

Update on the Molecular Epidemiology and Diagnostic Tools for *Blastocystis* sp

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Abstract

Blastocystis is the most common enteric protist found in humans. Due to the recent advancements in molecular technologies, up to 17 subtypes have been identified from humans and animals. Molecular epidemiological studies have shown the large range of subtype (ST) distribution within geographical locations with ST1-9 being identified in humans. ST3 has been identified as the predominant subtype in most epidemiological studies with a considerable absence of ST4 noted in Africa and the Middle East compared to Europe and Australia where this subtype is fairly common. This review summarises the molecular tools used for diagnosis and speciation of Blastocystis and comments on advancements in the area over the last 15 years and what future trends may be. This review also describes the geographical distribution of Blastocystis and comments on possible intra-subtype evolutionary relationships.

Keywords Blastocystis sp.; Epidemiology; Subtyping; Diagnosis

Introduction

For a protist that was discovered over 100 years ago there is still so much unknown about Blastocystis in regards to its pathogenicity, transmission, lifecycle and susceptibility to antimicrobials. Symptoms associated with Blastocystis infection include diarrhoea, abdominal pains and bloating [1,2]. Until recently it was not possible to differentiate between the subtypes of Blastocystis and many different molecular techniques have been developed to distinguish between them. In turn this has resulted in different terminologies being used for the identification and designation of Blastocystis subtypes. So far 17 subtypes have been identified within the Blastocystis clades existing in mammals, birds and reptiles. Due to recent studies it has been shown that no group exclusive to humans exists and that human isolates predominantly lie within subtype 1-4 [3-5]. Until recently, all human isolates of Blastocystis were commonly referred to as Blastocystis hominis and all other animal isolates were called Blastocystis sp. but these recent molecular results now show that all samples identified through only microscopy should be called Blastocystis sp. due to there not being a single subtype specific to humans. From many early studies completed on the subtyping of Blastocystis, lack of consistency in the designation of subtypes resulted in confusion and made it difficult for comparison and collaboration within studies. A study on the consensus for the terminology of Blastocystis compared all the previously published data which showed many discrepancies with subtype allocation [6]. This paper proposed that all isolates fall within nine subtypes and concluded that a standardisation within the nomenclature would make future epidemiological and clinical studies much easier. Since this paper was published, a further eight subtypes have been identified from animals [7-9]. Recently the whole genome sequence for subtype (ST) 7 was completed. This new information has shed some light on many of the aspects of Blastocystis including the metabolic pathways, virulence factors and possible drug resistance mechanisms.

Typing Methods

Clark and colleagues were among the first to employ molecular techniques to distinguish the various subtypes of Blastocystis present in human stool samples. They used Restriction Fragment Length Polymorphism (RFLP) analysis of Polymerase Chain Reaction (PCR) amplified small subunit (SSU) rDNA for the rapid comparison of multiple isolates. Through this technique they identified seven different Blastocystis ribodemes, and suggested that Blastocystis was a zoonosis [10]. In the last 15 years there has been extensive work done in developing molecular techniques to distinguish the different subtypes of Blastocystis and 17 subtypes have been identified from humans and animals. Using PCR and RFLP analysis, which relies on the digestion of PCR products with restriction endonucleases to yield variable gel banding products, up to five different subtypes were identified from different geographical locations [11-14]. The use of RFLP can be problematic in that mixed infections may be missed and if there is a mutation at any of the bases that make up the restriction recognition sequence isolates could be incorrectly identified [15].

The application of subtype-specific primer pairs was developed to distinguish between seven different subtypes but as more subtypes have been identified this method for typing is not reliable as it does not cover all the subtypes and therefore infections may be missed or incorrectly assigned to a different subtype [16-18]. PCR with subsequent dideoxy sequencing of amplified PCR products, targeting typically the 18S SSU rRNA, is now the most common way for identifying the different subtypes isolated from samples as this method allows for all subtypes to be identified, given that correct primer pairs have been chosen that do not show subtype preference [4].

Pyrosequencing technology combined with a nested PCR was developed to differentiate between seven subtypes [15]. This system is based on the pyrophosphate released when a nucleotide is introduced to the DNA-strand and is suited for detecting single or multiple nucleotide polymorphisms within short-read DNA sequences [19,20]. This technique shows a high specificity and sensitivity and is a fairly

fast typing method. This technique does have problems though with possible nucleotide miscalling in short reads and is also not possible to discriminate between subtypes found in mixed infections.

There have only been three studies reported which have developed a real-time (RT) PCR for *Blastocystis*. The first study [21] was able to detect ST1, ST3 and ST4 but it is unknown whether other subtypes are able to be detected using this assay and therefore the specificity and sensitivity is unknown. The second study designed a genus-specific PCR which was able to detect all known subtypes so far identified in humans [22]. This test only had 95% specificity though, and the amplicon size of 339 bp is much longer than usually desired for RT-PCR sensitivity. The most recent study developed a TaqMan assay which is highly sensitive and specific for subtypes 1-9 of *Blastocystis* [23]. This appears to be the most sensitive and specific RT-PCR so far developed but still does not differentiate between the subtypes. At the moment it appears that RT-PCR could be the most useful tool for the screening of *Blastocystis* but subtyping of isolates by conventional PCR is still the only way to designate subtypes. The development of RT-PCR techniques which have the ability to discriminate between the different subtypes within a sample will be beneficial for screening and epidemiological studies for *Blastocystis*. RT-PCR would also prove to be more time and cost effective with extraction and RT-PCR theoretically being able to be completed within only a few hours.

More recently Multilocus Sequence Typing (MLST) of the Mitochondria-Like Organelle (MLO) of *Blastocystis* has been used to show the differences in diversity within subtypes [24]. There were five loci of ST3 MLST sequences studied and six loci for the ST4 MLST. The authors showed that most human ST3 infections occur through human-human transmission as most human infections were restricted to a single clade (MLO clade 1) within the ST3 group. Animal infections and people exposed to animals (in particular animal handlers) were found to lie within three other clades which show the result of zoonotic transmission. This study showed the intra-subtype genetic variability within ST3 and ST4. The study also suggested that ST4 has a more recent history of colonising humans than ST3 due to the lack of variation within the sequences and also because ST4 is common in European populations but is rarely found elsewhere.

The use of the “barcode region” for sequencing appears to be the most effective for the designation of *Blastocystis* subtypes and is the best technique used if phylogenetic information is to be obtained [4]. DNA barcoding refers to a method proposed to produce a unique identifier for all living species and has been used previously to study a variety of animals [25,26]. The *Blastocystis* barcode region amplifies the 5’ 600 bp region of the *Blastocystis* SSU-rDNA using the primer pair of RD5, a primer with broad eukaryotic specificity, and BhRDr which is more specific to *Blastocystis* and was designed for subsequent sequencing. This PCR does have the possibility for false positives though if only used for *Blastocystis* screening. The PCR has the ability to detect other eukaryotes particularly fungi which makes this technique not desirable when sequencing of positives is not available.

A substantial number of sequences in Genbank use the “barcode region” and have been submitted to the *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database (www.pubmlst.org/blastocystis) which makes comparison and designation of subtypes simpler and keeps integrity within the subtype allocations. The advantage of the *Blastocystis* Subtype (18S) and Sequence Typing (MLST) Database is that it uses the consensus subtype nomenclature (discussed below), unlike GenBank, and also assigns the allele to the SSU-rDNA barcode

sequence [27]. This database currently has 255 sequences and 64 MLST profiles and is continually updated.

The uses of matrix-assisted laser desorption/ionisation time-of-flight mass spectrometry (MALDI-TOF MS) has recently revolutionised the clinical microbiology laboratory with accurate, rapid results available for the identification of microorganisms. A recent study showed the accuracy of MALDI-TOF MS for the speciation of five different *Blastocystis* STs [28]. Axenic cultures speciated by SSU-rDNA analysis were used to create profiles for the different *Blastocystis* subtypes. It was found that speciation was better after extraction using the ethanol/formic acid procedure. Axenic cultures were shown to be the preferred sample used with xenic broth cultures not having as high ID scores. This study did have a small study number though and showed the limitations of the xenic cultures. This does however highlight an exciting area of research for the future which could substantially cut costs for *Blastocystis* detection and speciation.

Before 2007 there was not a standardised subtype nomenclature and several studies assigned the same subtypes to different groups creating some confusion and meaning an overall global picture of *Blastocystis* molecular epidemiology was not possible. A consensus was agreed upon in 2007 and since then a greater picture of subtype infection has been possible [6]. Although there is now a standardised nomenclature, there is still not a standardised method for subtyping with people doing sequencing on different regions of the SSU rDNA or using Sequence-Tagged-Site PCR for subtype designation. Through the application of these different techniques it has been shown that there is substantial antigenic and genetic heterogeneity among *Blastocystis* isolates within and among geographical regions. Special care must be taken when choosing the primers used in PCR where certain primers can have an effect on the final result. Some primers have specific subtype amplification preferential, which could result in other subtypes being missed in the analysis. Also some primers have been developed from cultured samples, whereas others work better with direct extraction of DNA from the stool so this needs to be taken in to consideration when using molecular techniques for diagnosis. The primers that are being used may have different sensitivity depending on what sort of sample is being used and may give an incorrect result. There is still much more work needed to develop a simple and effective method for the detection and typing of *Blastocystis* sp. A RT-PCR that is able to detect and allocate all of the subtypes would be the most useful for diagnosis as well as epidemiological studies whereas the “barcode region” may be the most effective technique for phylogenetic studies and showing evolutionary relationships.

Epidemiology

The prevalence of *Blastocystis* infection was stated as being higher in developing countries than in developed countries [29]. Due to varying modes of diagnostic techniques used in epidemiological studies of *Blastocystis*, it is difficult to determine the actual prevalence of *Blastocystis* due to some studies relying solely on microscopy for diagnosis. It was shown that molecular techniques have the highest sensitivity rates for diagnosis of this organism [30,31] and the use of only microscopy in diagnosis and epidemiological studies limits interpretation of data on prevalence of *Blastocystis* infections.

Through the use of molecular techniques for epidemiological studies, ST3 was found to be the predominant subtype isolated in humans with a range of 40-92% of isolates belonging to this group

[10,13,32]. There have been two studies where ST1 was the predominant subtype with 51.4% of positive from this group belonging to this group in China, 41% in Brazil [17,33,34] and three others where ST4 was the predominant subtype with 63% in France, 84% in Nepal and 94.1% in Spain, with no ST3 identified in the Spain population [22,35,36]. From South American countries it is seen that there is a predominance of ST1, ST2 and ST3 with no other STs being isolated. ST1 appears to be the predominant ST from these South American studies followed by ST2 then ST3 with no ST4 noted [34,37].

In Singapore the prevalence of *Blastocystis* is at 3.3% of the studied population with ST1 and ST3 being the only subtypes isolated. There was a 78% incidence of ST3 and a 22% incidence of ST1. This prevalence of ST3 in humans in an urbanised area such as Singapore is consistent with the argument that ST3 is of human origin [38].

An investigation of isolates of *Blastocystis* in Japan, Pakistan, Bangladesh, Germany and Thailand showed that ST3 was the most common varying from 41.7-92.3% positives with the second most common being ST1 (7.7-25%) and ST4 (10- 22.9%). In Bangladesh an almost equal number of symptomatic and asymptomatic isolates were tested but there was no major percentage difference between ST1 and ST3 so it is not possible to comment on the pathogenicity of these two subtypes [18].

The incidence of *Blastocystis* in a province in China showed 32.6% of people studied were positive for *Blastocystis*. ST3 was the predominant subtype in 70.5% of the positive population, followed by ST1 with 20.5%. The significance of hygienic practices was shown to have an effect on the type of infection with ST3 corresponding to the drinking of unboiled water and ST1 relating to the consumption of raw water plants [39].

In Lebanon there was a 19% incidence of *Blastocystis* from a population of symptomatic and asymptomatic people [40]. Four subtypes were identified with almost equal numbers of ST3 (33.3%), ST2 (33.3%) and ST1 (30.6%) and only one patient with ST4 (2.8%). There was an association seen between ST1 and symptoms.

A study performed to show the incidence of intestinal parasites among immigrants in food handling and housemaid jobs in Qatar showed that of the 1337 people surveyed from the Philippines, Indonesia, Indian sub-continent and Africa over two years, there were 588 individuals positive for at least one of the parasites studied. Of all the parasites studied, *Blastocystis* was shown to have the second highest incidence rate at 13.2% for all the nationalities put together with only a slightly higher incidence in African samples than the other

nationalities. There was a much higher incidence in women than men [41].

A study of an aborigine community in Pahang, Malaysia showed that *Blastocystis* had the highest incidence rate (52.3%) followed by *G. lamblia* (29.2%). Overall there was a 72.3% positive parasitic infection rate for this community. These high rates of parasitic infection can be attributed to the low standard of sanitation and hygiene in this area with there being no electricity, running water or toilets inside their dwellings and water from the river being used for all purposes including cooking, bathing, swimming and washing vegetables [42].

In a Zambian school, 53.8% of children were reported to be infected with *Blastocystis* with some being co-infected with *Endolimax nana* (which is another parasite not considered to be pathogenic to humans) with almost half the children suffering from diarrhoea. This study supports the argument that *Blastocystis* infections are associated with inadequate sanitation and low hygienic standards and can contribute to diarrhoea in children in developing countries [43].

For travellers, it was shown that there is an increased rate of infection while travelling to tropical and under-developed countries, with 30% infection rates found in tourists in Nepal. It is frequently identified in stools from foreign workers in South-east Asia and Taiwan, with the incidence of infection being attributed to poor personal and environmental hygiene, lack of water supply and poor sewage and rubbish removal [44]. For 36 Peace Corps volunteers (PCV's) in Guatemala for a length of two years or greater there was a 38.8% infection rate with an intestinal parasite. *Blastocystis* was the most common parasite in these samples at 77.8% with a progressive increase in infection from 11.1% of PCV's at the start to 37.9% and then 53.1% and then 77.8% at the end of the 2 year study. Most of the *Blastocystis* infections were found to be asymptomatic and last several weeks. The prevalence of intestinal parasites found in these PCV's in Guatemala could be attributed to them being repeatedly exposed to potentially risky diets and diarrheal illness is the most common medical disorder encountered by travellers from developed countries to developing countries [45].

In an orphanage in Thailand over a one year period the rate of infection varied between 6.6%- 27.4% with samples being taken every two months [46]. These varying results could have been due to the shedding effect seen in many cases of parasitic infection as well as the diagnostic technique (microscopy of a permanent smear and culture) being used not being sensitive enough. These results also highlight the easy transmission of *Blastocystis* in places with poor hygiene. Table 1 summarises the different subtypes isolated in different countries.

Country/region (no. Of individuals studied)	No. Of positive isolates	Subtypes identified (%)											References
		1	2	3	4	5	6	7	8	9	unknown/ mixed		
Australia (513)	91	28	5	40	12	0	6	1	2	0	0		[47]
Australia	12	41	0	33	16	0	8	0	0	0	8		[48]
Bangladesh	26	7.7	0	92.3	0	0	0	0	0	0	0		[18]
Brazil (382)	66	41	32	17	0	0	0	0	0	0	10		[34]
China, Yunnan (239)	78	20.5	1.3	70.5	1.3	0	0	0	0	0	5		[39]
Colombia	125	56	32	12	0	0	0	0	0	0	0		[37]

Denmark	29	3.4	20.7	51.7	24.1	0	0	0	0	0	0	[49]
Denmark (126)	24	8.3	12.5	25	37.5	0	0	0	0	12.5	0	[50]
Denmark (444)	25	4	16	4	76	0	0	0	0	0	0	[51]
Egypt	44	18.2	0	54.5	0	0	18	9.1	0	0	0	[52]
Egypt	100	15	0	49	0	0	33	13	0	0	10	[53]
France	40	25.6	9.3	53.5	9.3	0	0	2.3	0	0	7.5	[32]
Germany (67)	12	25	0	41.7	0	17	0	17	0	0	0	[18]
Germany	171	23.4	0.6	69	7	0	0	0	0	0	3	[12]
Greece	45	20	13.3	60	2.2	0	2.2	2.2	0	0	0	[54]
Italy (34)	34	8.8	20.6	47.1	17.7	0	0	2.9	2.9	0	4	[55]
Iran	40	50	10	40	0	0	0	0	0	0	12	[56]
Iran	141	34	23	37	0	0	0	5	0	0	23	[57]
Japan	64	15.6	20.3	48.4	10.9	0	0	0	0	0	4.7	[13]
Japan (2,037)	50	8	0	52	4	0	22	10	0	4	0	[18]
Lebanon (220)	42	30.6	33.3	33.3	2.8	0	0	0	0	0	0	[40]
Liberia	25	28	28	32	12	0	0	0	0	0	20	[9]
Libya	38	50	7.9	39.5	0	0	0	2.6	0	0	0	[9]
Malaysia	40	12.5	5	50	27.5	0	0	0	0	0	2	[58]
Nepal (82)	20	20	20	60	0	0	0	0	0	0	0	[59]
Nepal (241)	63	63.5	12.7	0	84.1	0	0	0	0	0	70	[36]
Netherlands (442)	103	22	22	42	0	0	1	1	0	0	0	[60]
Nigeria	22	45.5	0	40.9	13.6	0	0	0	0	0	0.4	[9]
Pakistan	10	20	0	70	0	0	10	0	0	0	0	[18]
Philippines	5	20	0	80	0	0	0	0	0	0	0	[61]
Singapore (276)	9	22.2	0	77.8	0	0	0	0	0	0	0	[38]
Spain	51	2	3.9	0	94.1	0	0	0	0	0	0	[35]
Sweden	63	15.9	14.3	47.6	20.6	0	0	1.6	0	0	0	[62]
Thailand (30)	5	40	0	40	0	0	20	0	0	0	20	[18]
Thailand (675)	127	77.9	22.1	0	0	0	0	0	0	0	0	[63]
Turkey (286)	92	20	24	59.7	0	0	0	0	0	0	4	[64]
Turkey	87	9.2	13.8	75.9	1.1	0	0	0	0	0	0	[65]
Turkey	25	36	24	10	0	0	0	0	0	0	0	[66]
UK	55	5.4	16.4	40	31	0	0	1.8	5.4	0	0	[4]
UK	271	12.5	9.6	42.1	31.4	0.7	0.4	1.5	1.8	0	0	[9]
USA	21	11.1	0	66.7	0	0	0	0	0	0	22.2	[2]

Table 1: Subtype classification of human *Blastocystis* sp. isolates from different countries

From all the epidemiological studies a pattern can be seen where there is a much higher incidence of ST4 in Europe and Australia compared to Africa, the Middle East and South America where very low numbers, if any, are seen. Why this is occurring is unknown, but it could be due to several reasons. The use of diagnostic tool may have an effect on the STs identified, as previously discussed, with some primer pairs showing ST preferential. Another reason may be due to the recent evolution of ST4. There is a much higher homogeneity among ST4 samples compared to ST3, which is the closest related ST from phylogenetic studies, suggesting that this may be a relatively new ST [24].

Conclusions

The future of *Blastocystis* diagnostics and epidemiology is an exciting area. With the possibility of new genomes being available for the different subtypes, this may help improve targets for RT-PCR that will be beneficial for both diagnosis and differentiation between subtypes. As diagnosis and speciation becomes easier, more epidemiological studies will be possible which may potentially show the large range of *Blastocystis* distribution around the world. As more samples are studied, the relationship between geographical location and subtype might become apparent as well as intra-subtype evolutionary relationships.

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