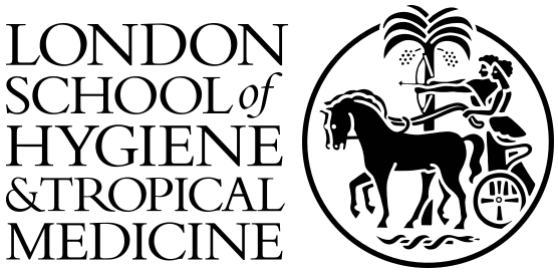

Running Title: Blastocystis *update*

Title: Current status of *Blastocystis*: a personal view

C. Rune Stensvold[a, *], C. Graham Clark[b]

[a] *Statens Serum Institut, Copenhagen, Denmark*

[b] *London School of Hygiene and Tropical Medicine, London, UK*

*Corresponding author: CR Stensvold - run@ssi.dk

Abstract

Despite *Blastocystis* being one of the most widespread and prevalent intestinal eukaryotes, its role in health and disease remains elusive. DNA-based detection methods have led to a recognition that the organism is much more common than previously thought, at least in some geographic regions and some groups of individuals. Molecular methods have also enabled us to start categorizing the vast genetic heterogeneity that exists among *Blastocystis* isolates, wherein the key to potential differences in the clinical outcome of *Blastocystis* carriage may lie.

In this review we summarize some of the recent developments and advances in *Blastocystis* research, including updates on diagnostic methods, molecular epidemiology, genetic diversity, host specificity, clinical significance, taxonomy, and genomics. As we are now in the microbiome era, we also review some of the steps taken towards understanding the place of *Blastocystis* in the intestinal microbiota.

Keywords: parasite; gut; Stramenopiles; public health; clinical microbiology

1. INTRODUCTION

It is now over 100 years since Alexeieff [1] first described the intestinal eukaryote *Blastocystis* but, despite the efforts of numerous researchers (especially in recent years), there are still many unknowns surrounding this organism. Most important of these is whether *Blastocystis* causes disease in humans. For every report linking *Blastocystis* with gastrointestinal or other symptoms there is another that finds no such link. There are a number of factors that have contributed to this apparent lack of progress and these will form the basis of this review. We would like to warn the reader at this early stage that we ourselves are convinced only that there are no definitive data yet available to resolve this issue.

2. TAXONOMY AND EVOLUTION

In culture, *Blastocystis* is generally spherical with no obvious surface features. When stained, the most common morphological form seen has a large central vacuole of unknown function and the cytoplasm with all the organelles is visible as a thin peripheral layer between the vacuole and the cell membrane (Figure 1). While many morphological forms have been described, the significance of most is unclear, the boundaries between them are not discrete, and some may well represent degenerating forms [2]. We refer the reader to earlier reviews for more details [3-5]. The life-cycle is typical of most gut protists, with a resistant cyst form for transmission and a trophic form that divides by binary fission. More complex and alternative life-cycles have been

68 described (discussed in [5]) but in our opinion there is no conclusive evidence
69 for anything other than this simple two-stage cycle.
70
71 *Blastocystis* has a complicated taxonomic history. It has been viewed as a
72 fungus, a sporozoan and even the cyst of another organism at various points
73 in its history, until 20 years ago [6] when it was finally placed among the
74 Stramenopiles. This is one of the major groups of eukaryotes [7], but one that,
75 to date, contains only a single other human-infective eukaryote, *Pythium*.
76 *Blastocystis* has none of the typical features of a stramenopile, which is in part
77 why identifying its correct relationships took so long.
78
79 Since its classification as a Stramenopile further data have emerged
80 regarding the closest relatives of *Blastocystis*. These turn out to be poorly
81 known flagellated or ciliate-like organisms that live in vertebrate intestines.
82 While most Stramenopiles are free-living and aerobes, *Blastocystis* and its
83 relatives are gut-living and anaerobes, although they do have mitochondrion-
84 like organelles (see later). *Blastocystis* is related specifically to the
85 Proteromonadidae and Slopalinida [8], but these cannot be considered close
86 relatives. However, it seems likely that the common ancestor of these groups
87 of organisms was already living in a gut and an anaerobe.
88
89 The simple spherical morphology of *Blastocystis* mentioned above applies to
90 all members of this genus. This means that morphology is of no use in
91 defining species. Traditionally, *Blastocystis* species have been defined by the
92 identity of their host, with all human *Blastocystis* being assigned to

Blastocystis hominis. However, even before DNA sequences identified *Blastocystis* as a Stramenopile it had become clear that significant heterogeneity existed among human *Blastocystis*. Using serology, isoenzymes and karyotyping, human *Blastocystis* were being divided into subgroups [4], and this picture of variation was reinforced by direct and indirect DNA sequence analyses [9]. Subsequent data have only added to the diversity and have refined our understanding of this genus.

Analyses of human *Blastocystis* by different researchers always resulted in the detection of variation, but each group came up with its own nomenclature for the groupings it identified. To resolve this confusion a consensus terminology was agreed [9] and this classification of human *Blastocystis* into numbered subtypes has simplified communication among workers in this field. At the time of the consensus two things were clear: 1. that humans were host to a number of distinct small subunit rRNA gene (SSU-rDNA)-based subtypes of *Blastocystis*, and 2. that most of these subtypes were also found in other mammalian or avian hosts. This meant the host-linked binomial species names were untenable, as the same organism was being called by multiple names. For example, one grouping of *Blastocystis hominis* proved to be genetically indistinguishable from *Blastocystis ratti*; both are now known as *Blastocystis* subtype 4 (ST4).

The current taxonomy of *Blastocystis* follows a distinct structure for mammal and bird organisms compared to all others [10]. The mammalian/avian *Blastocystis* are subdivided into seventeen subtypes (STs), nine of which

(ST1–ST9) have been found in humans. There is host range overlap observed for many of these organisms (Figure 2). *Blastocystis* from reptiles, amphibia and invertebrates retain Linnean binomial names for the most part. This is largely because little investigation of diversity and host range of these *Blastocystis* has been undertaken to date and so the same impetus to change the nomenclature has not existed. Whether a similar situation involving broad host-range and large genetic diversity will be uncovered in those organisms remains to be seen; it seems likely, and therefore the nomenclature of *Blastocystis* in those hosts may require a similar solution.

3. GENETIC DIVERSITY AND HOST SPECIFICITY

Subtypes of *Blastocystis* are discrete and no intermediate variants have been uncovered to date despite extensive sampling from around the world. However, many host species remain to be sampled, so this picture may change. Guidance on how and when to define a new subtype has been published [11]. The recommendation is that a minimum of 5% sequence divergence from the SSU-rDNA of known subtypes is required before defining a new subtype is appropriate. One of the reasons for establishing this boundary is that *Blastocystis* subtypes are often assigned based on the sequence with the closest similarity in sequence database searches, without taking into account the degree of similarity. So a sequence that actually represents a new subtype may be assigned to an existing subtype. This misattribution has been a problem in some existing cases, for example ST13, as discussed in reference [10]. Unfortunately, information attached to entries

143 in GenBank databases are rarely corrected and this can result in
144 misidentifications being propagated forward in the literature.
145
146 The 5% level of divergence to define a new subtype was chosen in part
147 because variation within subtypes can also be substantial, up to at least 3%
148 [11]. Therefore a single ‘outlier’ sequence that appears to be distinct and
149 potentially a new subtype could eventually merge into an adjacent subtype as
150 more sequences become available. Only as more subtyping data accumulate
151 will the validity of this arbitrary threshold be tested. Note that 5% divergence is
152 the recommendation for establishing new subtypes, where sampling is likely
153 to be limited. The divergence between some existing subtypes (for example,
154 ST6 and ST9) is actually less than 5%. However, sampling is sufficient to give
155 us confidence that these are indeed distinct lineages rather than variants of
156 the same subtype. In other words, 5% divergence has been chosen as quite a
157 stringent criterion and more data may lead to the revision of new subtype
158 definitions in the future..
159
160 As mentioned earlier, nine distinct subtypes have been found in humans
161 (Figure 2). However 95% of human infections sampled belong to one of just
162 four of these subtypes (STs 1-4; [12]) and only one of the human subtypes
163 has not yet been found in another host: ST9 can claim (at present) to be
164 restricted to humans. The four most common STs in humans have also been
165 detected in other hosts. Most frequently these hosts are other primates, but
166 they have also been found in various hoofed mammals, rodents and even
167 birds [10]. Conversely, the rarer subtypes in humans (STs 5-8) are more

commonly found in other hosts: ST5 in hoofed animals, STs 6 and 7 in birds, and ST8 in non-human primates. It has been suggested that these rarer subtypes in humans are of zoonotic origin and there is some evidence to support this: ST8 has frequently been found in zookeepers that work with non-human primates [13], and ST5 is prevalent in piggery workers in Australia [14], for example. However, there is no reason to suspect that human infections involving the common STs (STs 1-4) originate from non-human sources except in rare cases.

Exposure to *Blastocystis*-infected animals alone is not sufficient to result in an infection. For example, ST10 is very common in livestock [10] but is yet to be reported in humans. This suggests that variables other than just body temperature are determining the ability of *Blastocystis* to colonize the human gut; the gut flora may have an impact, for example.

The degree of genetic diversity within subtypes is quite variable. ST3 is probably the most diverse of the well-studied subtypes – varying by ca. 3% in the SSU-rDNA sequences - while ST4 shows the least variation, especially in humans [15]. Diversity in these subtypes has been further explored using a multi-locus sequence typing approach based on variation in several regions of the mitochondrion-like organelle’s genome [15]. MLST data are not yet published for other subtypes. How genetic variability within a subtype is reflected in phenotypic and functional variability is as yet unclear. However, differences in adhesion and drug resistance between strains of *Blastocystis* ST7 have been reported [16].

Intra-subtype variation has provided further insight into host specificity. For example, ST3 is common in both humans and non-human primates [13]. However, MLST analysis divided ST3 into four clades and almost all human samples fell into only one of these clades [15]. Where this was not the case, the individuals concerned had work exposure to non-human primates, again suggesting zoonotic transmission had occurred [15]. It would be interesting to know whether such host specificity exists between variants within other subtypes that are found in a wide range of mammals and exhibit genetic diversity, like ST10 for example [17].

MLST has the potential to provide insight into geographic aspects of genetic variation as well. However, this could be confounded by the increasing population mobility in today's world: geographic differences will be starting to break down. To date, it is only subtyping that has provided evidence of geographic differences in *Blastocystis* distribution. Specifically, it has become clear that ST4 has a restricted distribution, being rare or absent in South America, North Africa, and the Middle East, while being the second most common subtype in Europe (summarized in [12]). The reasons for this are obscure, but when combined with the relatively low genetic diversity of ST4 in humans the evidence suggests that ST4 may only have entered the human population relatively recently (perhaps in Europe) and is yet to spread around the world [12]. ST4 is also found in other hosts [10], but there is no link between these hosts and Europe.

4. DIAGNOSIS AND MOLECULAR CHARACTERIZATION

For most parasites, both direct and indirect diagnostic methods have been developed. Direct methods include those based on morphology (microscopy) and detection of DNA (typically PCR) or antigens (IFA, antigen ELISA, etc.), while indirect methods are based mainly on detection of antibodies [18]. While the potential utility of serology in the indirect detection of *Blastocystis* infections remains unclear, some studies have used serology to look for quantitative differences in antibody responses between symptomatic and asymptomatic individuals ([19-20]; see also below).

With regard to direct detection methods, the use of diverse diagnostic modalities of varying sensitivity may very well have impaired attempts to define the role of *Blastocystis* in health and disease [21-23]. Molecular methods developed to detect *Blastocystis* in genomic DNA extracted directly from fresh stool have highlighted the sensitivity shortcomings of diagnostic methods such as the traditional ‘ova and parasites’ (O&P) work-up (used to detect cysts of protozoa and larvae and eggs of helminths), culture methods, and permanent staining of fixed fecal smears [24-26].

Simple stains like Lugol’s iodine can be used as a quick aid to the identification of *Blastocystis* in fecal smears or concentrates; the organism is otherwise difficult to differentiate from other structures seen in unstained preparations due to the lack of diagnostic morphological features. Trichrome

staining is one of several permanent stains used for detection of trophic forms of protozoa in feces. *Blastocystis* stains characteristically with Trichrome, and this method had a specificity and sensitivity of 100% and 82%, respectively, in a study by Stensvold et al. [24].

Despite being the primary diagnostic tool worldwide, the use of microscopy to detect *Blastocystis* has limited utility in clinical microbiology laboratories and in generating data for clinical and epidemiological purposes: 1) Microscopy of fecal concentrates - the commonly applied O&P method - has very low sensitivity in detecting *Blastocystis* [24, 27]; 2) there is no consensus on the importance of the cell numbers (see below) or the various morphological forms reported; and 3) microscopy cannot distinguish between genetically highly dissimilar organisms (STs), which may differ in their clinical significance, a situation potentially similar to *Entamoeba histolytica* and *Entamoeba dispar*. Nevertheless, there are situations in which microscopy may serve a purpose, such as those aiming to verify the presence of *Blastocystis* in various types of non-human samples, including those of environmental and animal origin, to inform hypotheses on transmission. For instance, a recent study used microscopy to identify *Blastocystis* in various environmental samples, including food, water, and fomites [28].

Xenic *in vitro* culture (XIVC) is defined as culture in the presence of an undefined bacterial flora. *Blastocystis* can be grown and propagated xenically in a variety of media [29, 30]. Perhaps due to its simplicity and low cost, Jones' medium has been popular for both detecting and maintaining

Blastocystis; another medium often used for isolation is Robinson's [29], while we have also used LYSGM (a variant of TYSGM-9; [31]) for propagation when large numbers of cells are needed. XIVC as a diagnostic tool using Jones' medium has a sensitivity ranging from 52%—79% compared with real-time PCR assays [26, 32].

The diagnostic utility of Ag-ELISA and immunofluorescent antibody staining methods for the detection of *Blastocystis*, including commercial kits such as ParaFlor B (Boulder Diagnostics, Boulder, CO, USAa), coproELISA™ Blastocystis (Savyon Diagnostics, Ashdod, Israel), and Blasto-Fluor (Antibodies Inc., Davis, CA, USA), is as yet unclear, since these assays have been used in only a limited number of studies and applied to only a very limited number of samples [33-37]. The utility of such assays remains unknown as the range of subtypes they detect is unclear.

The first diagnostic PCR for *Blastocystis* was introduced in 2006 [25] but it was later suspected to exhibit preferential amplification of some subtypes over others. Since then, three diagnostic real-time PCR assays have been reported. A real-time PCR based on an unknown *Blastocystis* target using FRET probes was validated against ST1, ST3, and ST4 [38]. A SYBR green real-time PCR used the SSU rRNA gene for detection of *Blastocystis*-specific DNA (ST1–ST9), and subsequent subtyping was performed by melting curve analysis [26]. The relatively large PCR product used (320 to 342 bp, depending on the subtype) may impair the sensitivity of this test—especially when DNA quality is not optimal—and the specificity of the assay was 95%.

The third real-time assay, using a hydrolysis probe based on the SSU rRNA gene, was characterized by 100% specificity and ability to detect all nine subtypes identified in humans so far [32]. The use of real-time PCR in large-scale surveys would assist in identifying whether the development of symptoms is related to infection intensity by simple analysis of threshold cycle (C_t) values for individual samples, as this enables quantitation of the amount of *Blastocystis*-specific DNA present. The same DNA samples may also be used for subtyping and MLST protocols, hence allowing the detection and evaluation of genetic diversity as well as the simple presence of *Blastocystis* [22]. *Blastocystis* has also been included as a diagnostic target in commercial gastrointestinal pathogen diagnostic panels such as Feconomics® (Salubris Inc, Boston, USA), EasyScreen™ Enteric Parasite Detection Kit (Genetic Signatures, Sydney, Australia), and NanoChip® (Savyon Diagnostics, Israel).

While the potency of DNA-based methods is evident, they do not allow the evaluation of whether differences in morphotypes are important. Several different forms of *Blastocystis* have been described, including the avacuolar, vacuolar, multivacuolar, granular, ameboid, and cyst stages. Although there are a few reports of ameboid stages being detected only in symptomatic *Blastocystis* carriers [eg. [39]), there is no consensus regarding the significance of the different forms. Moreover, as mentioned earlier, it is not clear whether some of these forms represent life-cycle stages, or are artifacts resulting from exposure to oxygen or other stresses [2]. Relatively few studies on the cyst stage are available [40-42], which is remarkable given that this is

the stage that allows survival of the parasite in the environment and transmission to a new host.

The high sensitivity of qualitative PCR for detection of *Blastocystis* DNA in stool was reinforced by a recent study of *Blastocystis* in Senegalese children [43], where the prevalence of *Blastocystis* among 93 children with and without gastrointestinal symptoms was 100%. When prevalence is so high there will be little incentive for including *Blastocystis* PCR as a screening tool in the clinical microbiology laboratory. However, where treatment of a patient with *Blastocystis* has been undertaken, PCR methods are useful in post-treatment follow-up to evaluate treatment efficacy.

This leads to one of the fundamental questions for clinical microbiology labs: When is testing for *Blastocystis* appropriate? Data currently emerging indicate that *Blastocystis* can be more common in individuals with a healthy GI system than in patients with organic and functional bowel diseases (see below). Therefore, the inclusion of *Blastocystis* as a specific target in screening panels, alongside known pathogens such as *Giardia*, *Cryptosporidium*, and *Entamoeba histolytica*, currently appears to make little sense in the clinical microbiology laboratory. The presence of *Blastocystis* in stool samples most likely implies that the carrier has been exposed to fecal-oral contamination, which should prompt the laboratory to look more closely for the presence of pathogens transmitted in the same way. However, since *Blastocystis* may colonize the human colon for more than 10 years [44], it may be impossible to identify when this contamination happened. This has important implications

342 for the interpretation of clinical microbiology lab results. *Blastocystis* is
343 sometimes detected in stool samples of patients with diarrhea or other
344 gastrointestinal symptoms and in the absence of proven pathogens, so
345 clinicians might conclude that *Blastocystis* could be the cause of the
346 symptoms. If it is known that the infection is recent, the organism could
347 certainly be viewed as a potential cause of the symptoms; however, in most
348 cases it will be impossible to rule out that it has been present in the gut for
349 months - even years - and therefore is an incidental finding.
350
351 Another dilemma is the question of whether or not to report the presence of
352 *Blastocystis* in stool samples given that it is so common. Several studies have
353 sought to address this by setting a threshold number of *Blastocystis*
354 organisms detected microscopically per visual field at a specified
355 magnification before scoring the sample as positive; usually this has been set
356 at 5 organisms per 40x field (see references in [5]). However, the rationale for
357 this is unclear. It is known that shedding of both trophic and cyst forms of the
358 organism is irregular [45]. Moreover, several factors may influence the
359 number of organisms seen per visual field, including whether or not the
360 sample was fresh or preserved prior to analysis, and if preserved whether or
361 not the sample was fresh at the time of fixation. Real-time PCR would be
362 more sensitive and less affected by some of these variables.
363
364 In the event that symptoms are eventually linked to specific subtypes,
365 including those individual subtypes as specific targets in diagnostic panels
366 would be more relevant than including a general target for *Blastocystis*.

Subtype-specific PCRs already exist, and barcoding of *Blastocystis* DNA amplified by generic primers can also be performed [46, 47]. To date, diagnostic PCR methods have been developed and validated only for human clinical samples; no validated PCR method for detecting *Blastocystis* in environmental samples is yet available to the knowledge of the authors.

Given the extensive cryptic genetic diversity of *Blastocystis* [10, 15, 48], a number of tools have been developed to map its molecular epidemiology. Among these tools, two in particular have been widely used. A PCR assay for detecting subtypes using sequence-tagged-site (STS) primers was developed and refined in the early 1990s [49]. This approach involves the use of seven PCR reactions, one for each of subtypes 1—7, and should be viewed as comprising a diagnostic method for each of these subtypes, circumventing the need for sequencing. The other method involves analysis of SSU rDNA variation. This approach has been developed independently by several groups, each of which used different regions of the SSU rRNA gene as markers [24-25, 50-56]. The barcoding method mentioned above, developed in 2006 by Scicluna et al., is one such example [46]. A comparison of the STS method and barcoding showed that barcoding should be preferred where possible for a variety of reasons [47]. First and foremost, barcoding enables the detection of subtypes beyond STs 1—7 and further scrutiny of genetic diversity. The barcode region has also been validated as a marker of overall genetic diversity of *Blastocystis* [15].

391 Barcoding uses the primers RD5 and BhRDr, which amplify ~600 bp at the 5'-
392 end of the SSU rRNA gene. Comparison of phylogenetic trees obtained by
393 analysis of barcoding sequences with those obtained using concatenated
394 sequences obtained by MLST (reflecting loci in the genome of the
395 mitochondrion-like organelle) demonstrated the appropriateness of using the
396 barcode region as a surrogate marker for overall genome diversity in this
397 particular organism [15]. The drawbacks of barcoding compared to the STS
398 method are that sequencing is required and that mixed subtype infections
399 may not always be evident in sequence chromatograms, and, even if they are,
400 they may prove difficult to decipher [47]. On the other hand, barcoding
401 enables more subtle analyses, namely SSU rDNA allele analysis [15]. A
402 public database is available (http://pubmlst.org/blastocystis/) that includes a
403 sequence repository for barcode sequences and those obtained by MLST. It
404 also has a BLAST facility, where individual or bulk fasta files can be uploaded
405 and analyzed for rapid identification of subtype and allele number, hence
406 eliminating the need for phylogenetic analysis. To date, 35 SSU rDNA alleles
407 within ST3 have been identified, whereas the number of SSU rDNA alleles for
408 ST4 and some other subtypes remains much more limited. However, some of
409 the allelic variation included is the result of sequencing of cloned DNA;
410 intragenomic SSU rDNA polymorphism has been reported [57, 58], and such
411 polymorphism will likely go unnoticed when sequences obtained directly from
412 PCR products are studied.
413
414 There is no doubt that DNA-based methods now enable us to carry out large
415 and well-designed research studies that are dependent on accurate detection

and molecular characterization of *Blastocystis*. Such studies are required to produce data that can shed light on the role of this organism in human health and disease with a view to potentially developing diagnostics, biomarkers, and therapies, including antimicrobial or probiotic agents, as appropriate.

5. CLINICAL SIGNIFICANCE AND EPIDEMIOLOGY

Even after more than 100 years, the role of *Blastocystis* in human health and disease remains obscure. While *Blastocystis* has been speculated to be involved in a range of organic and functional bowel diseases, it is clear that asymptomatic carriage is common. This does not mean that *Blastocystis* does not cause disease. The situation may resemble that for *Giardia*, where many infections are asymptomatic (for example [59]), and *Entamoeba histolytica*, where the proportion of symptomatic infections is at most 10% [60]. Case reports and surveys continue to be published with regularity, mostly indicating a link between *Blastocystis* and symptoms, although not always. We do not propose to evaluate all the evidence here. However we do wish to highlight two common issues: 1. Identification of an appropriate control group for survey studies can be problematic; and 2. Excluding all other possible etiologic agents or non-infectious causes of intestinal symptoms is almost impossible.

While distinctive intestinal pathology has been clearly linked to the intestinal protists *Giardia*, *Cryptosporidium*, and *Entamoeba*, there is little – if any – evidence for direct pathology caused by *Blastocystis*. Phagocytosis of red

441 blood cells is a well-known feature of *Entamoeba histolytica* that correlates
442 with virulence; there is only one study reporting phagocytosis in *Blastocystis*
443 [61]. No *Blastocystis* proteins such as glycoproteins or lectins that could
444 facilitate attachment to the gut epithelial layer have been identified, although
445 Denoeud et al. [57] have speculated that *Blastocystis* hydrolases might be
446 able to alter the colonic mucus layer (see below). It is generally accepted that
447 *Blastocystis* is non-invasive as well as lacking the ability to phagocytize the
448 microbiota or host-derived material.
449
450 When examining tissue sections from pig intestines, Fayer et al. [62] found
451 *Blastocystis* primarily in the lumen, usually associated with digested food
452 debris, and although sometimes in close proximity to or appearing to adhere
453 to the epithelium, there were no cells penetrating to the epithelium or the
454 lamina propria. These observations were confirmed by Wang et al. [63], who
455 did not observe any obvious pathology in histological sections of porcine gut
456 mucosal biopsies. In the latter study, *Blastocystis* cells were observed as
457 vacuolar/granular forms found within luminal material or in close proximity to
458 epithelial cells, with no evidence of attachment or invasion. When *Blastocystis*
459 is observed adhering to the epithelium in histological preparations it should be
460 kept in mind that histological procedures are likely to dissolve and eliminate
461 the mucus layer that is potentially separating *Blastocystis* from the mucosa in
462 vivo.
463
464 Despite the absence of invasion, discrete non-specific colonic inflammation
465 has been reported in a patient with both urticaria and what was characterized

as ‘heavy *Blastocystis* colonization’; *Blastocystis* eradication resulted in symptom resolution [64]. There are also some reports of *Blastocystis* having been found extra-intestinally, but in those cases it has not been possible to rule out that the presence of *Blastocystis* at these sites was merely a result of incidental or secondary colonization resulting from damage generated by other microorganisms or anatomical anomalies [65-68].

Blastocystis is one of several organisms to have been linked to Irritable Bowel Syndrome (IBS), including post-infectious IBS [69-71]. Genome analysis by Poirier et al. [72] identified various genes encoding hydrolases and serine and cysteine proteases, and the authors speculated that these potential virulence factors could be triggers of IBS by alteration of the mucus layer and interaction with tight junctions.

Cross-sectional studies testing the hypothesis that *Blastocystis* is linked to IBS mostly assume that, if the organism is associated with the disease, it should be more common in patients with IBS symptoms. The outcomes of such studies have been mixed, with some finding a higher prevalence of *Blastocystis* in IBS patients and some finding no difference or even lower prevalence (summarized in [12]). A few have looked at the subtype distribution, but although they have generally found differences between IBS and non-IBS patients, there is no consistency regarding the subtypes associated with IBS (summarized in [12]). IBS itself presents a diverse picture, with patients having diarrhea, constipation or a mixture of symptoms

[69]. Even fewer investigations have been performed to look at potential links between *Blastocystis* and subgroups within IBS.

IBS patients are likely to have multiple tests performed before a diagnosis is made and, because of this, a common finding may well be *Blastocystis* in the stool, which might then be suspected of being the agent responsible for the symptoms if no other candidates have been uncovered. So *Blastocystis* may be more commonly detected in IBS patients simply because the investigations are more thorough. Post-infectious IBS - a term describing the development of IBS following treatment of an infection with antimicrobials [71] – adds another complication, as the actual trigger for IBS may have been eliminated by antimicrobial treatment, leaving *Blastocystis* behind to take the blame. It is also impossible to exclude that *Blastocystis* was the initial trigger of IBS even if it is no longer present. The potential links, if any, between *Blastocystis* and IBS may be impossible to prove or disprove without large longitudinal cohort studies.

One of the most interesting recent findings is that *Blastocystis* could be a marker of gastrointestinal health rather than a cause of disease. This may in fact not be surprising, given that we have been unable to reach a consensus on a role for the organism in disease despite the large number and wide range of investigations undertaken. A recent study identified *Blastocystis* as a common member of the healthy human gut microbiota, with greater than 50% of the healthy background population colonized [44]. Moreover, long-term colonization trends were also noted; the same strains were present in the

same hosts for up to 10 years [44]. A lower prevalence of *Blastocystis* in IBS patients (n = 189) compared with healthy controls (n = 297), 14.5% versus 22% respectively (p = 0.09), was also highlighted in a recent study [73]; the prevalence of *Dientamoeba fragilis* also differed significantly between the two groups, with *D. fragilis* being similarly more common in individuals without gastrointestinal symptoms. Another study, this time involving 96 healthy controls and 100 patients with Inflammatory Bowel Disease (IBD) - a disease affecting about 12,000 individuals in Denmark alone, 0.2% of the population - detected a significantly lower prevalence of *Blastocystis* in IBD patients compared with healthy controls ($p < 0.05$), with only 5/100 IBD patients being colonized by *Blastocystis* compared with 18/96 controls [74-75]. Interestingly, four of the five positive IBD patients were in an inactive stage of the disease; only 1/42 patients with active IBD was a carrier.

Whether it is linked to gastrointestinal health or disease, it is clear that *Blastocystis* is much more common than previously reported, reaching a prevalence of 100% in some cohorts [43]. Individuals in communities with high prevalence may become and remain infected from a very young age, while in other communities, particularly where the overall prevalence is low, many individuals may acquire *Blastocystis* later in life. For now, it is uncertain whether the age at colonization - including whether *Blastocystis* becomes a stable member of the intestinal microbiota from early on - is of any clinical importance. It could be that in some regions of the world, *Blastocystis* might be an ‘emerging pathogen’.

While recent observations suggest that *Blastocystis* colonization may be inversely correlated with intestinal disease [44], we now know that the bacterial component of the gut microbiota in IBS, IBD, and other intestinal diseases is significantly different to that of the healthy human gut [69, 76]. Importantly, this may in fact indicate that *Blastocystis* is dependent on other components of the microbiota to colonize and maintain a stable colonization in the human gut. To test this prediction, we recently obtained access to data from the MetaHIT Consortium (http://www.metahit.eu/), originally generated to identify associations between intestinal bacterial communities and disease patterns, including obesity, diabetes, and IBD [77]. From the data, we were able to extract *Blastocystis*-specific DNA signatures, which enabled us to (1) identify the relative prevalence of *Blastocystis* in each of the study groups, and (2) to perform a preliminary investigation of the association between *Blastocystis* and bacterial communities, in this case the so-called 'enterotypes' [77]. Our analysis [78] showed that: 1) *Blastocystis* was indeed negatively associated with disease and absent in all 13 patients with Crohn's disease (although not all studies have found this; [79]); and 2) very intriguingly, *Blastocystis* was negatively associated with the *Bacteroides* enterotype ($p < 0.0001$, unpublished data). This finding may be linked to the fact that the *Bacteroides* enterotype—compared with the *Prevotella* and the *Ruminococcus* enterotypes—is characterized by low microbial diversity, and this could therefore indicate that *Blastocystis* requires high overall microbial diversity to become established in the human colon. However, it could also be that some other unknown feature(s) of the enterotype may be responsible for determining *Blastocystis* colonization, such as bacterial metabolic by-

565 products. There is no doubt that studies of *Blastocystis* in the context of
566 intestinal bacterial communities and host physiology and immunity are likely to
567 advance our understanding of the clinical significance of *Blastocystis*. The
568 apparent impact of the gut flora on *Blastocystis* colonization may also mean
569 that standard animal models may be of limited use in exploring the effects of
570 *Blastocystis* on the human gut.
571
572 Comparing both bacterial and eukaryotic microbial communities in samples
573 from 23 individuals from agrarian communities in Malawi following traditional
574 lifestyles and from 13 individuals residing in Pennsylvania and Colorado,
575 USA, following a modern lifestyle, Parfrey et al. [80] recently showed that the
576 Malawi population harbored a diverse community of protists, including
577 *Blastocystis*, when compared to the North American populations, and that the
578 overall organismal diversity in the Malawian human gut is comparable to that
579 in other mammals. These, and other, data could indicate that the declining
580 diversity of the human bacterial microbiota identified in the West compared
581 with populations with traditional agrarian lifestyle has led to a reduced
582 prevalence of *Blastocystis* in Western populations [81].
583
584 It is also clear that geographical differences in subtype distributions may result
585 in geographical differences in the clinical significance of the parasite. There is
586 precedent in *Entamoeba* for cryptic genetic differences underlying differences
587 in the clinical outcome of infection (the *E. histolytica/E. dispar* story; [60]). So
588 a working hypothesis over the past few years has been that differences
589 between the clinical outcome of *Blastocystis* infection may reflect genetic

differences in the organism. Hence, dozens of studies from all over the world have sought to identify *Blastocystis* STs in both healthy and symptomatic individuals (summarized in [11]). The distribution of subtypes across the major geographical regions is depicted in Figure 3. So far, no particular subtype has been linked consistently to disease. However, such a finding might not be unexpected if the distribution of subtypes is uneven. While ST1, ST2, and ST3 appear to have a global distribution, current data suggest that ST4 is confined mainly to Europe. ST4 was the only subtype identified in Danish patients with acute diarrhea, but the overall prevalence of the parasite was also lower in this group of patients than in others that have been studied in Denmark [82]. ST4 also dominated in symptomatic patients in Spain [83].

A significant gap in clinical *Blastocystis* research is the lack of large randomized controlled clinical treatment trials [84-87]. To date these have produced inconsistent and indeed contradictory results. It appears that no single drug or drug combination currently in use consistently results in reliable *Blastocystis* eradication [88-90]. Metronidazole has traditionally been used to treat anaerobic microorganisms, including *Entamoeba* and *Giardia*; however, its effect on *Blastocystis* has in some studies been minimal, with an eradication rate as low as 0%. Even the use of combinations such as diloxanide furoate, secnidazole, and trimethoprim/sulfamethoxazole or nitazoxanide may not result in consistent eradication [90].

6. GENOMICS

615

616 With the advances in sequencing technology in recent years it has become
617 possible to sequence eukaryotic genomes quickly and relatively inexpensively
618 compared with even a few years ago. Perhaps surprisingly, the published
619 *Blastocystis* nuclear genome sequences at the time of writing are for ST7,
620 obtained by 'traditional' Sanger sequencing [57], and ST4, obtained by next
621 generation sequencing [91]. Others have not yet appeared in print despite
622 anecdotal evidence that suggests a flood of new data is about to arrive.

623

624 However, *Blastocystis* has two genomes. In addition to the nuclear genome it
625 also contains an organelle genome. In contrast to most anaerobic eukaryotes,
626 *Blastocystis* has mitochondrion-like organelles that have a quite normal
627 appearance under the transmission electron microscope (see [4]). It was
628 known for many years that these organelles contained DNA, based on
629 staining properties, but it was not until 2007 that the coding potential of these
630 molecules was uncovered. Two groups published sequences of the genomes
631 present in the mitochondrion-like organelle in three subtypes – STs 1, 4 and 7
632 [92-93]. The gene content and gene order of the 27-29 kilobasepair circular
633 molecules was identical, although the sequence divergence was
634 considerable. Subsequently, mitochondrion-like organelle genomes from
635 additional subtypes have been obtained (unpublished data) and these initial
636 observations have been upheld.

637

638 The gene content of the genome of the mitochondrion-like organelle is distinct
639 from the more familiar ones from mammals and yeast. Particularly notable is

640 the absence of any genes encoding cytochrome and ATPase subunits and
641 the presence of a number of ribosomal protein genes. In common are the
642 genes encoding ribosomal RNAs and several tRNAs plus NADH
643 dehydrogenase (Complex I) subunits. The nuclear genomes and expressed
644 sequence tag (EST) surveys that are available confirm that the *Blastocystis*
645 mitochondrion-like organelle has only retained complexes I and II of the
646 electron transport chain, a characteristic shared with certain other anaerobic
647 eukaryotes. However, many other features of mitochondrial metabolism are
648 also present [31, 57]. This is in contrast to the situation in, for example,
649 *Giardia* and *Entamoeba* where the genome has been lost completely and the
650 function of the resulting organelles (known as mitosomes) has become highly
651 reduced. Whether the *Blastocystis* organelle would follow a similar path given
652 enough time is impossible to predict.
653
654 The only published nuclear genomes at this time are for ST4 and ST7.
655 However, a recently published report on polyadenylation in *Blastocystis* also
656 includes data on a ST1 genome, suggesting its publication is imminent. The
657 polyadenylation report uncovered a unique situation in *Blastocystis*, where
658 around 15% of the stop codons in messenger RNAs are created through the
659 cleavage of a precursor and addition of the poly A tail to the mRNA [94]. This
660 is unprecedented outside of mitochondria. Given the degree of genetic
661 divergence between subtypes, comparative genomics may well reveal
662 significant differences between features of their nuclear genomes as well as
663 confirming genus-wide peculiarities, as in this case.
664

Overall, the *Blastocystis* nuclear genome is quite small (under 19 Mb) with relatively few genes (just over 6,000), quite a few of which appear to have been acquired by horizontal gene transfer. Introns are numerous and small, but repetitive DNA is rare. Of note is the fact that individual ribosomal RNA cistrons are sometimes present in subtelomeric regions of the genome rather than being exclusively found in long tandem arrays as in many other eukaryotes [57].

7. CONCLUSION

Blastocystis is one of the most successful intestinal eukaryotes identified to date, being able to infect a wide range of host species. It may reside in the gut for years on end and appears to show remarkably little susceptibility to standard chemotherapeutic interventions, although analysis of biochemical pathways identified through genome sequencing may generate some new directions for drug interventions. However, the recognition of a high prevalence of *Blastocystis* in healthy populations, identified using sensitive molecular diagnostic tools, has heralded a paradigm shift in clinical *Blastocystis* research. Studies of the gut microbiota in people with and without *Blastocystis* are likely to provide valuable - if not critical - information to help determine the role of *Blastocystis* in human health and disease.

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691 9. REFERENCES

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Figure 1. Light microscopy images of *Blastocystis*. A. *Blastocystis* in culture. Using Robinson's and other media [29], *Blastocystis* often reaches high density in xenic culture. This stage is typically reported as 'vacuolar' due to the large central region of uncertain function. Organelles are seen as 'dots' along the periphery of the cell. B and C. *Blastocystis* in fecal smears, stained using iron-hematoxylin. Prominent nuclei are seen in the periphery of the cells as the most conspicuous morphological hallmark, along with the large central 'void'. Other organelles can be discerned as smaller peripheral 'dots', which will include the mitochondrion-like organelles, etc. However, these can only be positively identified by transmission electron microscopy. Images courtesy of John Williams (A) and Claire Rogers (B, C), Diagnostic Parasitology Laboratory, London School of Hygiene and Tropical Medicine.

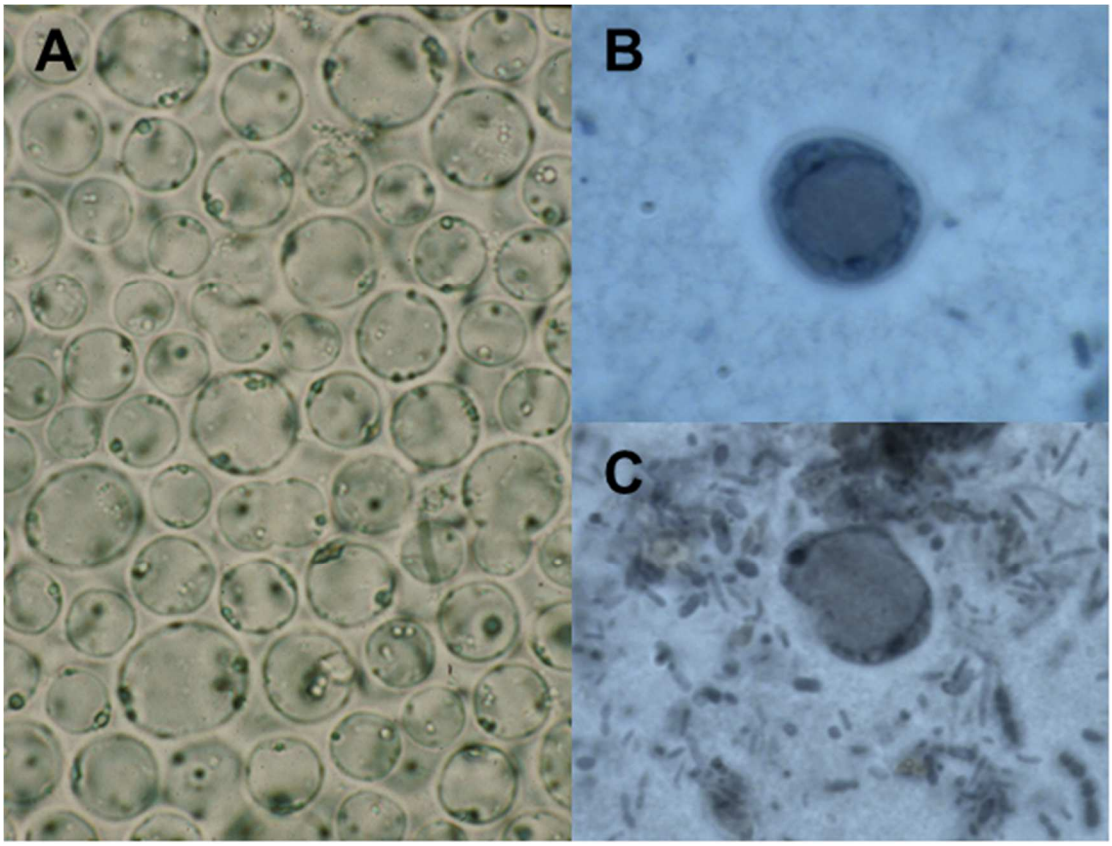

Figure 2. Host range and relative prevalence of *Blastocystis* subtypes. In this schematic, the range of subtypes reported for four major host groups (humans, non-human primates, ungulates and birds) is shown. In the circle, the numbers are those of the most common subtypes found in the respective host, with the integer font size proportional to its prevalence. Numbers in the magnified boxes represent those subtypes that each constitute less than 5% of the total samples subtyped to date. Derived from the numbers presented in reference [10]. As an indication, prevalence figures for STs 1-4 in humans are 28.0%, 10.9%, 44.4% and 10.0% respectively.

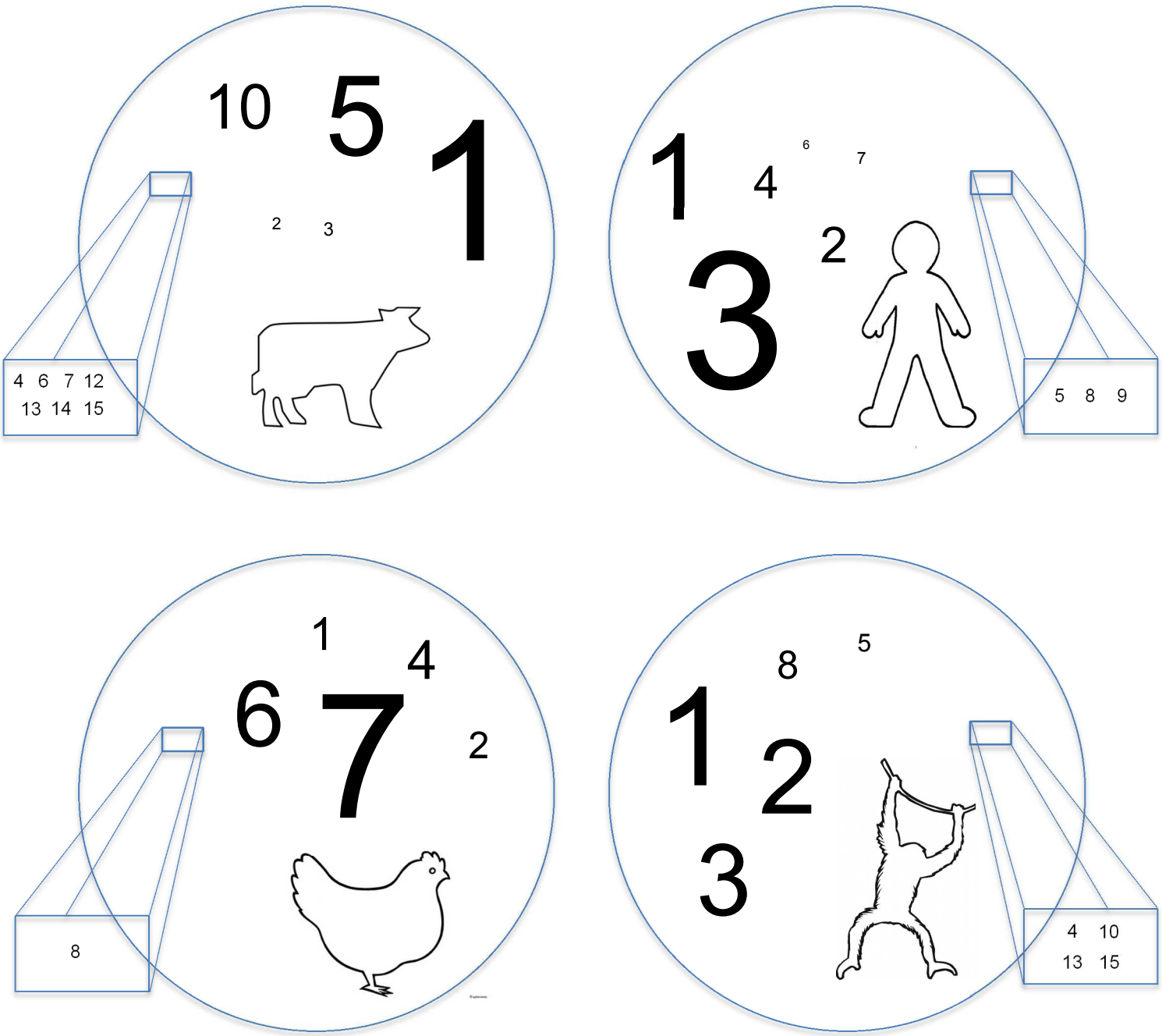

Figure 3: Pie charts of human *Blastocystis* subtype distributions in Europe (A) and the rest of the world (B). These were produced from the data presented in Alfellani et al. [12]. Of note is the fact that although ST4 accounted for 10% of the samples across the world (*N* = 318), 87% of these (278) were from Europe, suggesting that ST4 is more or less geographically restricted to Europe.