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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.**

Blastocystis is a single-celled intestinal parasite of humans and a vast array of animals. Based on small-subunit (SSU) ribosomal DNA (rDNA) analysis, the genus comprises at least 13 subtypes (STs), 9 of which have been found in humans (29, 32, 34, 36); it is very likely that each subtype represents a separate species (36). In humans, ST3 appears to be the most common subtype, followed in prevalence by ST1, ST2, and ST4 (19, 23, 30–32, 35–38). There apparently is a geographical component to variation in global subtype distribution; for instance, ST4 is rarely reported outside Europe.

Besides *Blastocystis* being associated with irritable bowel syndrome (IBS) (32), recent data indicate that at least one subtype may be associated with gastrointestinal illness (6, 31). In studies aiming to further explore and clarify the epidemiology and pathogenicity of *Blastocystis*, accurate identification of carriers and noncarriers in screening situations is essential (34). Recently, various diagnostic methods, including conventional PCR, xenic *in vitro* culture (XIVC), permanently stained preparations of fixed feces, and microscopy of fecal concentrates, were compared, and PCR and culture were found to be the most sensitive methods and to be almost equally sensitive (30). However, culture results are available only 48 to 72 h after sample submission. Although it involves DNA extraction, molecular detection is faster and enables subsequent subtyping by analysis of sequences obtained from specific PCR products.

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.

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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-

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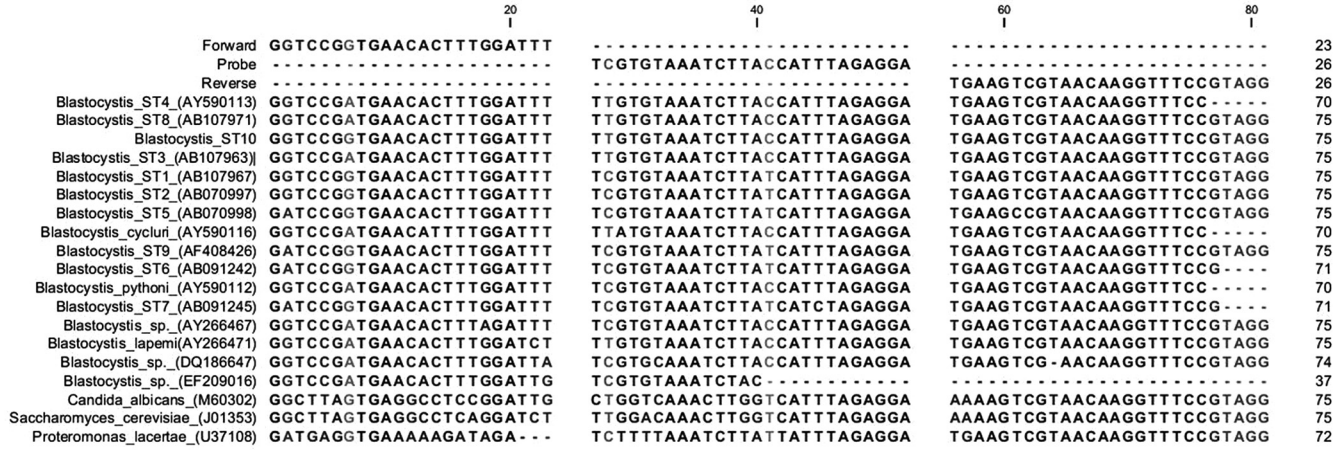

FIG 1 Alignment of *Blastocystis*-specific oligonucleotides (forward, probe, and reverse) and SSU rDNAs from *Blastocystis* sp. ST1 to ST10, other *Blastocystis* spp., *Proteromonas lacertae*, *Candida albicans*, and *Saccharomyces cerevisiae*. Polymorphic bases are highlighted in gray. Dashes indicate missing or nonexisting bases.

signed by eye and using Primer Express 2.0 (Applied Biosystems) and Generunner 3.01 (http://www.generunner.net/). All sequences were downloaded from GenBank (http://www.ncbi.nlm.nih.gov/GenBank/), except for the sequence of *Blastocystis* sp. ST10, which was kindly provided by Graham Clark. Oligonucleotides are shown in Table 1. The forward primer had a mismatch range of 0 to 1 bp in *Blastocystis* subtypes, 1 to 2 bp in other *Blastocystis* species, and 10 to 11 bp in non-*Blastocystis* species. The probe exhibited a mismatch range of 1 to 2 bp in *Blastocystis* subtypes, 0 to 2 bp in other *Blastocystis* species, and 4 to 8 bp in non-*Blastocystis* species. Finally, the reverse primer showed a mismatch range of 0 to 1 bp in *Blastocystis* subtypes, 0 bp in other *Blastocystis* species, and 0 to 2 bp in non-*Blastocystis* species (Fig. 1).

Real-time PCR assay, standard curves, and controls. To enable the detection of *Taq* DNA polymerase inhibitors or suboptimal reaction conditions, an internal process control (IPC) was constructed as described previously (7, 8). Briefly, primers for amplification of parts of the phage lambda genome were synthesized with a tail that included the sequence of each of the *Blastocystis* primers added to the 5′ end of the corresponding phage lambda primer (Table 1). PCR products of 190 bp thus containing the binding sites of the *Blastocystis* primers were obtained by amplification of 1 ng of purified phage lambda DNA. The amplicons were gel purified, and a 10-fold titration of the IPC was added to separate master mixtures. A dilution of the IPC that had no influence on the cycle threshold (C_T) number for purified *Blastocystis* DNA was used in the assay. The optimum dilution of the IPC was found to be 1×10^{-8}. The IPC probe (TAG Copenhagen, Copenhagen, Denmark) was 5′ labeled with 6-carboxytetramethylrhodamine (TAMRA) and quenched with Black Hole Quencher 2 (Table 1). *Blastocystis* DNA from a strain available in culture was used to generate standard curves.

Real-time PCRs were performed in 50-μl volumes, and the following reagents were used: 1 μM each of the primers (Table 1), 300 nM each of the two probes (*Blastocystis* and IPC), 5 U/μl Platinum *Taq* polymerase (Invitrogen, Taastrup, Denmark) in 20 mM Tris-HCl (pH 8.0), 40 mM sodium chloride, 2 mM sodium phosphate, 0.1 mM dithiothreitol (DTT), stabilizers, 50% (vol/vol) glycerol, 10× PCR buffer minus $MgCl_2$ (200 mM Tris [pH 8.4], 500 mM KCl) (Invitrogen, Taastrup, Denmark), 5 mM $MgCl_2$, dUTP mix (12.5 mM dUTP, 50 mM dGTP, 50 mM dATP, 50 mM dCTP), 5 μl of the appropriate dilution of IPC, 50% glycerol, and water. Samples were processed on an ABI 7500 real-time PCR system instrument with a 96-well block (Applied Biosystems, Nærum, Denmark). The PCR profile consisted of 50°C for 1 s, 95°C for 2 min, and 50 cycles of denaturation at 95°C for 15 s followed by annealing and extension at 60°C for 1 min.

Evaluation and validation of real-time PCR. DNAs from various subtypes (ST1 to ST9) were tested in the assay to enable confirmation of genus sensitivity. Assay sensitivity was determined by testing a 10-fold dilution series of DNA extracted from 1 million *Blastocystis* organisms isolated from xenic *in vitro* culture by gradient centrifugation (38) and eluted in 200 μl (Table 2). Hence, 5 μl of DNA from the undiluted sample was equivalent to 25,000 organisms.

TABLE 1 Primers and probes used in the TaqMan assay for amplification of *Blastocystis* and the internal process control

Primer or probe	Sequence (5′→3′)[a]	Nucleotide position
Primers for TaqMan assay		
Blasto FWD F5	GGTCCGGTGAACACTTTGGATTT	1641–1663 in AY244621 sequence[b]
Blasto R F2	CCTACGGAAACCTTGTTACGACTTCA	1734–1759 in AY244621 sequence[b]
Primers for construction of IPC		
Blasto FWD F5 IPC	GGTCCGGTGAACACTTTGGATTT**CCGGGACGTATCATGCT**	13918 in U39284 phage lambda sequence
Blasto REV F2 IPC	CCTACGGAAACCTTGTTACGACTTCA**ACCGCTCAGGCATTTGCT**	14061 in U39284 phage lambda sequence
Probes		
Blastocystis probe	FAM-TCGTGTAAATCTTACCATTTAGAGGA-MGBNFQ	1705–1730 in AY244621[b] sequence
IPC probe	TAMRA-TCCTTCGTGATATCGGACGTTGGCTG–BHQ2	14011 in phage lambda sequence

[a] 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).
[b] Sequence may exhibit polymorphism compared to oligonucleotide.

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

Probe	C_T values for duplicates at dilution of[a]:								
	1×10^{0}	1×10^{-1}	1×10^{-2}	1×10^{-3}	1×10^{-4}	1×10^{-5}	1×10^{-6}	1×10^{-7}	1×10^{-8}
Blasto	21.90/21.89	25.15/25.22	28.67/28.67	31.04/30.97	34.84/35.11	36.82/40.91	39.21/38.12	40.93/UD	UD/UD
IPC	UD/UD	UD/UD	UD/47.26	36.56/38.64	35.17/35.11	35.18/35.70	36.76/36.03	36.70/34.95	35.98/37.15

[a] DNAs were tested in duplicates to test for reproducibility. UD, undetermined (i.e., signal absent).

The assay was specificity tested against panel dilutions of fungal DNAs from *Candida albicans* (ATCC 64548), *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 parahaemolyticus*, 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*, and 1 for *Entamoeba dispar* by in-house real-time PCR assays used for detection of *Giardia*, *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 using primers described by Scicluna et al. (25) and Stensvold et al. (38). All products were sequenced unidirectionally. 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_T) values were calculated and a two-tailed Student *t* test for comparison of means carried out using software available at http://qudata.com/online/statcalc/ and http://studentsttest.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_T value of 21.89/21.90 in duplicate determinations, and reproducible C_T values were obtained down to a 10^{-4} 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 TaqMan assay (Table 3), with C_T values ranging from 14.03 to 39.52, a mean C_T value of 20.48 (standard deviation [SD], 5.85) and a median C_T value of 18.76 (interquartile range [IQR], 17.23 to 21.46). Thirteen samples negative by XIVC were positive by the TaqMan assay (Table 3), with C_T values ranging from 16.25 to 40.26, a mean C_T value of 28.93 (SD, 4.99) and a median C_T 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 TaqMan 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. Using primers amplifying a product of ~600 bp (25) or ~300 bp (38), it was possible to amplify 9/13 samples by conventional PCR (ST1, 3 samples; ST2, 1 sample; and ST3, 5 samples); the mean C_T 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_T 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_T 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 3 Comparison of test results for real-time PCR and XIVC and distribution of *Blastocystis* subtypes

Test results	No. of samples with ST:					
	1	2	3	4	—[a]	Total
Real-time PCR negative						
XIVC negative	0	0	0	0	89	89
XIVC positive	0	0	0	0	2	2
Total	0	0	0	0	91	91
Real-time PCR positive						
XIVC negative	3	1	5	0	4	13
XIVC positive	18	13	11	5	2	49
Total	21	14	16	5	6	62
Total	21	14	16	5	97	153

[a] —, 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 to the data presented by Poirier et al. (22), who found that the sensitivity of XVIC 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.

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