasites was detectable; however, since the number of SSU rRNA gene copies per cell is not known, the absolute detection level of the PCR cannot currently be ascertained.

Forty-nine samples were positive by both XIVC and the Taq-Man assay (Table 3), with C<sub>T</sub> values ranging from 14.03 to 39.52, a mean C<sub>T</sub> value of 20.48 (standard deviation [SD], 5.85) and a median C<sub>T</sub> value of 18.76 (interquartile range [IQR], 17.23 to 21.46). Thirteen samples negative by XIVC were positive by the Taq-Man assay (Table 3), with C<sub>T</sub> values ranging from 16.25 to 40.26, a mean C<sub>T</sub> value of 28.93 (SD, 4.99) and a median C<sub>T</sub> value of 29.33 (IQR, 26.02 to 31.89). A comparison of the two means gave a P value of 0.00067, which means that samples negative by XIVC and positive by the TaqMan assay were generally characterized by having a smaller amount of Blastocystis-specific DNA than that present in samples positive by both methods.

The sensitivity and specificity of XIVC compared to the Taq-Man assay were 79% and 98%, respectively. Cohen’s kappa index was 0.79, indicating substantial intertest agreement.

The 13 samples positive by real-time PCR and negative by XIVC were tested by conventional PCR and sequencing. Sequencing of the 10 samples positive by both methods resulted in amplification of 9/13 samples by conventional PCR (ST1, 3 samples; ST2, 1 sample; and ST3, 5 samples); the mean C<sub>T</sub> value for the 4 samples not amplifiable by conventional PCR was 33.48.

In total, unambiguous sequences were obtained in 56/62 real-time PCR-positive cases. ST1 was seen in 21 cases, ST2 in 14, ST3 in 16, and ST4 in 5. The mean C<sub>T</sub> values (SDs) for individual subtypes were 19.33 (3.64) for ST1, 19.76 (6.80) for ST2, 23.52 (6.03) for ST3, and 24.36 (9.06) for ST4. Samples positive for ST1 had lower C<sub>T</sub> values than samples positive for ST3 (P = 0.022).

The two samples positive by XIVC and negative by real-time PCR tested negative by the conventional PCR after repeated ef-

### TABLE 2 Cycle threshold values for the 10-fold dilution row of DNA from 1 million Blastocystis organisms/200 μl elution buffer

<table>
<thead>
<tr>
<th>Probe</th>
<th>1 × 10&lt;sup&gt;9&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-1&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-2&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-3&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-4&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-5&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-6&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-7&lt;/sup&gt;</th>
<th>1 × 10&lt;sup&gt;-8&lt;/sup&gt;</th>
</tr>
</thead>
<tbody>
<tr>
<td>Blasto</td>
<td>21.90/21.89</td>
<td>25.15/25.22</td>
<td>28.67/28.67</td>
<td>31.04/30.97</td>
<td>34.84/35.11</td>
<td>36.82/40.91</td>
<td>39.21/38.12</td>
<td>40.93/UD</td>
<td>UD/UD</td>
</tr>
<tr>
<td>IPC</td>
<td>UD/UD</td>
<td>UD/UD</td>
<td>UD/47.26</td>
<td>36.56/38.64</td>
<td>35.17/35.11</td>
<td>35.18/35.70</td>
<td>36.76/36.03</td>
<td>36.70/34.95</td>
<td>35.98/37.15</td>
</tr>
</tbody>
</table>

<sup>a</sup> DNAs were tested in duplicates to test for reproducibility. UD, undetermined (i.e., signal absent).

### TABLE 3 Comparison of test results for real-time PCR and XIVC and distribution of Blastocystis subtypes

<table>
<thead>
<tr>
<th>Test results</th>
<th>No. of samples with ST:</th>
<th>—&lt;sup&gt;a&lt;/sup&gt;</th>
<th>Total</th>
</tr>
</thead>
<tbody>
<tr>
<td>Real-time PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>negative</td>
<td>XIVC negative</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>XIVC positive</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>Real-time PCR</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>positive</td>
<td>XIVC negative</td>
<td>3</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>XIVC positive</td>
<td>18</td>
<td>13</td>
</tr>
<tr>
<td></td>
<td>Total</td>
<td>21</td>
<td>14</td>
</tr>
</tbody>
</table>

<sup>a</sup> —, not applicable.
forts with multiple DNA dilutions. Results from IPC analysis showed that inhibition in these two samples was not an issue.

**DISCUSSION**

Accurate diagnostic tools are of vital significance in clinical and epidemiological studies of Blastocystis. So far, PCR has been used mostly for characterization purposes (1, 3–6, 12–14, 16–21, 23–27, 29, 31, 32, 35, 37, 40–42), although a few diagnostic PCRs have been published, two of which are based on real-time PCR technology (11, 22).

A major challenge in the development of genus-specific Blastocystis PCRs is the genetic diversity seen within the genus, which limits the number of potential targets in the SSU rRNA gene. The pairwise genetic distance of Blastocystis subtypes amounts to at least 14.8% across the SSU rRNA gene (29), and some conserved regions are likely to be conserved in other genera as well, which hampers identification of oligonucleotide target regions.

Compared to previously published real-time PCR assays (11, 22), the present one has the advantage of probe-based detection, which increases assay specificity. Based on confirmatory sequencing, the TaqMan assay did not produce any false positives, and this is probably due to the fact that primers and probe sequences were highly specific. The real-time PCR assay developed by Poirier et al. (22) generated 8/186 false positives and had a specificity of 95%. Specificity testing of previously published diagnostic PCRs has included testing against other intestinal parasites, such as Entamoeba, Dientamoeba, Giardia, and Cryptosporidium (22), or even bacteria (11), but it is also highly relevant to evaluate the assay against a panel of fungi such as Candida, Geotrichum, and Saccharomyces, which are common components of the fecal flora (9, 15) and which differ from Blastocystis by only about 20% at the SSU rDNA level.

A previous comparison between XIVC and conventional PCR (amplifying 550 bp) revealed a nonsignificant difference in sensitivity in favor of PCR (30). Although the sensitivity of the TaqMan assay is higher than that of the XIVC, it is not immediately comparable because the specificity is not as high. The TaqMan assay by Poirier et al. (22) generated 8/186 false positives and had a sensitivity of 95%.

Specificity testing of previously published diagnostic PCRs has included testing against other intestinal parasites, such as Entamoeba, Dientamoeba, Giardia, and Cryptosporidium (22), or even bacteria (11), but it is also highly relevant to evaluate the assay against a panel of fungi such as Candida, Geotrichum, and Saccharomyces, which are common components of the fecal flora (9, 15) and which differ from Blastocystis by only about 20% at the SSU rDNA level.

A previous comparison between XIVC and conventional PCR (amplifying 550 bp) revealed a nonsignificant difference in sensitivity in favor of PCR (30). Although the sensitivity of the TaqMan assay is higher than that of the XIVC, it is not immediately comparable to the data presented by Poirier et al. (22), who found that the sensitivity of XIVC was only 53% compared to their SYBR green assay. Importantly, Poirier et al. (22) used Jones’ medium supplemented with antibiotics (100 IU/ml penicillin and 100 μg/ml streptomycin), while we used Jones’ medium without adding antibiotics. It is not unlikely that these antibiotics will indirectly suppress the growth of Blastocystis by a reduction of bacteria, despite the fact that anaerobic chambers were used.

Samples with \( C_T \) values of ≤35 are most likely indicative of active, ongoing infestation. \( C_T \) values of >35 possibly represent samples with relatively few Blastocystis organisms, and it could be speculated that there is no active Blastocystis infection going on in the patients from whom those samples came. It is possible that these patients had been exposed to nonviable Blastocystis (detectable by real-time PCR but not by XIVC) or that they were clearing an infection. \( C_T \) values of >40 may primarily reflect unspecific amplification of a target present in the DNA samples that is of non-Blastocystis origin.

Whether parasite intensity is linked to clinical outcome of Blastocystis infections remains unclear. It is known that Blastocystis shedding exhibits day-to-day variation (39). The present data obtained by real-time PCR analysis confirmed that Blastocystis-positive fecal samples exhibit a range in \( C_T \) values from 12 to 40. Such a span of \( C_T \) values likely reflects vast differences in relative parasite load. Using real-time PCR, Poirier et al. (22) did not find any correlation between high intensity and symptoms, but the study was limited with regard to sample size. In the present study, all samples were from patients submitting stools for parasitological analysis due to travel-associated or persistent diarrhea. Future studies should aim to investigate whether differences in symptoms and the severity of these are associated with differences in \( C_T \) values. If low \( C_T \) values are associated with diarrhea and/or other symptoms, epidemiological cutoff values could be determined and used in the clinical management of Blastocystis-positive patients.

The overall subtype distribution reflected the usual subtype distribution seen in Danish cohorts (23, 30, 32, 35, 37, 38) and indicates that assay detection is independent of subtype. ST1 samples had lower \( C_T \) values than ST3 samples, indicating that ST3 infections might be lighter in parasite load. However, larger data sets are needed to confirm this hypothesis and allow speculation on its clinical implications.

The present assay has a built-in IPC, which distinguishes it from previously published PCR assays. In the current evaluation, inhibition or suboptimal conditions appeared not to be a problem. The assay does not enable accurate subtyping by sequencing of PCR products; although the amplicon spans a hypervariable region, it is relatively small compared to the amplicon size usually recommended for subtyping (25, 30).

In conclusion, we have developed a highly applicable TaqMan assay for sensitive and specific screening for Blastocystis ST1 to ST9 of large numbers of DNAs extracted directly from human fecal samples. We believe that this method will prove to be an invaluable tool in all studies aiming at accurately identifying carriers and noncarriers of Blastocystis. Once DNAs have been found to be positive, these can be subjected to the genus-specific PCR published by Scicluna et al. (25) for subtyping.

**ACKNOWLEDGMENTS**

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**REFERENCES**


