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Running Title: Recent *Blastocystis* Research

Recent developments in *Blastocystis* research

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Abstract:

Blastocystis is a common parasite of the human large intestine but has an uncertain role in disease. In this review, we appraise the published evidence addressing this and its weaknesses. Genetic diversity studies have led to the identification of numerous subtypes within the genus *Blastocystis* and, recently, methods for studying variation within subtypes have been developed, with implications for our understanding of host specificity. The geographic distribution of subtypes is summarised and the impact this may have on investigations into the role of the organism in disease is discussed. Finally, we describe the organelle and nuclear genome characteristics and look to future developments in the field.

Key words:

Blastocystis, small subunit ribosomal RNA gene, phylogeny, multilocus sequence typing, taxonomy, Irritable Bowel Syndrome, genome

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1. INTRODUCTION

Organisms assigned to the genus *Blastocystis* are the most common eukaryotes reported to colonise humans, yet remain an enigma on many levels. Despite having been described more than 100 years ago (Alexeieff, 1911; Brumpt, 1912), the question of whether *Blastocystis* causes disease or is a commensal of the human gut still has no definitive answer. Our understanding of its taxonomy has improved but our knowledge of its genetic diversity, host specificity and geographic distribution remains very incomplete. This review will critically evaluate the information that has recently become available on the pathogenicity of this organism, summarise our understanding of its prevalence, diversity and distribution, and give an overview of the data emerging from genome projects.

2. BACKGROUND

After bouncing between many taxonomic 'homes' during most of the 20th century, *Blastocystis* finally came to rest among the stramenopiles in 1996 (Silberman et al., 1996), a grouping that did not exist before 1989 (Patterson, 1989). *Blastocystis* is an atypical stramenopile as this group is named for the straw-like tubular hairs on the flagella and sometimes the cell body – *Blastocystis* has no flagella or tubular hairs. The link was made using phylogenetic analysis of small subunit ribosomal RNA gene (SSU-rDNA) sequences and has been confirmed using other gene sequences (Arisue et al., 2002). Within the stramenopiles, *Blastocystis* is specifically related to the Proteromonadidae and Slopalinida (Kostka et al., 2007), which are mostly commensal flagellated or

ciliated organisms found in reptiles and amphibia. They include the genera *Proteromonas*, *Opalina*, *Protoopalina*, *Karotomorpha* and *Cepedea*. The absence of typical stramenopile morphology in *Blastocystis* is clearly the result of secondary loss.

In fact, *Blastocystis* morphology is not well understood. A large number of morphological cell types have been described (Zierdt, 1991) but, in the opinion of the present authors, many are likely to be artifactual and due to oxygen exposure rather than actually occurring in vivo (Stenzel et al., 1991; Vdovenko, 2000). Nevertheless, it can be stated confidently that *Blastocystis* is normally a spherical cell of ca. 5–10 μm in diameter that is multinucleated and contains multiple mitochondrion-like organelles, Golgi apparatus and other typical eukaryotic cellular features. Transmission of infection is via a small cyst stage that is difficult to detect in stool samples (Stenzel and Boreham, 1991) – when *Blastocystis* is observed by light microscopy, it is primarily the vegetative form that is noted. Under the electron microscope, the nucleus has a distinctive appearance, with a crescent of dark-staining chromatin being seen on one side (Zierdt, 1991).

The secondary loss of morphology has been responsible for much of the confusion surrounding species names and host ranges, because all small spheres look much alike. As a result, species names for *Blastocystis* were until recently linked to the host in which they were found – the prime example being *Blastocystis hominis* as the name applied to all *Blastocystis* seen in humans. The advent of nucleic acid-based analyses in the mid-1990s quickly revealed two

things: 1. The SSU-rDNA from *Blastocystis* in humans is genetically extremely diverse; 2. SSU-rDNA from *Blastocystis* in other hosts can be indistinguishable from that in humans (Böhm-Glönig et al., 1997; Clark, 1997). This meant that host origin was not a reliable indicator of organism identity and that some other means of identifying types of *Blastocystis* would be necessary.

Over the next 10 years, molecular analyses of *Blastocystis* became quite popular and a number of groups in different parts of the world were working independently to understand the significance of genetic diversity in *Blastocystis*. This had an unfortunate consequence, namely that each group came up with its own nomenclature to denote the *Blastocystis* molecular types that they detected. In addition, two distinct methods of analysis were being employed: SSU-rDNA sequencing and PCR amplification of sequence-tagged sites (STS). The former has the advantage of generating quantitative data and has the ability to detect new molecular types as they are uncovered. The latter (Yoshikawa et al., 2004b) has the advantage of better detection of mixed infections since a separate PCR reaction is performed to detect each major type, but has the limitation of only detecting seven known types.

By 2006, the literature had become almost impenetrable for anyone not intimately involved in *Blastocystis* typing, and even then it required an ability to cross-reference between nomenclatures to interpret newly published data. Adding to the confusion, the identifiable variants were given different names – genotype, clade, group, subgroup, subtype and ribodeme. As a result, a consensus was developed and was published in 2007 (Stensvold et al., 2007b). The

consensus relied on multiple types of data being available for some strains (eg. ribodeme + sequence or STS + sequence) to allow extrapolation of the nomenclature to other strains that shared the same characteristics. Today, for the most part (but not always) new publications follow the consensus nomenclature for 'subtypes' of *Blastocystis*. The proposed replacement of all names for avian and mammalian species, including *Blastocystis hominis*, with the identifier '*Blastocystis* sp.' followed by the subtype number is also widely employed. This consensus terminology will be followed throughout this review.

It is perhaps appropriate here to propose a standard approach to subtyping. While we recognise that DNA sequencing will not be easily available to everyone, the advantages greatly outweigh those of STS. The method used to develop STS was sequencing of randomly amplified genome fragments followed by the development of specific primers. These were then validated by testing them against a panel of isolates of various subtypes. Now that much more is known regarding diversity of *Blastocystis*, we feel that re-validation would be appropriate. In our hands, the STS primers for subtype 4 only amplify one of the two clades in this subtype, at least in faecal DNA. As mentioned above, only known subtypes can be detected and, as more have been named, the specificity of STS has not been further explored nor the range of detectable subtypes expanded. STS is certainly of limited use if non-human samples are of interest. STS typing is also more dependent on interpretation – size and specificity of bands, for example – than is sequence analysis.

In contrast, SSU-rDNA sequencing is a pan-*Blastocystis* technique and not limited to known subtypes. It has been shown that sequencing of the complete gene is not necessary for accurate subtype classification, as long as a diagnostic region is used that is known for all subtypes. Several regions of the gene (Fig. 1) have been used by different authors for this purpose (Parkar et al., 2010; Santín et al., 2011; Scicluna et al., 2006; Stensvold et al., 2006) but in our hands the specificity of amplification and ease of sequencing of the 'barcode' region (Scicluna et al., 2006) at the very 5' end of the gene make it the region of choice for subtyping. That is not to say it is universally successful. It is likely that all of the primer pairs used occasionally produce non-specific amplicons, especially when screening DNA extracted directly from faeces and when the sample is actually negative for *Blastocystis*.

The barcode region is by far the best represented in the databases, and the correct subtype can be identified by BLAST analysis in either GenBank or the new *Blastocystis* MLST database (www.pubmlst.org/blastocystis; (Jolley and Maiden, 2010; Stensvold et al., 2012a). The latter has the added advantages of automatically assigning allele types to the SSU-rDNA as well as using the consensus subtype nomenclature (unlike GenBank where the subtype is included only if one was part of the accession submission and no attempt to impose a standard nomenclature is made). Because of the occasional problem of non-specific amplification, it is recommended that samples be screened first for positivity, where possible, using Real-Time PCR (Stensvold et al., 2012b) before undertaking subtype identification by sequencing.

3. SUBTYPES

3.1 Current status

The use of numbers has the advantage of allowing new subtypes (STs) to be assigned to novel sequences as they are discovered. However, this requires a consensus, which does not as yet exist, on what the requirements are for designation of new subtypes. We suggest that new subtype assignments be based on complete or essentially complete SSU-rDNA sequences, not on just a small piece of the gene like the barcode region, even though this may well be how their novelty is first identified. In most cases, this should not be a burdensome requirement, especially if cultures exist, but occasionally primary material might be limited and inhibitors/non-specific amplicons can interfere with successful sequencing of some products.

An example of how necessary this approach can be exists in the case of ST13. The subtype was found in a Quokka (a marsupial) and named by Parkar et al. (2010) in Australia, who showed it to cluster near ST5 in phylogenetic trees. Their sequence consisted of the 3' two-thirds of the SSU-rDNA, over 1,000 bases (Fig. 1). Later, Petrášová et al. (2011) identified an infection in a Colobus monkey in Tanzania as ST5 based on the sequence of the 5' one-third of the gene – the barcode region defined by Scicluna et al. (2006). The Colobus sequence was not identical to previously-known ST5 sequences but that was the most closely related subtype in the databases. In actual fact, the Colobus sequence belonged to ST13. This only became apparent when we compared the Colobus and Quokka sequences to a complete ST13 sequence we had obtained independently (from a

deer; Alfellani et al., submitted). The overlap between the 'barcode' region and the Parkar sequence is short and shows little variation among related subtypes. Hence the link between the Petrášová et al. Colobus sequence and the available ST13 sequence was not obvious.

Nevertheless, agreement on how different a new sequence needs to be before being considered a novel subtype does not exist at present. In 2007, only nine subtypes were known and all had been identified in humans. All formed discrete clades in phylogenetic trees that were supported by maximum posterior probabilities in Bayesian analyses and very high bootstrap values in maximum likelihood analyses. The minimum divergence between sequences assigned to different STs was around 5%. As more hosts are sampled and more sequence variants are discovered, two things are happening. The amount of known diversity within existing subtypes is increasing and more potentially new subtypes are being uncovered that differ by less than 5%. In our opinion, when the divergence from known subtypes is less than this arbitrary figure, care needs to be taken before assigning a new subtype number unless substantial sampling has taken place. The reason for this is exemplified by ST3; in this subtype (and some others), the most divergent SSU-rDNA sequences differ by almost 3%, yet the clade itself is strongly supported. If a single new sequence is found that differs from others in a known clade by just over 3%, one of two things can happen – 1. Additional sampling may 'fill in' the gap between the new branch and the existing clade, indicating that it is part of the same subtype; or 2. Additional sampling will identify sequences that are specifically related to the new variant and do not fall in-between, in which case it can be considered a new subtype.

Initially, new variants will often be represented by a single example and so the problem remains of what to call them. In our opinion, a new sequence type that differs by 4% or more can be considered a new ST with confidence. A sequence that differs by less than 1.5% is most likely to fall within the range of variation of an established subtype. Those that fall between these cut-off values can be tentatively assigned new ST numbers subject to confirmation by further sampling – in a way this resembles the ‘Candidatus’ species names used for bacteria although we would prefer not to use such a prefix for *Blastocystis* STs! Ultimately, like species names, *Blastocystis* ST designations will be accepted by those in the field or rejected as synonyms based on further data (see (Boenigk et al., 2012)).

One thing rarely mentioned is that the *Blastocystis* ST nomenclature refers exclusively to organisms infecting birds and mammals, as these two host groups share many of the same subtypes even though some host specificity exists. *Blastocystis* is common in reptiles and amphibia, and has been reported from other hosts, such as insects. The assumption exists that such organisms are unlikely to overlap with the bird/mammal subtypes because of the different body temperatures of the hosts. However, this need not always be the case – one toad sequence reported belongs to ST5 (Yoshikawa et al., 2004a). Nevertheless, most non-bird/non-mammal *Blastocystis* have their own species names or at least do not cluster with bird/mammal subtypes. Should interest lead to greater sampling of such hosts, perhaps a similar approach to that outlined above will prove necessary to prevent the nomenclature from becoming unwieldy.

When the consensus subtype nomenclature was developed, nine STs were recognised. Sampling from a wider range of hosts has led to five additional STs being published; several additional unpublished STs are known to us. We have no doubt that many more remain to be uncovered as more hosts and more individuals within already-sampled hosts are studied. Therefore, to discuss host specificity at this stage is certainly premature in that we know the picture is incomplete. However, some general trends are starting to emerge that are worth commenting on.

Of the nine definite subtypes detected in humans, only four are common – ST1, ST2, ST3 and ST4. Together, these make up around 90% of all human *Blastocystis* in surveys that involve subtyping (Alfellani et al., 2013). These will be discussed further below. The other subtypes are only sporadically reported in humans and may well prove to be the result of zoonotic transmission, as they are mostly much more common in non-human hosts. ST5 is prevalent in livestock, ST6 and ST7 occur frequently in birds, and ST8 is common in some non-human primates (NHPs). Of the STs that are rare in humans, only ST9 is yet to be reported from non-human sources.

Although ST5 is rare in humans, it is found commonly in captive apes, although not in other NHPs (Stensvold et al., 2009a). Its highest frequency seems to be in livestock, particularly cattle, pigs, sheep and camels (Stensvold et al., 2009a). Subtypes 6 and 7 have been detected primarily in ground-dwelling birds, with only single ST7 samples from a goat and a NHP having been found in non-human mammalian hosts (Alfellani et al., in press and submitted). Other than in humans,

ST8 appears to be restricted to arboreal NHPs from Asia and South America – it has not been reported from African NHPs (Alfellani et al., in press). The STs with numbers above 9 are, as far as is known at present, confined to non-human hosts. Little experimental work on host-specificity has been performed (Iguchi et al., 2007) and given that diversity within subtypes may be linked to host range (see below), the results in this respect must be seen as preliminary.

3.2 Intra-subtype diversity

As discussed above, analysis of SSU-rDNA sequences within certain subtypes has revealed substantial genetic diversity (Scicluna et al., 2006; Yoshikawa et al., 2009). Since SSU-rRNA genes are generally highly conserved within species, this finding suggested that the study of variation within subtypes might lead to further insights into host range and transmission patterns, as well as potentially identifying surrogate markers for virulence. In particular, it would help in determining the relevance of the subtyping system, which could be too crude a classification tool. Investigations into genetic diversity using non-SSU rRNA genes have therefore started. A multilocus sequence typing (MLST) system has been developed for ST3 and ST4 (Stensvold et al., 2012a). This is based on the sequencing of 5–6 loci in the genome of the mitochondrion-like organelle (MLO; see below) chosen for the presence of polymorphism. MLST systems for ST1 and ST2 are currently in development, also based on markers in the MLO. MLST has been used primarily in bacteria – its utility in eukaryotes is restricted by the fact that most are diploid or have higher ploidy. The existence of heterozygotes makes interpretation of the data much more difficult than in haploid organisms. The organelle genome in *Blastocystis* is effectively haploid, but this characteristic

was only part of the reason for selecting the MLO as the target for our MLST – very few nuclear gene sequences were available for *Blastocystis* at the start of the project so options were limited!

Application of the MLST system to samples from humans and non-human primates has already led to some important observations. The phylogenetic tree obtained from analysis of the SSU-rDNA sequence (the barcode region – Fig. 1; (Scicluna et al., 2006) is congruent with the one inferred from sequences of loci in the MLO for both ST3 and ST4 (Stensvold et al., 2012a). Hence, the MLST data have so far validated the use of the barcode region as a suitable marker for inter- and intragenetic diversity.

The levels of intragenetic diversity in ST3 and ST4 differ dramatically. MLST analyses of ST3 isolates showed a high discriminatory index compared to the one obtained for ST4; in other words most strains can be distinguished by MLST in ST3 while that is not the case for ST4. ST4 from humans shows a surprising degree of genetic homogeneity, with most SSU-rDNA and MLO loci sequences being completely identical between samples (Stensvold et al., 2012a).

Conversely, many SNPs in MLO loci of ST3 are shared by only a few strains or are unique. Importantly, the differences between ST3 and ST4 are not attributable to the fact that ST4 samples were almost exclusively from humans, since the vast majority of the ST3 alleles were detected within the human population. Due to the homogeneity of ST4, and perhaps also because of the fact that ST4 appears to be absent or at least very rare in some parts of the world, we speculate that ST4 entered the human population relatively recently compared to ST3. ST4 is

common in Europe, but is rarely reported from Asian, Middle Eastern and South American populations; however, in many regions comparatively little sampling has been undertaken.

ST3 is the most common subtype in humans worldwide, and its occurrence is a frequent finding in analyses of subtype distribution, irrespective of the geographic origin of the population (Forsell et al., 2012; Malheiros et al., 2011; Meloni et al., 2011; Nagel et al., 2011; Souppart et al., 2010; Souppart et al., 2009; Stensvold et al., 2009a; Stensvold et al., 2009b; Stensvold et al., 2011b). The high discriminatory index of the ST3 MLST system makes it useful for surveillance of ST3 strains (re-infection or recrudescence; longevity of colonisation; patterns of transmission), whereas that of ST4 would not be suitable for these purposes. Nevertheless, two clades can be detected in ST4 SSU-rDNA and MLST analyses, one of which consists mostly of non-human samples. Little is known about the host range of ST4 but, in addition to humans, it has been found in rodents and occasionally in non-human primates (lemur (Santín et al., 2011; Stensvold et al., 2009) and woolly monkey (Alfellani et al., in press)).

Interestingly, MLST analysis of ST3 isolates from humans and non-human primates revealed that NHP ST3s are significantly more diverse than human ST3s, most of which fell into one clade. Human ST3 samples are found only rarely in the 'NHP clades' and are likely a result of zoonotic transmission, illustrated by the fact that one of the few such human isolates was from a NHP keeper. In contrast, NHP samples were also detected in the clade containing the vast majority of the human sequences. Together, these results suggest that ST3 has

largely co-evolved with humans, but that either this co-evolution has been going on for a long time or 'human clade' ST3 has entered the human population repeatedly from another source. This is in contrast to ST4, where the same restriction of human samples to one clade exists but little sequence diversity is detected, a finding that implies a single and relatively recent origin in humans followed by clonal expansion. ST3 has also been reported from a number of non-primate hosts and MLST analysis of such ST3s is needed to increase our understanding of the currently observed cryptic host specificity and thus the transmission and epidemiology of *Blastocystis*. Both clades of ST4 have been detected in rodents but relatively little sampling has been reported, so it is not yet clear whether these hosts are a significant reservoir for human colonisation.

4. GEOGRAPHIC VARIATION IN *BLASTOCYSTIS* PREVALENCE

The prevalence of *Blastocystis* is reported in many parasite surveys performed across the world. Published infection rates fall anywhere between 0.5% and 62%. A serious problem with such data is the often highly selected nature of the population studied. Only rarely are the surveys large enough or the population examined diverse enough to really conclude that the infection rate reported is representative of the country/cohort as a whole, yet that is often how the data are interpreted. A good illustration of this is where more than one survey has been performed in the same country. For example, two studies carried out in Malaysia found prevalences of 15% (Suresh et al., 2001) and 52% (Noor Azian et al., 2007), while two in Turkey reported 2% (Köksal et al., 2010) and 14% (Ostan et al., 2007). In Turkey, the studies were undertaken in different cities. One

surveyed school children (Ostan et al., 2007), many of whom lived in a shanty town, while the other surveyed adults. The diagnostic techniques used, however, were basically the same. In Malaysia, the age range of the populations was similar but one population lived in apartments in the capital (Suresh et al., 2001) while the other (Noor Azian et al., 2007) was in an aboriginal village settlement. In one of these Malaysian studies, a variety of techniques - including culture - were used for diagnosis of infection, while the other involved microscopy only. In both countries, the populations were demographically quite different in several ways and whether either could be viewed as truly representative of the country as a whole is debatable. The diagnostic technique used is potentially another significant variable that can influence the reported prevalence (fresh stool vs. fixed and concentrated; bright field vs. stained) as microscopy is generally thought to be less sensitive than culture, although the skill of the microscopist is also a significant factor. In the future, it seems likely that diagnostic PCR will become the tool of choice where it can be afforded, which will make comparison of results with studies using only microscopy even more difficult.

5. LINKING *BLASTOCYSTIS* TO DISEASE

5.1 Prevalence and intensity of infection

Like the simple population prevalence surveys, many comparisons of *Blastocystis* prevalence in symptomatic and asymptomatic individuals have been published. Approximately equal numbers of papers report significantly higher prevalences of *Blastocystis* in symptomatic individuals and no significant difference at all. Here, some of the same variables are notable confounding factors – how can

studies be compared in which researchers have variously used fixed material or fresh, direct or concentrated samples, iodine, Trichrome, or haematoxylin as the stain for microscopy? Furthermore, shedding of *Blastocystis* may be cyclical (Vennila et al., 1999), yet it is unusual for more than one stool sample to be examined. However, when the aim is to explore links to disease, the main complication is again the selection of the populations – in this case, the definitions of symptomatic and asymptomatic and comparability of the symptomatic group and the asymptomatic controls. Most, if not all, investigations have been cross-sectional, meaning that carriers will have harboured *Blastocystis* for different periods of time, which may affect whether they are experiencing symptoms – if acquired immunity to *Blastocystis* plays a role in symptom resolution.

Since *Blastocystis* is a faecal-orally transmitted parasite, carriers will also have been exposed to other intestinal organisms, some of which may be pathogens, at the time of *Blastocystis* colonisation. Most association studies do not exclude all other possible origins of the symptoms. Likewise, most control populations are not case-matched and there is some suggestion that ‘asymptomatic’ individuals who volunteer for such studies do not represent a random selection but may have a history of intestinal problems (Stensvold et al., 2011a; Stensvold et al., 2009b). However, one of the most peculiar variables used is the fact that some studies define colonisation with *Blastocystis* as having more than 5 organisms per microscope field. The rationale for this is unexplained – why would having 4 vs. 6 organisms per field be a significant difference? The recent descriptions of Real-Time PCR diagnostic tools for *Blastocystis* (Poirier et al., 2011; Stensvold et

al., 2012b) will make detection of the organisms easier as well as allowing exploration of any role for infection intensity in symptomatology.

One might assume that animal models are an obvious way of potentially establishing a link between *Blastocystis* and pathology. However, the studies performed to date are, in our opinion, inconclusive. For example, experimental infections of laboratory mice (Elwakil and Hewedi, 2010) resulted in tissue invasion – something never reported in humans. Another study showed increased oxidative stress in *Blastocystis*-infected rats (Chandramathi et al., 2010), again something not linked to human colonisation. Studies that provided evidence for induction of cytokines, contact-mediated apoptosis, and barrier disruption all used axenic *Blastocystis* and in vitro mammalian cell cultures, with no evidence provided that these effects occur in vivo. One other issue is the use of appropriate controls – for example, experimental infection of animals with *Blastocystis* from cultures growing in the presence of bacteria need to have the appropriate controls – namely, exposure to the accompanying bacterial flora alone – before it can be concluded that *Blastocystis* is responsible for any effects seen (Hussein et al., 2008). It has to be said that, to date, animal models are not showing much promise in resolving the question of the pathogenic potential of *Blastocystis*.

5.2 Links to Irritable Bowel Syndrome

One of the popular associations made in the literature is between *Blastocystis* and Irritable Bowel Syndrome (IBS; (Poirier et al., 2012). There are two reasons for this. The most telling is that people diagnosed with IBS appear in several

studies to have a much higher infection rate with *Blastocystis* – often twice as high or more (Giacometti et al., 1999; Jimenez-Gonzalez et al., 2012; Yakoob et al., 2010; Yakoob et al., 2004). The second is that many of the symptoms ascribed to *Blastocystis* infection are very similar to those defining some types of IBS (diarrhoea, vomiting, abdominal cramps and bloating), suggesting either that *Blastocystis* colonisation may be a differential diagnosis or that *Blastocystis* is the causative agent in some cases of IBS. Alternatively, it could mean that *Blastocystis* colonises the IBS gut more efficiently than a healthy gut. As mentioned above, the presence of *Blastocystis* means exposure to faecal organisms and so superficially at least the data support a link between faecal exposure and IBS rather than a specific link to *Blastocystis*.

A diagnosis of IBS should include the exclusion of other potential causes of the symptoms, but it is not always possible to tell from publications whether this has been done and what pathogens have been excluded – it is unlikely that every possible intestinal disease agent has been tested for. IBS is also not a disease with a unique and specific diagnosis; it is a syndrome having several different forms. Diagnosis is currently based on the Rome III criteria: “Recurrent abdominal pain or discomfort at least 3 days per month in the last 3 months associated with 2 or more of the following

- Improvement with defecation
- Onset associated with a change in frequency of stool
- Onset associated with a change in form (appearance) of stool”

The “change in frequency” covers diarrhoea (IBS-D) and constipation (IBS-C) or alternation of the two (IBS-M), yet in several *Blastocystis* studies these are not differentiated.

One illustration of the problem can be found in the study by Giacometti et al. in Italy (1999). The authors compared *Blastocystis* prevalence in IBS patients (15/81) to that in patients with other gastrointestinal complaints (23/307) and found it to be significantly different ($p=0.006$). However, when the IBS patients were followed up 6 months later, 53/72 returning patients no longer met the criteria for a diagnosis of IBS. Since IBS is considered a chronic disease, this calls into question the original diagnosis as such a high rate of ‘cure’ would not be expected.

In a study in Pakistan, Hussain et al. (1997) found that levels of antibody against *Blastocystis* were higher in patients with IBS than in controls but, surprisingly, levels in IBS patients were the same whether the parasite was detected concurrently or not. In Mexico and Thailand, three studies failed to show a higher prevalence of *Blastocystis* in IBS patients (Ramirez-Miranda et al., 2010; Surangsrirat et al., 2010; Tungtrongchitr et al., 2004).

One of the factors that make the interpretation of all these studies of *Blastocystis*/IBS prevalence and association difficult is the existence of nine subtypes in humans that, as judged by molecular criteria, could be considered distinct species. More than 20 countries have been surveyed for the range of subtypes present and this number is gradually increasing. Differences in the

subtypes present in a country could be responsible for the differing conclusions from research investigating links between *Blastocystis* and disease. However, only a small number of studies to date have compared distribution of subtypes in symptomatic and asymptomatic individuals.

From the geographic subtype surveys (Table 1), several interesting observations have emerged. The first is a technical one. Surveys that use the STS method of subtyping detect ST6 and ST7 at a much higher frequency on average than those using SSU-rDNA sequencing. It is difficult to interpret this, however, as the STS method is most widely used in continental Asia and there are no comparable sequencing studies in the same countries that can be compared to STS. The exception to this disjunction is Egypt, where two STS studies found many ST6 and ST7 (48/144) but sequencing did not (0/20). Nevertheless, it is possible that ST6 and ST7 are simply more common in humans in Asia.

The second observation has to do with the distribution of ST4. This subtype is very common in Europe (often the second most common ST found after ST3), but apparently absent in Egypt, Libya, Iran, Nepal, the Philippines, Thailand, Malaysia and Brazil, and rare in many other countries (see summary in Alfellani et al., 2013). Again, there may be some link to the method used (STS in Asia) but not in all countries as sequencing was used in the studies from Libya and Brazil, for example. One possible explanation would be an inability of the STS method to amplify certain clades within subtypes (as discussed earlier). However, if this is the case, more unidentified *Blastocystis* isolates would be expected in STS analysis, and these are not commonly reported. Thus, it appears that ST4 has a

very uneven distribution across the world, with the focus being in Europe – perhaps that is where this subtype entered into the human population in the recent past – while STs 6 and 7 are rare in humans outside of Asia. There is no immediately obvious reason why humans in Europe might be more exposed to rodents and those in Asia to birds, so zoonotic transmission seems an unlikely explanation. Perhaps some dietary, cultural or other demographic variables are responsible – at present, it is not useful to speculate.

Three studies have investigated the possibility of a link between *Blastocystis* subtypes and IBS. However, the results are very inconsistent. In Pakistan (Yakoob et al., 2010) and Egypt (Fouad et al., 2011) significant differences in subtype distribution were found, with ST1 being much more common in IBS patients than controls. A study in the UK found ST4 to be more common and ST1 less so in patients from IBS clinics than in other diagnostic laboratory samples, but not reaching statistical significance (Alfellani et al., 2013). Again, there was a difference in methodology (STS vs. sequencing) as well as many other variables, not the least of which is geography and the presence of ST4, so the potential relationship needs further investigation. What can be concluded is that no single subtype is found in IBS patients. Perhaps this is not surprising, given the variability in the combination of symptoms that can lead to a diagnosis of IBS.

5.3 Case studies

The other source of information on the link between subtype and symptoms has been individual case studies. In such reports, an individual with gastrointestinal symptoms, *Blastocystis* infection, and no other identifiable cause is investigated

and treated. Often the report correlates clearance of the parasite with resolution of symptoms and, in the recent past, the subtype of the organism present has often been determined. There are two problems with such reports. The first is that the drugs used to 'eliminate' *Blastocystis* are many and varied but have no known specificity for the parasite. It seems equally likely that the treatment perturbs the intestinal flora, indirectly making it a poor or unsuitable habitat for *Blastocystis*. The second problem is that all common subtypes and certain others have been linked with symptoms, which seems an unlikely situation (Dogruman-Al et al., 2008; Domínguez-Márquez et al., 2009; Jones et al., 2009; Stensvold et al., 2008; Stensvold et al., 2011a; Vassalos et al., 2010; Vogelberg et al., 2010). Treatment of *Blastocystis* colonisation is contentious and no widely accepted drug regimen exists (see (Stensvold et al., 2010)). It is certainly not surprising that subtypes differ in their susceptibility to drugs, given the large genetic differences that exist between them (Mirza et al., 2011), and in vitro drug resistance has been generated successfully in the laboratory (Dunn et al., 2012).

5.4 The way forward?

To summarise current data on the relationship between *Blastocystis* and disease is not easy, since we feel that the definitive investigations are yet to be carried out. Because of the valid argument that if disease is associated with one subtype, the signal may be masked when the overall prevalence in a population is considered, we believe that: it is necessary for subtyping to be performed; sequencing should be used if possible in preference to the indirect STS method; and DNA extracted directly from faeces should be used in preference to cultures, in case cultivation selects in favour of certain subtypes. It is possible that

Blastocystis is a pathogen but that this is unlinked to the subtype of the organism. Nevertheless, subtyping should be done if only to rule this out as a variable. It is also important that studies be carried out in more than one country, given the apparent geographic differences in subtype distribution. For example, ST4 is prevalent in Europe and has been linked to symptoms in more than one investigation. If ST4 is the only subtype linked to disease, a study in a country where the subtype has not been reported or is rare will not reveal an association of *Blastocystis* with disease even if one exists.

The appropriate study population is difficult to define. The presence of 4 common subtypes (in Europe at least) means that more individuals are needed in order to have the same power to detect an association. Most crucial of all are the faecal samples themselves. An advantage of using IBS patients is that they have usually had an extensive microbiological workup to eliminate other pathogens as a cause of their symptoms, which is a deficiency in many other studies. However, it seems unlikely that if *Blastocystis* causes IBS, it is responsible for both IBS-D and IBS-C. The question remains of how to obtain a suitable control group for IBS patients.

In many cultures, people are very happy to provide blood samples but reluctant to donate faecal specimens unless they are ill, which has impaired the ability of controlled studies to be undertaken in many countries. Nevertheless, research has been successfully undertaken where a large number of individuals with gastrointestinal symptoms and a similarly large asymptomatic population of individuals have been sampled for investigation of intestinal pathogens (eg.

(Tam et al., 2012). Unfortunately, *Blastocystis* has not been included in those studies.

An alternative approach is to identify a smaller number of individuals infected with *Blastocystis* but no other potential pathogen, and then match these as closely as possible with asymptomatic controls. Recruitment of individuals from web panels (for instance, the 'YouGov' panel in Denmark) has previously been used successfully to obtain data on the prevalence of gastrointestinal symptoms in the background population (Reimer and Bytzer, 2009). Again, the presence of 4 common subtypes means that the sample numbers needed might make this approach difficult. It is also possible that symptoms resulting from *Blastocystis* infection are acute but resolve, and we have no knowledge of how long *Blastocystis* colonisation may persist for afterwards. Until appropriate studies are performed and a clear answer obtained, the question of whether *Blastocystis* is a pathogen will continue to be contentious.

Whatever the ultimate outcome, it must be emphasised that infection with *Blastocystis* is a surrogate marker for exposure to faecal contamination, and in an individual with symptoms and *Blastocystis* colonisation, an infectious agent (whether it is *Blastocystis* or not) seems the most likely cause.

6. GENOME STUDIES

Other than investigations into *Blastocystis* diversity and the role of the organism in disease, the most important recent advances have been those involving genome studies. These consist of projects that have given rise to the sequences of one nuclear genome and several mitochondrial genomes. The latter represent a number of different subtypes and, as mentioned earlier, these sequences have been the basis for development of MLST schemes for the most common subtypes.

6.1 *Blastocystis* MLO genomes

The presence of mitochondrion-like organelles in *Blastocystis* was for many years an enigma as it was unclear why a strictly anaerobic eukaryote needs an organelle traditionally associated with aerobic metabolism. Nevertheless, it was shown quite early on using DNA stains that these organelles contain DNA and this attracted the attention of those interested in organelle evolution. The presence of mitochondrion-derived organelles had been the subject of investigation for some years but it became clear that the mitosomes of *Entamoeba*, microsporidia and *Giardia* and the hydrogenosome of *Trichomonas* are actually quite different end products of reductive evolution from the mitochondrial endosymbiont – and none have retained any trace of a genome in the organelle (van der Giezen, 2009). The possibility that the *Blastocystis* MLO represents an intermediate step in the ‘degeneration’ of the mitochondrion is intriguing.

Three MLO genomes were published almost simultaneously, representing by chance three different subtypes, one from each of the three main clades of human *Blastocystis*: ST1, ST4 (Pérez-Brocal and Clark, 2008) and ST7

(Wawrzyniak et al., 2008). All three encode the same 27 proteins and 18 RNAs, and the gene order is identical. The 27.7–29.2kb genomes were found to contain several *nad* genes, encoding proteins of mitochondrial Complex I, and ribosomal protein genes; none of the genes encoding cytochromes and ATPase subunits found in other stramenopile genomes are present. A reduced set of tRNA genes identified in the *Blastocystis* MLO genome implies that tRNAs for some codons must be nuclear encoded and imported from the cytoplasm.

6.2 *Blastocystis* nuclear genome

Of all stramenopile nuclear genomes sequenced to date, the one from *Blastocystis* strain B (ST7) is the smallest. It is just under 19 Mb in size and contains about 6,000 genes, which is just over half the number in the stramenopile genome with the next lowest number of genes (see Table 2). It is also relatively intron rich, but its introns are by far the smallest found among stramenopiles as the median size is only 32 bp (Denoeud et al., 2011). Although it is useful to make comparisons with other stramenopile genomes, those genomes available are of relatively distant species and none are for human pathogens. Although the *Phytophthora* and *Pythium* genomes can provide some useful reference material because of the pathogenic nature of these organisms (in plants), it should not be forgotten that *Blastocystis* is the only anaerobe among the sequenced stramenopiles, and hence the special features of its genome could have been driven by environmental factors as well as being reflective of its evolutionary distance from the other sequenced stramenopiles.

Nonetheless, it might be useful to look at the presence of potential effector proteins encoded in the *Blastocystis* genome and other proteins that might play a role in pathogenesis. Such analyses might provide useful avenues for exploring the potential pathogenicity of this organism. Many eukaryotic pathogens use effector proteins to remodel their host's cells/tissues into more suitable niches for proliferation. If *Blastocystis* is truly a pathogen, then one might expect it to use strategies similar to those of other eukaryotic pathogens, perhaps in particular those used by *Phytophthora* species. Effectors are molecules that either facilitate infection (virulence factors or toxins) or that trigger host defence (avirulence factors or elicitors) (Kamoun, 2006). In order to be able to affect the host, these effectors need to be secreted by the pathogen, and analysis of the *Blastocystis* genome using SignalP has suggested that 307 proteins contain secretion signals (Denoeud et al., 2011). Whether these potentially secreted proteins contain any additional host cell targeting signals, such as the *Plasmodium* RxLxE/D/Q motif or the *Phytophthora infestans* RxLR motif (Haldar et al., 2006) still needs to be investigated. Among the proteins making up the putative *Blastocystis* secretome are hydrolases, proteases and protease inhibitors. The latter are generally involved in protecting parasite proteins from degradation. The *Blastocystis* genome encodes a cystatin A homologue, a type-1 proteinase inhibitor and an endopeptidase inhibitor-like protein (Denoeud et al., 2011). However, only one of these seems to encode a putative secretion signal, using SignalP, and none contain the Kazal-like domains that are often found in secreted protease inhibitors of eukaryotic parasites (Haldar et al., 2006). The genome is predicted to encode several hydrolases that might be involved in attacking host tissue (Denoeud et al., 2011), although tissue invasion by

Blastocystis has never been observed in humans. The most likely possible effectors found in the *Blastocystis* genome are cysteine proteases, considering that these genes are also present in large numbers in the pathogenic protist *Entamoeba histolytica* (Bruchhaus et al., 2003). Nonetheless, these predictions all need further elucidation in the laboratory in order to determine whether they have any role in pathogenesis and disease and to prove that they are secreted. Two secreted proteases have recently been characterised (Wawrzyniak et al., 2012). It is anticipated that more subtypes will have their genomes sequenced in the near future. This will help to confirm that the oddities of the ST7 genome apply to all *Blastocystis* and are not subtype-specific, and so are relevant to the common human-infective subtypes.

In common with other protistan genomes (Carlton et al., 2007; Loftus et al., 2005), *Blastocystis* seems to contain a number of genes that may have been acquired by lateral gene transfers (LGT). Two possible red algal genes might hint at a lost chromalveolate plastid while others might be involved in some aspects of anaerobic fermentation (Denoeud et al., 2011). The anaerobic nature of *Blastocystis* combined with the presence of MLOs with cristae that are capable of taking up active dyes such as Rhodamine 123 (Nasirudeen and Tan, 2004) has sparked an interest in the nature of these organelles (Denoeud et al., 2011; Lantsman et al., 2008; Stechmann et al., 2008). Unlike classic mitochondria, the *Blastocystis* organelles contain the enzymatic capability to convert pyruvate into CO₂ and H₂ using enzymes normally encountered in hydrogenosomes (van der Giezen, 2009). Although hydrogen production has not been detected (Lantsman et al., 2008), the enzyme hydrogenase does localise to the organelle (Stechmann

et al., 2008). Furthermore, the organelle contains the unusual acetate: succinate-CoA transferase (ASCT) shuttle, which allows for the production of ATP via substrate-level phosphorylation. The absence of cytochromes had been reported early on (Zierdt, 1986) so the lack of mitochondrial Complex III and IV components in the genome came as no surprise. Many genes encoding proteins that make up Complex I, possibly involved in proton pumping, have been detected, as has a complete Complex II (Denoeud et al., 2011; Stechmann et al., 2008). As no further downstream electron transport chain components have been found, the question whether Complex II functions as a succinate dehydrogenase, as in classical mitochondria, or as a fumarate reductase, is still open. Because of the anaerobic nature of *Blastocystis*, a fumarate reductase using rhodoquinone seems a plausible possibility. Currently, the terminal electron acceptor (Denoeud et al., 2011) seems to be the alternative oxidase (Standley and van der Giezen, 2012) but the choice of molecular oxygen as a substrate seems odd for an intestinal organism.

Overall, the complete genome of *Blastocystis* (Denoeud et al., 2011) has confirmed many previous studies (Lantsman et al., 2008; Stechmann et al., 2008; Zierdt, 1986; Zierdt et al., 1988) with respect to the biochemical nature of the MLO but several questions still remain. Perhaps the most significant one relates to the anaerobic status of this organism (Zierdt, 1986), as its genome suggests that it most likely is not a strict anaerobe after all.

7. FUTURE DEVELOPMENTS

It is probable that new genomes will become available in the immediate future, both from MLOs and from nuclei of different subtypes, and hopefully also from non-bird/non-mammalian *Blastocystis* as well, so that features common to *Blastocystis* can be distinguished from those that may be lineage-specific adaptations. We fully expect that surveys of *Blastocystis* subtypes from previously unsampled regions of the world will be forthcoming. The current impression of subtype distribution being geographically disjunct might be affirmed by such studies, or they may result in subtype prevalence numbers being seen as part of a continuum from common to absent. Further sampling of an increasing range of non-human hosts will also help confirm or refute the current impression of partial host-specificity of certain subtypes and genotypes.

The situation regarding the role of *Blastocystis* in disease is more difficult to predict as the necessary investigations will be expensive and time-consuming to conduct in a way that will give unambiguous answers. They will also have to be carried out in different parts of the world, given the observed geographic variation in subtype prevalence. Nevertheless we feel that these are the most important types of study to perform, because until the uncertainty surrounding the role of *Blastocystis* in disease is settled, it seems likely that it will continue to be dismissed by many clinicians as an organism of no importance. In the meantime, screening by Real-Time PCR and barcode-sequencing of *Blastocystis* in human cohorts with varying symptoms and different geographic origins will continue to provide useful prevalence, subtype and parasite load data.

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Country of samples	Technique*	No. of samples	Subtype distribution											Reference
			ST1	ST2	ST3	ST4	ST5	ST6	ST7	ST8	ST9	Mixed ST	Unknown ST	
Germany	RFLP	166	35	1	110	12	—	—	—	—	—	8	—	Böhm-Gloning et al., 1997
UK	RFLP	29	2	1	22	4	—	—	—	—	—	—	—	Clark, 1997
Japan	STS	32	1	—	30	—	—	1	—	—	—	—	—	Yoshikawa et al., 2000
Japan	RFLP	64	11	13	30	7	—	—	—	—	—	—	3	Kaneda et al., 2001
Thailand	RFLP	153	138	—	7	—	—	—	2	—	—	6	—	Thathaisong et al., 2003
Japan	STS	50	4	—	26	2	—	11	5	—	2	—	—	Yoshikawa et al., 2004b
Bangladesh	STS	26	2	—	24	—	—	—	—	—	—	—	—	Yoshikawa et al., 2004b
Pakistan	STS	10	2	—	7	—	—	1	—	—	—	—	—	Yoshikawa et al., 2004b
Germany	STS	12	3	2	5	2	—	—	—	—	—	—	—	Yoshikawa et al., 2004b
Thailand	STS	4	1	—	1	—	—	1	—	—	—	1	—	Yoshikawa et al., 2004b
Philippines	RFLP	12	10	—	—	—	—	—	—	—	—	—	2	Rivera and Tan, 2005
Denmark	Sequencing	29	1	6	15	7	—	—	—	—	—	—	—	Stensvold et al., 2006
UK	Sequencing	49	2	8	20	16	—	—	1	1	—	1	—	Scicluna et al., 2006
China	STS	35	13	—	14	—	—	—	2	—	—	5	1	Yan et al., 2006
Denmark	Sequencing	28	5	9	13	1	—	—	—	—	—	—	—	Stensvold et al., 2007a
China	STS	192	47	9	116	1	—	1	—	—	—	10	8	Li et al., 2007
Egypt	STS	44	8	—	24	—	—	8	4	—	—	—	—	Hussein et al., 2008
Greece	SSCP	45	9	6	27	1	—	1	1	—	—	—	—	Menounos et al., 2008
Malaysia	STS	20	9	1	10	—	—	—	—	—	—	—	—	Tan et al., 2008
Ireland	Sequencing	14	1	6	4	3	—	—	—	—	—	—	—	Scanlan and Marchesi, 2008

Iran	RFLP	45	20	4	16	—	—	—	—	—	—	—	5	Motazedian et al., 2008
Turkey	Sequencing	87	8	12	66	1	—	—	—	—	—	—	—	Özyurt et al., 2008
Singapore	RFLP	9	2	—	7	—	—	—	—	—	—	—	—	Wong et al., 2008
Turkey	STS	92	17	20	51	—	—	—	—	—	—	4	—	Dogruman-Al et al., 2008
Spain	RFLP	51	1	2	—	48	—	—	—	—	—	—	—	Domínguez-Márquez et al., 2009
France	Sequencing	40	8	4	20	4	—	—	1	—	—	3	—	Souppart et al., 2009
Nepal	STS	20	4	4	12	—	—	—	—	—	—	—	—	Yoshikawa et al., 2009
Malaysia	STS	40	5	—	20	—	—	11	2	—	—	—	2	Tan et al., 2009
Turkey	STS	32	20	3	9	—	—	—	—	—	—	—	—	Eroglu et al., 2009
Denmark	Sequencing	99	20	15	39	16	—	1	—	1	—	7	—	Rene et al., 2009
Denmark	Sequencing	116	21	22	21	20	—	—	5	—	1	26	—	Stensvold et al., 2009b
Turkey	STS	19	0	8	10	—	—	—	—	—	—	1	—	Dogruman-Al et al., 2009a
Turkey	STS	66	10	9	38	—	—	—	—	—	—	9	—	Dogruman-Al et al., 2009b
Egypt	Sequencing	20	3	4	12	—	—	—	—	—	—	1	—	Souppart et al., 2010
Pakistan	STS	179	87	10	49	8	7	6	10	—	—	—	2	Yakoob et al., 2010
Turkey	STS	25	9	6	10	—	—	—	—	—	—	—	—	Eroglu and Koltas, 2010
France	Sequencing	27	1	1	4	17	—	1	3	—	—	—	—	Poirier et al., 2011
Colombia	Sequencing	12	4	3	4	-	—	—	—	—	—	1	—	Santín et al., 2011
Denmark	Sequencing	25	1	4	1	19	—	—	—	—	—	—	—	Stensvold et al., 2011a
Denmark	Sequencing	22	9	11	—	—	—	—	—	—	—	2	—	Stensvold et al., 2011b
Italy	Sequencing	30	2	5	13	6	—	—	—	—	—	4	—	Meloni et al., 2011
Brazil	Sequencing	66	27	21	11							7		Malheiros et al., 2011
Egypt	STS	100	15	—	39	—	—	23	13	—	—	10	—	Fouad et al., 2011
Sweden	Sequencing	63	10	9	30	13	—	—	1	—	—	—		Forsell et al., 2012
	Total	2299	608	239	987	208	7	66	50	2	3	106	23	

Table 1. Subtype geographic distributions. Reports using different techniques and older terminologies have been translated into the consensus terminology of subtypes (Stensvold et al., 2007a). *Technique: RFLP = Restriction Fragment Length Polymorphism; SSCP = Single Strand Conformation Polymorphism; Sequencing = partial or complete SSU-rRNA gene.

Species	Size (Mb)	Chromosomes	Genes	Average gene length	GC content	Introns	Average intron length	Introns/gene	Reference
<i>Blastocystis</i> sp.	18.8	15	6020	1299	-	18 560	32 bp	3.1	(Denoëud et al., 2011)
<i>Ectocarpus siliculosus</i>	195.8	-	16 256	6859	54%	113 619	704 bp	6.98	(Cock et al., 2010)
<i>Thalassiosira pseudonana</i>	34.3	24	11 242	992	47%	15 739	-	1.4	(Armbrust et al., 2004)
<i>Phytophthora infestans</i>	240	8-10	17 797	1523	51%	-	125 bp	-	(Haas et al., 2009)
<i>Phytophthora sojae</i>	95	-	19 027	1613	54%	-	124 bp	-	(Tyler et al., 2006)
<i>Phytophthora ramorum</i>	65	-	15 743	1625	54%	-	123 bp	-	(Tyler et al., 2006)
<i>Phaeodactylum tricornutum</i>	27.4	33	10 402	-	-	8169	-	0.79	(Bowler et al., 2008)
<i>Pythium ultimum</i>	42.8	-	15 290	-	52%	24 464	115 bp	1.6	(Lévesque et al., 2010)

Table 2. Comparison of several features of stramenopile genomes. Several more stramenopile genome projects are currently ongoing but not all information for inclusion in this table is readily available. '-' = data not available.

Figure legend:

Figure 1. Schematic representation of the *Blastocystis* SSU-rRNA gene. Examples of the regions of the gene used for subtype identification by various authors are indicated (Parkar et al., 2010; Santín et al., 2011; Scicluna et al., 2006; Stensvold et al., 2006).