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Blastocystis: getting to grips with our guileful guest

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***Blastocystis*, a common single-celled intestinal parasite of humans and animals, continues to puzzle clinical microbiologists, gastroenterologists, and general practitioners who are still unsure of the clinical significance of the organism. Here we consider some less well-addressed areas of *Blastocystis* research, which, facilitated by recent technological advances, could potentially turn out to be significant pathways to knowledge. First and foremost we discuss new trends in *Blastocystis* research, including the 'omics' perspectives, and then highlight some aspects of *Blastocystis* research in the context of host coevolution, its potential as a biomarker of intestinal functionality, and its relationship to other components of the human intestinal microbiota.**

Expanding our perceptions of *Blastocystis*

Blastocystis (see Glossary) is one of the most common intestinal parasites of humans and animals (Box 1). Extrapolating from published prevalence data, the parasite is estimated to colonize between 1 and 2 billion people on a global scale. Whether pathogenic or not, the parasite is remarkable in that it is capable of establishing chronic infections, for which there is no known eradication strategy [1]. Based on small subunit rDNA analysis (SSU rDNA), at least 17 subtypes (STs) (arguably separate species) are known to colonize a range of hosts including humans, other mammals, birds, reptiles, and insects [2]. Some STs exhibit cryptic host specificity [3], and emerging evidence indicates that animals may not in fact be a significant source of human *Blastocystis* carriage as previously suspected [2–4]. Humans are colonized mainly by ST1–ST4, but the relative prevalence of these STs appears to differ substantially across regions [5], and evidence of differential ST virulence is limited and equivocal [6]. Because *Blastocystis* exhibits remarkable intra-ST genetic diversity, an understanding of the variation in both genetic and phenotypic diversity between isolates of *Blastocystis* and how this variation impacts directly and indirectly on human intestinal disease and human intestinal homeostasis, via interactions with the host environment, viruses, and the intestinal microbiota, is one of the central challenges to the future of *Blastocystis* research.

Candidate virulence factors and beyond: can an 'omics' approach help?

Classifying an organism as pathogenic is not always straightforward as is the case for *Blastocystis* [6–8]. Despite evidence supporting a role for *Blastocystis* in gastrointestinal and extraintestinal disease [7], a direct causal link is yet to be demonstrated. Furthermore, the high prevalence of different *Blastocystis* STs in asymptomatic hosts may also suggest that not all humans are susceptible to disease even when infected by a potentially virulent organism or/and that not all *Blastocystis* genotypes are pathogenic.

In the simplest scenario, genetic variation in a specific trait, such as the presence or absence of specific virulence factors or genes (e.g., those encoding toxins), can result in observed differences in pathogenicity in otherwise highly related organisms. There is evidence to support a role for cysteine proteases as the virulence factors responsible for observed variation in *Blastocystis* pathogenicity. Cysteine proteases are enzymes essential for host invasion and infection and are well recognized virulence factors of pathogenic protozoa [9]. A direct correlation between protease activity and virulence has been demonstrated for a range of parasites including isolates of *Blastocystis* [10]. Using the Caco-2 cell line as an *in vitro* model system, researchers demonstrated the ability of *Blastocystis* cysteine proteases to reduce epithelial barrier resistance and increase epithelial permeability [11]. Crucially, inhibition of cysteine protease activity mitigated these cytological effects. Cysteine proteases of *Blastocystis* can also induce the proinflammatory cytokine interleukin-8 (IL-8) [12] and degrade human immunoglobulin A (IgA) [13]. Marked differences in cysteine protease production are evident between *Blastocystis* STs [14], and together with experimental evidence highlighting a variation in pathophysiological effects and immunological responses to *Blastocystis* genotypes isolated from symptomatic and asymptomatic carriers [15,16], these data support the hypothesis that cysteine proteases may be essential virulence factors responsible for variation in disease symptoms observed across carriers. Future studies using whole genome sequencing and comparative

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Glossary

Blastocystis:: a single-celled intestinal parasite that colonizes a large range of vertebrates and invertebrates. It belongs to an extremely diverse (genetically and phenotypically) group of protists called Stramenopiles, but is distinguished within this group by an adaptation to an anaerobic, parasitic life cycle, and it is the only member of this group potentially associated with human intestinal pathology [6]. Contrary to most other intestinal parasites of humans, the clinical significance of *Blastocystis* remains unclear, and no effective therapeutic strategy has been identified [1].

Caco-2 cell line:: Caucasian colon adenocarcinoma cells; a human intestinal cell line derived from a human colon adenocarcinoma that has been used extensively over the past couple of decades as an *in vitro* model system to study (*inter alia*) intestinal barrier function [50].

Cryptic host specificity:: although STs commonly found in humans (e.g., ST3) are shared with a variety of other animals, emerging evidence [3] suggests that humans and non-human hosts are colonized by separate genotypes, implying that the mere existence of shared subtypes does not *per se* suggest zoonotic potential.

Dysbiosis:: although efforts to define what constitutes a normal intestinal microbiota are ongoing, it is clear that several key aspects, for example, level of diversity and species composition, of the microbiota in healthy individuals differ from those of patients suffering from many diseases. Significant differences in the microbial composition related to a disease is called dysbiosis, and specific dysbiosis has been identified in patients suffering from type 2 diabetes, IBS, IBD, *Clostridium difficile*-associated diarrhea, and colorectal cancer [47,51–53]. The intestine of healthy individuals is characterized by anaerobiosis. In individuals with IBD, intestinal dysbiosis is typified by a reduction in diversity and decrease of dominant obligate anaerobes in parallel with an increase of subdominant facultative anaerobes or the appearance of unusual aerobes [39].

Enterotypes:: the application of multidimensional cluster analysis and principal component analysis to fecal metagenomes revealed three distinct clusters, the so-called enterotypes [54]. Each of these three enterotypes is characterized by a dominance in abundance of one of three bacterial genera: *Bacteroides* (enterotype 1), *Prevotella* (enterotype 2), and *Ruminococcus* (enterotype 3) [54]. Although recent data have questioned the generalizability and utility of defining the microbiota of individuals on the basis of this concept of enterotypes [55,56], it remains clear that metagenomic approaches to characterizing the intestinal microbiota have vast potential for phylogenetic interrogation.

Genotype:: genotypes of *Blastocystis* refer to genetically distinct strains within a ST. Barcoding of *Blastocystis* [57] has proven useful in establishing 18S (SSU rRNA gene) alleles that can easily be identified using the database: www.pubmlst.org/blastocystis; 18S alleles serve as cost-effective proxies for sequence types obtained by multilocus sequence typing [3,58].

The Hygiene Hypothesis and Old Friends Hypothesis:: the 'Hygiene Hypothesis' proposes that a lack of exposure to certain parasites or microorganisms during childhood is linked to the increase in allergic and chronic inflammatory disorders [32]. This lack of exposure leads to improper immunoregulation and consequent disease state. An extension of this hypothesis is the concept of microorganisms and/or parasites as 'Old Friends'. The 'Old Friends Hypothesis' is based on the idea that we have a shared evolutionary history with parasites and microbes such that we have evolved dependence upon them, and they play an essential role in the establishment and maintenance of a competent immune system [33].

Irritable bowel syndrome (IBS):: is a common functional (i.e., with no known structural or biochemical cause) bowel disorder, affecting approximately 15% of the adult population in Western countries [59]. Several symptoms are associated with IBS, including bloating, cramping, nausea, and altered bowel movements (diarrhea or constipation or both), and diagnosis is based on the Rome III criteria. The Rome III criteria represent a system developed to help diagnose different bowel disorders including IBS. The system is based on the presentation of certain clinical symptoms with each disorder having its own specific diagnostic criteria. Although the etiology of IBS is unknown, the disease correlates in part with characteristic changes in the intestinal microbiota [60].

MetaHIT Consortium (Metagenomics of the Human Intestinal Tract Consortium):: MetaHIT is a project financed by the European Commission under the Seventh Framework Program. The project aims to investigate relationships or associations between the human intestinal microbiota and human health and disease using a metagenomic approach (http://www.metahit.eu/).

Microbiota:: the collective repertoire of microbes present in the intestinal tract of humans is referred to as the intestinal microbiota. In newborns, the gut is quickly colonized by facultative anaerobic bacteria, and after a few days the consumption of available oxygen favors a shift towards colonization by obligate anaerobes. In healthy humans, 90% of the intestinal bacterial flora, the gut microbiota, is represented by species of *Firmicutes* and *Bacteroidetes*.

Multidimensional cluster analysis:: a statistical approach used in the analysis of complex datasets to identify clusters or group data into clusters based on similarity.

Multilocus sequence typing:: a method used for molecular typing of, in particular, bacteria, but also parasites. Typically five to six loci, comprising multiple housekeeping genes, are amplified and sequenced. For each housekeeping gene, the different sequences present within a species are assigned as distinct alleles and, for each isolate, the alleles at each of the loci define the allelic profile or sequence type.

Pleomorphism:: variation in cell size or shape of an organism. Various morphotypes of *Blastocystis* have been described, including the vacuolar, avacuolar, multivacuolar, granular, amoeboid, and cystic forms, but little is known about the environmental conditions or cellular mechanisms responsible for transition between stages.

Principal component analysis:: a method of identifying patterns in data, and expressing the data in such a way as to highlight their similarities and differences. It uses orthogonal transformation to convert a set of observations of possibly correlated variables into a set of values of linearly uncorrelated variables called principal components; the analysis can be applied to visualize high dimensional data.

Short chain fatty acids (SCFAs):: are the byproducts of bacterial fermentation in the colon. These secondary microbial metabolites include butyric, acetic, and propionic acid. SCFAs have a range of diverse roles in maintaining gut ecology homeostasis; for instance, SCFAs regulate the size and function of the colonic regulatory T cells critical for regulating intestinal inflammation and hence protect against colitis [45]. Butyrate also serves as an energy source for colonocytes and also has inherent anti-inflammatory and anticarcinogenic properties [61].

Subtype (ST):: based on analysis of ribosomal genes, *Blastocystis* from humans, non-human primates, other mammals, and birds can be divided into a number of STs (currently 17), nine of which have been found in humans [2]. STs arguably represent separate species [6].

Synteny:: refers to the order or colocalization of genes or blocks of genes in chromosomes.

analysis of *Blastocystis* genotypes are required to provide information on the relative presence or absence and genetic diversity of such candidate virulence genes as cysteine proteases.

Although virulence may be attributed to specific genes such as cysteine proteases, the pathogenicity of a given organism may also result from a more complex genotype by host or genotype by host by environment interactions [17]. Such complexity in virulence makes it very difficult to predict and fully appreciate the pathogenicity of an organism in many instances. For example, variation in virulence gene expression in response to changes in environmental conditions has been shown for several pathogenic parasites, and the altered expression of virulence genes is often associated with specific environmental cues such as oxygen and iron availability [18,19]. A classic example of a complex virulence phenotype is *Entamoeba histolytica*, which sporadically becomes invasive presumably due to changes in its environment [20]. The host response to infection by *E. histolytica* results in a significant change in environmental conditions for the parasite, and several putative genes associated with virulence, including proteases, are upregulated to counteract the host response [21]. Changes in gene expression during host colonization have also been shown for other protists including *Trichomonas vaginalis*, a human urogenital parasite. Upon entry into the human host and contact with host cells, *T. vaginalis* undergoes a rapid morphological change from flagellated to amoeboid in a matter of minutes [22]. This morphological shift to the amoeboid form is accompanied by adherence to the host cell, and recent transcriptomic analysis of *T. vaginalis* has identified several genes upregulated in response to contact with a human vaginal epithelial cell line; these include cysteine proteases, actin, and ribosomal proteins [23].

These transcriptomic studies provide key insights into what genes are involved in virulence and what initiates