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Comparison of Sequencing (Barcode Region) and Sequence-Tagged-Site PCR for *Blastocystis* Subtyping

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***Blastocystis* is the most common nonfungal microeukaryote of the human intestinal tract and comprises numerous subtypes (STs), nine of which have been found in humans (ST1 to ST9). While efforts continue to explore the relationship between human health status and subtypes, no consensus regarding subtyping methodology exists. It has been speculated that differences detected in subtype distribution in various cohorts may to some extent reflect different approaches. *Blastocystis* subtypes have been determined primarily in one of two ways: (i) sequencing of small subunit rRNA gene (SSU-rDNA) PCR products and (ii) PCR with subtype-specific sequence-tagged-site (STS) diagnostic primers. Here, STS primers were evaluated against a panel of samples ($n = 58$) already subtyped by SSU-rDNA sequencing (barcode region), including subtypes for which STS primers are not available, and a small panel of DNAs from four other eukaryotes often present in feces ($n = 18$). Although the STS primers appeared to be highly specific, their sensitivity was only moderate, and the results indicated that some infections may go undetected when this method is used. False-negative STS results were not linked exclusively to certain subtypes or alleles, and evidence of substantial genetic variation in STS loci was obtained. Since the majority of DNAs included here were extracted from feces, it is possible that STS primers may generally work better with DNAs extracted from *Blastocystis* cultures. In conclusion, due to its higher applicability and sensitivity, and since sequence information is useful for other forms of research, SSU-rDNA barcoding is recommended as the method of choice for *Blastocystis* subtyping.**

Since the revelation of extensive genetic diversity in *Blastocystis* (1, 2), a microeukaryote infecting the large intestine of possibly more than 1 billion people, data on the distribution of *Blastocystis* subtypes (3) have accumulated primarily with a view to identifying any potential association between subtype and disease phenotype (4, 5). While the number of *Blastocystis* subtypes in animals is continually expanding (6, 7), the number of subtypes found in humans has remained stable; thus, humans are natural hosts of nine subtypes (ST1 through ST9), of which ST1 to ST4 are by far the most common (4). Analysis of subtype data is important not only in attempts to test for epidemiological association between subtypes and clinical outcome of *Blastocystis* infection (5, 8) but also to clarify transmission patterns and potentially the question of zoonosis. Supportive evidence for subtype-dependent differences in the clinical significance of *Blastocystis* is emerging (9, 10).

Although *Blastocystis* subtype nomenclature was standardized in 2007 (3), there is still no consensus as to the best method to use for subtyping. Subtyping has been approached mainly in two ways: (i) sequencing of small subunit rRNA gene (SSU-rDNA) PCR amplicons and (ii) direct assignment using diagnostic subtype-specific sequence-tagged-site (STS) primers (11). The latter were designed from random amplified polymorphic DNA sequences, with the nature of the DNA targets as well as their copy numbers remaining unknown.

For sequencing, several regions in the SSU-rDNA have been targeted (4); however, the "barcode region" (12) has been used extensively (10, 12–18). This region encompasses the 5′-most ~600 bp and is known to be a valid proxy for complete SSU-rDNAs and is a region for which many sequences are available in both GenBank and the *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database (www.pubmlst.org/blastocystis). The region is amplified by RD5, a primer of broad eukaryotic specificity, and the BhRDr primer of stricter specificity (see Table 2) (2, 12).

The STS primers are advantageous in that they are diagnostic, and hence no sequencing of the PCR product is required. Moreover, such an approach theoretically enables precise dissection of mixed subtype infections. However, the STS primers only target ST1 to ST7. Therefore, other subtypes go undetected or could potentially be amplified inappropriately by one or more of the primer pairs and assigned to subtypes to which they do not belong. It is also clear that some subtypes, for instance, ST3, exhibit substantial intrasubtype genetic diversity (17), and the ability of the STS primers to detect all of the genetic variants within subtypes has yet to be investigated.

Among surveys of *Blastocystis* STs, there are examples of significantly different findings from the same country that might stem from differences in the methodology used. Three studies carried out in Egypt used different techniques to identify *Blastocystis* STs. Hussein et al. (21) and Fouad et al. (22) used the STS technique and found similar *Blastocystis* ST frequencies even though samples came from different cities in Egypt (Cairo and Ismailia). Unusually, they identified 27 to 33% of *Blastocystis* as being ST6 or ST7. In contrast, Souppart et al. (15), also sampling in Cairo, used sequencing and reported quite different ST distributions from the other two studies: ST6 and ST7 were not detected at all. Whether the choice of methodology affected the results in any way is not clear, but it suggests that a comparative study is needed using both techniques on the same samples.

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Here, the results of a study comparing the STS method with the barcoding method are presented with a view to identifying which method should be recommended for *Blastocystis* subtyping.

MATERIALS AND METHODS

DNA samples. The majority of the DNAs included in the study were extracted directly from feces as described elsewhere (17, 20), while a few were from *Blastocystis* cultures (17) (Table 1). All samples had been barcoded according to the method of Scicluna et al. (12). The barcode region covers ca. 30% of the *Blastocystis* SSU-rDNA. The *Blastocystis* database at www.publmst.org/blastocystis holds hundreds of sequences covering the barcode region and can be used to assign subtype to barcode sequences as well as to identify the alleles within subtypes. In choosing the panel of samples for evaluation, efforts were made to include as many different alleles as possible from each subtype, which is why the number from each subtype varies. The number of samples representing each subtype also reflects sample availability. Although ST1 to ST4 are very common, other subtypes are relatively rare in humans (4). For this reason, most of the ST5s included in the study were from nonhuman hosts (Table 1). All barcode sequences were identified to allele level using the sequence query facility at www.pubmlst.org/blastocystis.

For STS primer specificity testing, a panel of fecal DNAs giving false-positive results by barcoding PCR was used (i.e., a product was produced but the sequence proved not to be from *Blastocystis*). These products were identified as coming from other eukaryotes commonly found in fecal samples (*Penicillium*, *Galactomyces*, and *Saccharomyces*) (Table 1). Eight samples positive for *Dientamoeba fragilis* detected using an in-house real-time PCR using primers previously described (19) were also included (Table 2). STS specificity was further tested by including a few DNAs from subtypes other than ST1 to ST7 (Table 1).

STS PCR. All samples were tested using all seven pairs of STS primers (11) with standard PCR conditions (35 cycles) and individual annealing temperatures to accommodate differences in the melting temperatures of the different primer pairs. Since no STS PCR product was expected to be longer than 600 bp, a 1-min extension time was used in each cycle for all primer pairs. In cases where negative results were unexpectedly obtained using the STS primers, a barcoding PCR was performed on the sample (same sample template concentration and reaction volume) to test for inhibition; samples were subsequently reprocessed where necessary at a template dilution where the barcoding PCR worked, and if amplicons were still not seen the STS PCR was scored as negative. In only a few instances were STS PCR products sequenced.

RESULTS AND DISCUSSION

STS PCR results are compared to barcoding in Table 1. The STS primers did not amplify non-*Blastocystis* DNA and were therefore highly specific. The reason for including subtypes other than ST1 to ST7 in the study was to test whether any of the STS primer pairs would nonspecifically amplify *Blastocystis* belonging to such subtypes, but no such amplification was seen.

While maximum specificity was observed, the sensitivity of the primers was significantly lower than expected. Most strikingly, only one of the two ST4 18S alleles, allele 91, was amplified by the SB337 primers. Compared to allele 42, allele 91 is extremely rare in humans (10, 17), at least in subtype surveys where sequencing was used. These data support the hypothesis proposed by Stensvold et al. (17) that ST4 may be underdiagnosed by the STS method.

While the STS primers were found to enable amplification of all intended subtypes, sporadic negative STS results were also observed for most subtypes. The explanation for negative results appears less straightforward than for ST4, since there were quite a few examples of samples with the same allele showing variable STS amplification results (Table 1). The major issue here is that little is known about the STS gene targets and how conserved we can expect them to be within subtypes; the levels of conservation may also vary between subtypes. The predicament is that the STS primers are intended to be diagnostic, which means that few sequences of STS products are available, and so we do not know the extent of variation in the different STS loci. For example, the ST5 primers by chance may target a highly conserved locus and so are positive for all samples tested, while the ST3 primers target a less conserved locus. It is more difficult to explain the variation within genotypes of ST3 and why some samples with alleles 34/36/37 are positive and some are negative. In two cases, STS PCR products were sequenced. In the case of sample MACA6 (ST5, allele 17), the sequence revealed a 10-bp deletion and 2 single nucleotide polymorphisms compared to GenBank sequence AY048751. Similarly, the STS PCR products for "T70600" (ST2, allele 9) only showed 97% identity to GenBank sequence AY048752. These very few observations indicate that significant genetic variation in the STS target sites exists. This hypothesis is supported by the variation seen in the sequences recently submitted to GenBank by Moosavi et al. (23) (AB714500 to AB714503). Although it therefore cannot be ruled out that inconsistent STS amplification is due to intrasubtype variability in the STS loci, there is also the possibility that STS primers work better with DNA extracted from culture rather than directly from feces. This could be explained by nonspecific annealing of the primers to DNA from various other organisms in cases where *Blastocystis* DNA is present at much lower levels than non-*Blastocystis* DNA, as is typical in fecal DNA. If DNA from cultures had been available for each of the 58 *Blastocystis* samples included in the present study, it would have been possible to answer this important question. It is worth noting that the three studies from Egypt all used DNA extracted from cultures established in Jones' medium, which indicates that the source of the DNA (feces versus culture) may not be the sole variable impacting the results. Even in the light of these observations, there is not enough information to explain the discrepancies among the three Egypt studies. It may be that the populations studied had been exposed to different subtypes of *Blastocystis*, or that they differed in terms of susceptibility to infection.

The BhRDr primer was designed to be combined with the RD5 primer, a primer of broad eukaryotic specificity, originally to characterize DNA from cultured *Blastocystis* (12), but the primer pair has subsequently also been applied directly to amplification from fecal DNA. It is clear that they are not fully *Blastocystis* specific since they appear to amplify *Blastocystis* SSU-rDNA if present and SSU-rDNA from other eukaryotes, mainly fungi, in the absence of *Blastocystis* (Table 1). In the event that these primers are used to screen fecal DNAs for *Blastocystis*, a certain false-positive rate must be expected, and positivity should always be confirmed by sequencing. However, since the amplicon is almost 600 bp, prescreening fecal DNAs using a *Blastocystis* real-time PCR (16, 24) instead may prove more sensitive.

In terms of *Blastocystis* genetic markers, the barcode region is by far the best represented in publicly available sequence databases, and subtypes can be identified by BLAST analysis in the sequence database at GenBank or the *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database site (http://pubmlst.org/blastocystis). Blasting against the latter database has the added advantages of using the consensus subtype nomenclature (unlike GenBank, where the subtype is included only if one was part of the accession submission and there is no attempt to impose a standard

TABLE 1 Sample overview and subtype results obtained by barcoding and STS PCR[a]

Sample[b]	Host	Genus	Barcoding	Allele(s)	STS PCR
T70666	*Homo sapiens*	*Saccharomyces*	*Saccharomyces*	NA	Neg
M24550	*Homo sapiens*	*Saccharomyces*	*Saccharomyces*	NA	Neg
M26556	*Homo sapiens*	*Saccharomyces*	*Saccharomyces*	NA	Neg
M27666	*Homo sapiens*	*Penicillium*	*Penicillium*	NA	Neg
M26195	*Homo sapiens*	*Penicillium*	*Penicillium*	NA	Neg
M27596	*Homo sapiens*	*Galactomyces*	*Galactomyces*	NA	Neg
M27798	*Homo sapiens*	*Galactomyces*	*Galactomyces*	NA	Neg
M29502	*Homo sapiens*	*Galactomyces*	*Galactomyces*	NA	Neg
M29643	*Homo sapiens*	*Galactomyces*	*Galactomyces*	NA	Neg
T1054	*Homo sapiens*	*Galactomyces*	*Galactomyces*	NA	Neg
S18243	*Homo sapiens*	*Dientamoeba*	Neg	NA	Neg
F43057	*Homo sapiens*	*Dientamoeba*	*Saccharomyces*	NA	Neg
T50024	*Homo sapiens*	*Dientamoeba*	Neg	NA	Neg
T48806	*Homo sapiens*	*Dientamoeba*	Neg	NA	Neg
F43051	*Homo sapiens*	*Dientamoeba*	Neg	NA	Neg
W4753	*Homo sapiens*	*Dientamoeba*	Neg	NA	Neg
H49273	*Homo sapiens*	*Dientamoeba*	*Saccharomyces*	NA	Neg
F41534	*Homo sapiens*	*Dientamoeba*	Neg	NA	Neg
H4338	*Homo sapiens*	*Blastocystis*	ST1	4	ST1
H4483	*Homo sapiens*	*Blastocystis*	ST1	4	Neg
H4582	*Homo sapiens*	*Blastocystis*	ST1	4	ST1
T2955	*Homo sapiens*	*Blastocystis*	ST1	4	ST1
M22539	*Homo sapiens*	*Blastocystis*	ST2	9	ST2
H1172	*Homo sapiens*	*Blastocystis*	ST2	9	Neg
T70600	*Homo sapiens*	*Blastocystis*	ST2	9	ST2
M24604	*Homo sapiens*	*Blastocystis*	ST2	10	ST2
M27646	*Homo sapiens*	*Blastocystis*	ST2	10	ST2
W19923	*Homo sapiens*	*Blastocystis*	ST2	11	ST2
S31622	*Homo sapiens*	*Blastocystis*	ST2	11	ST2
M22519	*Homo sapiens*	*Blastocystis*	ST2	11	ST2
M25210	*Homo sapiens*	*Blastocystis*	ST2	12	ST2
H3505	*Homo sapiens*	*Blastocystis*	ST2	12	Neg
H1380	*Homo sapiens*	*Blastocystis*	ST2	12	ST2
S32380	*Homo sapiens*	*Blastocystis*	ST3	34	Neg
S32319	*Homo sapiens*	*Blastocystis*	ST3	34	Neg
M29606	*Homo sapiens*	*Blastocystis*	ST3	34	ST3
M26030	*Homo sapiens*	*Blastocystis*	ST3	34	Neg
S32244	*Homo sapiens*	*Blastocystis*	ST3	34	ST3
T70252	*Homo sapiens*	*Blastocystis*	ST3	36	Neg
M27582	*Homo sapiens*	*Blastocystis*	ST3	36	ST3
T2853	*Homo sapiens*	*Blastocystis*	ST3	36	ST3
S32304	*Homo sapiens*	*Blastocystis*	ST3	36	ST3
H6344	*Homo sapiens*	*Blastocystis*	ST3	37	ST3
T2793	*Homo sapiens*	*Blastocystis*	ST3	37	ST3
H802	*Homo sapiens*	*Blastocystis*	ST3	37	Neg
W9615	*Homo sapiens*	*Blastocystis*	ST3	37	ST3
M27783	*Homo sapiens*	*Blastocystis*	ST4	42	Neg
H2565	*Homo sapiens*	*Blastocystis*	ST4	42	Neg
T70361	*Homo sapiens*	*Blastocystis*	ST4	42	Neg
T2785	*Homo sapiens*	*Blastocystis*	ST4	42	Neg
H6267	*Homo sapiens*	*Blastocystis*	ST4	42	Neg
DMP/10-212*	*Homo sapiens*	*Blastocystis*	ST4	94	ST4
Nille	*Gorilla gorilla*	*Blastocystis*	ST5	16	ST5
MA40*	*Pan troglodytes*	*Blastocystis*	ST5	16	ST5
PIG5.2*	*Sus scrofa*	*Blastocystis*	ST5	115	ST5
MA129*	Unidentified primate	*Blastocystis*	ST5	17	ST5
MA157*	*Homo sapiens*	*Blastocystis*	ST5	16	ST5
MA292*	*Pongo pygmaeus*	*Blastocystis*	ST5	17	ST5
MACA6*	*Camelus dromedarius*	*Blastocystis*	ST5	17	ST5
M66137	*Homo sapiens*	*Blastocystis*	ST6	134	ST6
S32277	*Homo sapiens*	*Blastocystis*	ST7	110	Neg

(Continued on following page)

TABLE 1 (Continued)

Sample[b]	Host	Genus	Barcoding	Allele(s)	STS PCR
W9483	*Homo sapiens*	*Blastocystis*	ST7	110	Neg
W11245	*Homo sapiens*	*Blastocystis*	ST7	111, 108	ST7
T67977	*Homo sapiens*	*Blastocystis*	ST7	112	Neg
M27606	*Homo sapiens*	*Blastocystis*	ST7	111, 108	Neg
M27857	*Homo sapiens*	*Blastocystis*	ST7	111, 108	Neg
MAGO40*	*Capra* sp.	*Blastocystis*	ST7	41	Neg
F3014	*Homo sapiens*	*Blastocystis*	ST8	21	Neg
W11359	*Homo sapiens*	*Blastocystis*	ST8	21	Neg
GiQui*	*Homo sapiens*	*Blastocystis*	ST4, ST8	133, 21	Neg
GiJoe*	*Homo sapiens*	*Blastocystis*	ST9	129	Neg
M27745	*Homo sapiens*	*Blastocystis*	Unknown[c]	Not yet established	Neg
W11396	*Homo sapiens*	*Blastocystis*	Unknown	Not yet established	Neg
MACO25*	*Bos taurus*	*Blastocystis*	ST10	43	Neg
MACA27*	*Camelus dromedarius*	*Blastocystis*	ST10	New allele (one SNP)	Neg
MACO3*	*Bos taurus*	*Blastocystis*	ST14	New allele (two SNPs)	Neg

[a] Sample overview and subtype results were obtained by barcoding (12) and STS PCR (11) as described previously. NA, not applicable; Neg, negative; SNP, single nucleotide polymorphism.
[b] *, DNA extracted from cultured isolates (and not directly from feces).
[c] Unknown, the barcode sequence is either ST9 or a new subtype. Complete SSU rDNA sequencing is ongoing.

nomenclature), as well as assigning the allele to the SSU-rDNA barcode sequence. While the STS primers constitute a convenient approach in situations where sequencing is not an option, the present study highlights at least three major limitations of the method. (i) If the STS primers are used, some subtypes will go undetected. For humans, this includes ST8 and ST9 but also the majority of ST4 strains. For animals, this includes many more subtypes (Table 1). (ii) The data strongly indicate that the STS method fails to detect some strains belonging to targeted subtypes as well. Hence, the sensitivity of each primer set is reduced, perhaps due to intrasubtype variability. However, the results may be different if only DNA from cultures is used, as suggested above. (iii) Finally, no intrasubtype resolution is given when using the STS method. Comparison of SSU-rDNA alleles belonging to the same subtype can help determine whether one strain is distinct from another, which has implications for our ability to identify potential zoonotic transmission and whether certain strains predominate in particular clinical settings.

However, there is little reason to question the epidemiological data reported in studies where the STS method has been used on DNAs from cultured isolates if (i) all subtypes of *Blastocystis* grow equally well in culture without preferential amplification of one subtype over another (in cases of mixed infection) and (ii) STS primers are more sensitive when using DNAs from cultures than fecal DNAs.

Redesigning STS primers is a potential option but will require extensive sequencing of primer target regions to enable evaluation of primer sensitivity and specificity. A validation study to evaluate the applicability of STS primers directly to fecal DNAs should also be carried out. Moreover, STS primer pairs should be designed at least for subtypes ST8 and ST9.

Nevertheless, due to better overall applicability and higher sen-

TABLE 2 Primers used in this study[a]

Primer pair	DNA target	Target organism	Primer		Reference(s)
			Type[b]	Sequence (5′–3′)	
SB83	Unknown	*Blastocystis* sp. ST1	F	GAAGGACTCTCTGACGATGA	11
			R	GTCCAAATGAAAGGCAGC	
SB340	Unknown	*Blastocystis* sp. ST2	F	TGTTCTTGTGTCTTCTCAGCTC	11
			R	TTCTTTCACACTCCCGTCAT	
SB227	Unknown	*Blastocystis* sp. ST3	F	TAGGATTTGGTGTTTGGAGA	11
			R	TTAGAAGTGAAGGAGATGGAAG	
SB337	Unknown	*Blastocystis* sp. ST4	F	GTCTTTCCCTGTCTATTCTGCA	11
			R	AATTCGGTCTGCTTCTTCTG	
SB336	Unknown	*Blastocystis* sp. ST5	F	GTGGGTAGAGGAAGGAAAACA	11
			R	AGAACAAGTCGATGAAGTGAGAT	
SB332	Unknown	*Blastocystis* sp. ST6	F	GCATCCAGACTACTATCAACATT	11
			R	CCATTTTCAGACAACCACTTA	
SB155	Unknown	*Blastocystis* sp. ST7	F	ATCAGCCTACAATCTCCTC	11
			R	ATCGCCACTTCTCCAAT	
Barcode	Small subunit rRNA gene	*Blastocystis* sp.	RD5	ATCTGGTTGATCCTGCCAGT	2, 12
			BhRDr	GAGCTTTTTAACTGCAACAACG	

[a] The 2007 consensus nomenclature is used here (Stensvold et al. [19]), which is different from that in the original primer description (Yoshikawa et al. [11]).
[b] That is the orientation (forward [F] or reverse [R]) or the name.

sitivity, SSU-rDNA-based subtyping of *Blastocystis* from humans and animals is unquestionably the method of choice, with barcoding being the most useful and relevant variant of this approach available.

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