



Review Article

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Recent insights into the genetic diversity, epidemiology and clinical relevance of *Blastocystis* species

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Abstract

Blastocystis is a widely prevalent intestinal protozoan of man and other animals. It is associated with gastro intestinal disorders and symptoms. Emerging literature suggests that several subtypes of this parasite exist and are responsible for virulence, pathogenicity and symptoms. A variety of new diagnostic methods have been developed which help to recognize genotypic variations, subtype relationship and determination of carrier state. In symptomatic patients, when the other etiological agents have been excluded, it is prudent to treat *Blastocystis* infection. Metronidazole remains the drug of choice for treatment. Further research is required to shed light on taxonomy, biology and virulence of this organism.

Keywords: *Blastocystis*, Taxonomy, Controversies, Treatment.

Introduction

Blastocystis is an unicellular, anaerobic, eukaryotic protist confined to the gastrointestinal tract of humans and other hosts. It is a highly controversial parasite in terms of its taxonomical status and pathogenic potential. Despite being a common enteric protozoan in developing countries, knowledge of its life cycle and virulence is incomplete and contradictory. It has remained a mystery for many decades to both microbiologists and clinicians.^{[1], [2]}

Taxonomy

Brittain and Swayne first observed the parasite in the year 1849 while investigating a cholera epidemic. It was thought to be the causative agent of cholera then. In 1900, Alexieff and Emile Brumpt proposed it to be harmless saprophytic yeast of the gastrointestinal tract. The name *Blastocystis hominis* was coined by the above two scientists. Much later, in 1967, Zierdt *et al* reclassified the organism as a protist based on the presence of more than one nuclei, mitochondria, golgi apparatus and endoplasmic reticulum.^{[1], [3]}

By molecular analysis of rRNA (SSU- rRNA) and elongation factor -1alpha, Silberman *et al* placed the organism under the Eukaryotic phylum Heterocontophyta or stramenophiles comprising of algae, diatoms, slime moulds and oomycetes, *Blastocystis* became the newer member of this complex group of "botanical Protists."^{[4], [5]} Since the stramenophiles have flagella surrounded by lateral hair like mastigonemes, that is characteristically absent in *Blastocystis*, this organism was designated a separate sixth kingdom named "Chromista".^[1]

Deducing the identity of the organism to the species level is still an unresolved challenge. There are several species which are host specific: *B. hominis* from humans and *B. ratti* from rats. But diversity in hosts is well known and human to animal and animal to human transmission is documented.^[6] Due to the above factors, there has been a move to classify the different species based on their ultra-structural electron microscopic morphology.^[7] The host specificity and the pathogenic potential of different isolates correlate with sequence variations in the SSU-rRNA.^[6]

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Conserved and variable regions within the 18SSU (small subunit) rDNA constitute the basis for identifying the phylogenetic relationship between species. The rRNA also serves as popular targets for diagnostic PCR assays with a high diagnostic sensitivity.^[8] The more recent classification into subtypes (ST) is equivalent to the earlier species classification. SSU-rDNA correlates with ST and to date 17 STs have been reported. Presently, the final characterization of *Blastocystis* species is possible only by molecular subtyping.^{[1], [8]}

Biology

Blastocystis colonises the intestinal tract of a wide variety of hosts such as insects, reptiles, birds and mammals. The ST types determine host specificity. ST 1 to 8 colonises / infects humans as well as non-human hosts. ST 9 is found only in humans and ST 10-17 are exclusive to non-humans.^{[6], [7]} The organism is a strict anaerobe. Though structures resembling mitochondria are observed intracellularly, they lack cytochrome enzymes. The intracellular organelles are involved in various metabolic pathways such as amino acid metabolism, iron sulfur biogenesis and tricarboxylic acid cycle. *Blastocystis* is capable of synthesizing certain essential cellular phospholipids and they usually accumulate them in storage vacuoles.^[1] The average generation time of *Blastocystis in vitro* is 17-22 hours, though it may depend on the culture media used. If cultures performed first on xenic media followed by axenic media shortens, the generation time is shortened. The generation time is also variable among the different ST types.^[9] The organism has been observed to undergo apoptosis when exposed to adverse conditions, like exposure to ambient air and antiparasitic agent such as metronidazole. This phenomenon serves as a mechanism to increase the number of viable cells during adverse conditions.^[10]

Morphology

The morphological forms exhibited by species are many. Cysts develop into vegetative forms. The well characterized forms are the vacuolar, granular and the amoeboid forms. Other vegetative forms such as avacuolar or multi-vacuolar also have been identified. Other rare forms observed include medusa head and chest nut burr cells especially in aging cultures and on exposure to oxygen. All these above forms can be viewed by phase contrast microscopy and bright field microscopy of wet mounts, stained smears and electron microscopy.^{[3], [11], [12]}

The vacuolar form has a large central vacuole filling the entire cell space and limiting the cytoplasm and its intracellular components to a thin peripheral rim. There is size variation from 3 µm to 120 µm, though most human isolates measure 5-15 µm. Subsequently, the central vacuoles were actually found to be the membrane bound bodies containing carbohydrate and lipid dispersed as flocculent or granular material. These central bodies are probably storage organelles and take part in apoptosis.^{[1], [4]} The cytoplasmic rim contains one or two nuclei and the mitochondria are observed as rosettes around the nuclei. These structures may bulge inwardly into the central body and appear as filaments. Rarely, in fresh clinical isolates a surface coat or capsule has been observed, which presumably

protects the organism from osmotic shock and trap bacteria for nutrition.^{[3], [4]}

The granular form resembles the vacuolar form except for the presence of granules both in the central body and cytoplasm. The granules may be metabolic, reproductive or lipid containing. The reproductive granules have a possible role in schizogony. The granular forms exhibit lesser degree of pleomorphism and range in size from 15-80 µm.^[12]

The amoeboid form is less frequently encountered. As its name implies, they are irregular in shape, possess one or two pseudopodia but are non-motile. The cytoplasm contains a single large vacuole and this form converts to cyst. This form is more frequently observed in symptomatic patients suggesting its pathogenic potential.^[13] Because they resemble neutrophils and macrophages, they can be easily missed in conventional stool examination. To identify them, Zierdt suggested simultaneous gram staining of an unfixed smear where these forms lyse on exposure to air, while the leucocytes remain intact.^{[3], [4]}

The cyst form is spherical or ovoid and is smaller in size (3-6 µm). Some cysts encountered in animals are larger.^[7] Each cyst has a thick multilayered wall with or without a surface coat. The condensed cytoplasm has several mitochondria and storage vacuoles. The number of intracystic nuclei may vary from one to four. The cyst can remain viable for up to one month at 25 °C and on exposure to air. These cysts serve as transmissible infective forms.^[4]

Vegetative forms transform into other vegetative forms of various morphology and hence these can be easily overlooked in fecal samples.^[1] Though the vacuolar and granular forms appear irregularly stained under the microscope, Vdovenco was able to demonstrate uniformly stained live organisms in fresh cultures. So the vacuolar and granular forms may represent degenerative changes in the organism. The avacuolar forms and the multivacuolar forms have recently been recognized to be the most predominant forms *in vivo* and also the forms which are frequently missed during microscopic examination.^[14]

Life Cycle and Mode of Reproduction

There are no animal models for the organism and hence the life cycle remains improperly elucidated. What is well known is that, the cysts are the transmissible forms and the mode of infection is faeco-oral.^[15] Infection is acquired by drinking untreated water or eating raw aquatic plants contaminated with cysts. Unclean hands can serve as fomites.^{[16], [17]} In a suitable host the cyst develops into vegetative forms by excystation in the large intestine to liberate the vacuolar form. Further continuation of life cycle depends on the compatibility of the ST type with the host. Other forms may also develop from the vacuolar form. After a certain period of infection the vacuolar forms encyst in the lumen of the intestine.^[14] Various modes of reproduction have been hypothesised for this organism namely binary fission, budding, plasmotomy, multiple fission, endodyogeny and schizogony. However, binary fission seems the most established.^[18]

Virulence and Clinical Significance

Studies to determine the pathogenicity of *Blastocystis* have remained inconclusive and debatable. More recently, there is some plausible explanation of its pathogenicity in relation to ST types and virulence.^[19] Symptomatic patients usually carry the ST1, 2, 3, 4 and 6 types. Among them, ST3 is the most common, followed by ST1 and ST2.^[20] Intrasubtype variations in pathogenicity has also been observed and this probably explains the differences between pathogenic and non-pathogenic potential.^[21] Generally, the amoeboid form is the most virulent and secretes proteases. This form is the predominant form in symptomatic patients. It is prudent to look for these forms while screening stool samples of symptomatic patients.^{[14], [22], [23]} Apart from proteases, other hydrolytic enzymes have also been identified by gel electrophoresis. *Blastocystis* culture lysates produce cytoskeletal alterations and induce apoptosis in epithelial cells resulting in increased permeability. The cysteine proteases stimulate the mucosal cells to produce interleukin-8. The above mechanism is responsible for fluid loss and intestinal inflammation in affected individuals. The proteases cleave the secretory IgA and help in immune evasion and parasite survival.^{[1], [24]} Whole genome sequencing has been done for ST7. Genes coding for proteins responsible for host protease inhibition have been identified. These proteins alter the intestinal homeostasis. Also identified are the genes responsible for the production of non-ribosomal peptides and polyketides which are antibacterial and cause intestinal dysbiosis. Gene targets coding for hydrolases which are capable of altering the intestinal mucus layer thus exposing the epithelium for adhesion have also been characterized. Expression of serine proteases and glycosyltransferases supposedly disrupt the tight junctions of the intestinal mucosal epithelium resulting in increased permeability.^{[1], [25]}

The molecules responsible for extra intestinal manifestations still remain elusive. *Blastocystis* antigens stimulate the T helper II cells producing IgE mediated allergic response. The organism probably also activates the complement cascade to release anaphylotoxins resulting in activation of mast cells. Iron deficiency anemia associated with *Blastocystis* infection still remains unexplained.^{[26], [27]}

In general, the virulent strains are large sized, sport a rough surface, grow slowly and exhibit increased binding affinity to lectins. The relationship between infection density and clinical symptoms is still not clear.^{[28], [29]}

Genetic Diversity and Subtyping of *Blastocystis*

Based on SSU-rDNA analysis *Blastocystis* genus comprises of at least 17 lineages, the so called "subtypes". While ST1 to ST4 account for 90% of human carriage, the remaining carriers harbour ST5 to ST9. ST 1 to 8 is also found in non-human hosts and birds.^[8] By amplification of *Blastocystis* DNA directly from fecal DNA template, the subtypes found in non-human hosts have been studied. ST-5 is found in amphibian host while ST 15 and ST 17 are harboured by other mammals and reptiles.^[30]

Amplification of parasite specific rDNA in feces without culture has offered new diagnostic avenues in the laboratory. It has

enabled detection of new ribosomal lineages. Molecular screening assays help the differentiation from other intestinal parasites and assist in determination of transmission patterns and outbreak investigations. The intragenetic diversity and their prevalence has become obvious only because of the molecular methods adopted.^{[8], [31]} Genetic variations within some STs in *Blastocystis* may be around 3-5%.^[32] Variation in the distribution of STs is seen across the world. ST4, ST1 and ST3 are common in some countries in Europe while being absent in many other countries. ST 6 is rare in Europe and America and is rather common in some Asian and Middle East countries.^{[20], [33]}

It has been postulated that the methodology used for subtyping may attribute to the variation in geographic distribution of the STs. Subtyping methods employed includes screening of fecal samples by PCR using ST specific primers or genus specific primers followed by sequencing for ST identification. Bar coding appears robust for the genetic characterization, combined with the use of a forward primer for broad Eukaryotic specificity and genus specific reverse primer. Multilocus sequence typing (MLST) helps to decipher mixed ST carriage and offers high resolution.^[8] By using the sequence tagged primer method, the need for sequencing PCR products is circumvented and detection of mixed of ST carriage is straightforward.^[34] Intra ST diversity appears to vary dramatically from ST to ST. ST 4 sequences from humans are strikingly homogenous. It accounts for 1-17% of *Blastocystis* carriage. Wide intra ST variation exists in ST 3 also. Currently research on intra ST diversity of ST 1 and ST2 is being undertaken by MLST analysis. Because of this diversity in the STs, the design of genus specific primers applicable to fecal DNA template amplification is much more challenging.^[8]

Epidemiology and Prevalence

Blastocystis is an extremely ubiquitous parasite. Prevalence varies widely from country to country and within various communities in the same country. Developing nations have a higher prevalence that is linked to poor hygiene, exposure to animals and consumption of contaminated food or water. Japan has a low prevalence of 0.5-1% and Singapore has a prevalence of 3.3%. In contrast, the prevalence is high in countries such as Argentina (27.2%), Brazil (40.9%), Cuba (38.5%), Egypt (33.3%) and Indonesia (60%).^[35] Prevalence has ranged from 1.9 to 32.6% in China, Thailand and Turkey. Variations within a population are attributed to different diagnostic approaches and the sub population studied.^[4]

PCR based genotype surveys have thrown light on the distribution of the genotypes in several countries. Subtype 3 is found to be the most dominant in Bangladesh, Germany, Pakistan, Japan, Singapore, Greece, Turkey and China. Subtype 1 to a lesser extent has been detected in Singapore, Greece and Germany. Though a majority of individuals harbour one subtype, mixed infections are also being reported. Most co-infections occur with subtype 1 and 3.^[35-38] The risk factors for *Blastocystis* acquisition include immunocompromised health, poor hygiene practices, travel to and from a developing tropical country and exposure to animals or contaminated food or water. In persons with intestinal disorders, the incidence is

noted to be higher as with patients with allergic skin disease. Collectively, there is an increasing body of evidence suggesting that *Blastocystis* is pathogenic or is an opportunistic pathogen.^{[4], [39]}

Laboratory Diagnosis

Microscopic identification poses considerable challenge. The polymorphic nature of the organism in wet mounts results in mistaken identity with yeast, *Cyclospora* species or fat globules. The classical vacuolar forms may not predominate in fresh fecal specimens. Multiple stool specimens may be required because of its irregular shedding. Wet mounts with Lugol's iodine, permanent stained smears with acid fast, Giemsa, Fields and Trichrome stains have been used. Trichromestain remains the most popular to be employed.^[4]

A short term (24-72 hours) *in vitro* culture increases the sensitivity of detection compared to microscopy. The formol ethyl acetate concentration technique results in very poor sensitivity for parasite detection especially for the subtype 3.^[40]^[41] Correlation between infection density and symptoms is done by counting the number of parasites in microscopy. Generally five or more parasites per high power field are considered significant.^[4]

Culture

Xenic or monoxenic cultures have been employed in the laboratory. The organism can be maintained in Jones or Boeck and Drbohlav's inspissated egg medium.^{[4], [41]} The organism can grow both in solid medium and liquid medium. Use of biphasic slant medium has been attempted for culture of *Blastocystis* from non-human sources. Luxuriant growth has been observed in a variety of media such as Iscove's modified Dulbecco's medium and Minimum Essential Medium. The doubling time of axenic isolates can be variable ranging from 6-23 hours. The colony growth of *Blastocystis* can be established in soft agar using the pour plate method. Cultures are incubated at a constant temperature of 37°C before sub-culturing into fresh medium once every 3 days. Addition of sodium thioglycolate as reducing agent and antibiotics for inhibiting the contaminating bacteria and yeasts makes the media selective. It has also been suggested that some isolates require the presence of bacteria to survive and therefore their absence results in death.^[3] The colonies of *Blastocystis* macroscopically resemble bacterial colonies and are viable upto two weeks. Some authors noted improved success in culture when physical methods were employed during axenisation namely, differential centrifugation and density gradient separation.^[4]

Serology

Infection with *Blastocystis* leads to immunoglobulin A and G responses as detected by indirect fluorescent antibody testing and ELISA. High titers of IgG antibodies are associated with symptomatic infections. IgA responses are poor or absent in asymptomatic population. The strongest reaction is encountered in individuals with chronic infections. Application of genotype specific antigens in ELISA or immunofluorescence will be useful for serological or epidemiological studies.

Comparison of acute phase sera and convalescent sera reveals a marked increase in titer in infected patients. Overall, there is little knowledge of the host immune response to *Blastocystis* species. Hence the role of serology in routine laboratory diagnosis is limited.^[4]

Molecular Screening Tools

The first diagnostic PCR for detection of *Blastocystis* was introduced in 2006. These were conventional PCR based on sequences in Genbank and did not take into account the ST specific polymorphism.^[42] We now have subtype specific diagnostic primers and these are useful to identify them distinctly.^[4]

Only two "pan-*Blastocystis*" real time PCR assays have been published so far. The first assay had low sensitivity and a specificity of 95 %.^{[8], [43]} Its positive predictive value was only 91%. The other assay published was based on Taqman technology including a specific probe and an internal process control to identify cases of PCR inhibition. Thus far, this method has not been adopted for screening surveys. Based on this method the mixed ST infections account for less than 10% of all *Blastocystis* infections studied. This assay also helps to delineate carriers for non-carriers.^{[8], [44]}

Short term xenic *in-vitro* culture has been reported to be more sensitive than conventional PCR. But in comparison to real time PCR, it has a sensitivity of only 79%.^{[43], [44]} Also under consideration, is the designing and application of real time PCR assay using molecular beacons or scorpion probes for ST /allele analysis by using single nucleotide polymorphism (SNP) detection. ST specific SNP analysis by pyrosequencing has been developed but has not come into use. Lately metagenomic approaches have been advocated for analyzing *Blastocystis* specific mitochondrion like organelle sequences (ST1 to ST4). This technique enables the characterization of the bacterial flora that accompanies *Blastocystis* colonization. While it is important to detect *Blastocystis* carriage in symptomatic and asymptomatic individuals, it is also equally important to investigate the function of *Blastocystis* genes. Comparative genomic and transcriptomic analysis should assist us in characterizing the effect or proteins of potential interest.^[8]

Impact of sampling and sample processing on *Blastocystis* detection and subtyping

Methodological issues may influence the extent of identification and distribution of *Blastocystis*. Xenic *in-vitro* cultures may favor one ST over another in case of mixed colonization. Moreover, different strains may have different requirements in terms of growth conditions, temperature and nutrients. Sample storage may vary from cold /freeze storage, ambient temperature in preservatives such as lyses buffer or ethanol. These above factors may influence the ability to detect *Blastocystis*. DNA extraction method, the size of PCR product and the amount of competing DNA template in fecal samples also are hypothesised to influence the results of PCR. The "state of the art" technology appears to be direct screening of fecal DNA templates by real time PCR or a conventional PCR to amplify a short PCR product with subsequent bar coding of positive samples.^{[8], [45], [46]}

Pathogen or Commensal?

There is still much debate about the pathogenicity of *Blastocystis* in humans. Though many authors have given credit to it as a pathogen, there are still many that doubt its role in human disease. Some researchers have suggested that certain populations may be more susceptible to infection. Several studies have cited it as a common parasite in HIV infection, cancer and other immune deficient states. Children in under developed countries have a higher incidence of infection. This parasite has also been described as a cause of traveler's diarrhea. Recently, the finding of potential markers of pathogenicity has allowed insights into its causal role. Clinical symptoms seemed to be subtype dependent. *Blastocystis* has met all the criteria for pathogenicity that are met by *Giardia* and *Entamoeba histolytica* such as fulfilment of Koch's postulates, demonstration of a treatment that eliminates the organism and the symptoms and an elevated immune response in symptomatic individuals. But, all persons infected are not symptomatic. Infection and symptoms are not uniform across geographic areas. Therefore, it is prudent to consider it as a pathogen and institute treatment for acute and chronic cases.^{[39], [47], [48]}

Infection and Diseases

There are equal numbers of studies that either implicate or exonerate, *Blastocystis* as a cause of disease. The clinical features of illness are nonspecific and include nausea, anorexia, abdominal pain, bloating, flatulence, acute or chronic diarrhea. Of the above, abdominal pain and diarrhea are the most consistent. Symptoms vary from mild, moderate and severe to acute or chronic. The parasite density determines the severity of symptoms.^{[4], [14], [39], [47]} Rarely patients have eosinophilia and cutaneous rashes particularly urticaria. Since the parasite is non-invasive, dysentery does not occur.^[49] Interestingly, extra intestinal Blastocystosis has been reported in a man with 5 days history of fever and dysentery who developed liver abscess, although its invasive potential is still to be proven.^[50] Accumulating evidence also suggests an association between *Blastocystis* and irritable bowel syndrome [IBS]. *Blastocystis* is detected more frequently in IBS patients than in control groups.^[25] Some studies also cite an association between *Blastocystis* infection and the use of non-steroidal anti-inflammatory drugs (NSAID) in the development of skin disorders.^[51] The presence of *Blastocystis* in symptomatic individuals in the absence of co-infecting enteric pathogens and the resolution of symptoms upon anti-protozoal treatment with the concomitant eradication of the parasite are strong pointers to its pathogenic role.^{[4], [52]}

Irritable Bowel Syndrome (IBS) and the Role of *Blastocystis*

Blastocystis infection and IBS have been linked for the last few years. The symptoms in both are similar. It is possible that the change in the intestinal environment caused by IBS allows the growth of *Blastocystis*. Low grade inflammation through persistent antigenic exposure in chronic *Blastocystis* infection may cause IBS like symptoms. Pro-inflammatory cytokines released by the organism might have a role in pathophysiology. Gene polymorphisms in IL8 and IL10 in IBS are linked to

Blastocystis carrier state.^{[25], [48], [53]} High levels of IgG2 directed against *Blastocystis* is found in patients having IBS patients.^[52]

Treatment of Blastocystosis

Treatment of *Blastocystis* infection remains a complicated issue. There is still much debate about the need for treatment. The pathogenic role and the antimicrobial efficacy on various subtypes still being determined. In symptomatic patients, isolation of cysts in stool specimens should trigger a thorough evaluation for other causes of GI complaints. Since co-infection with *Giardia intestinalis* and *Entamoeba histolytica* can occur, it is possible that response to treatment for *Blastocystis* with metronidazole or trimethoprim-sulphamethoxazole (TMP-SMX) may actually be due to treatment of the secondary pathogens. To date, a number of antimicrobial agents have been used for treatment. Metronidazole is considered as first line, but the success rates reported varies between 0-100%. Studies on metronidazole efficacy have not elucidated the relationship between the subtypes and treatment failures. TMP-SMX has been used as a second line agent in patients who do not respond to metronidazole. Eradication of the parasite from the stool has been observed in a considerable proportion of treated children along with resolution of symptoms. Other agents found to be effective include nitazoxanide, iodoquinol, tinidazole, emetine, pentamidine, iodochlorhydroxyquin and fluorozolidine. Natural compounds like garlic and dietary herbs, ginger, black pepper and white cumin have been tried for treatment potential. Probiotics like *Saccharomyces boulardii* has been found to be as effective as metronidazole in alleviation of symptoms. Genome analysis of the species and application of modern techniques including proteomics and bioinformatics are required to elucidate the difference in pathogenicity, virulence and response to treatment. If drug resistance is proven, new therapeutic options should be developed.^{[2], [52]}

Conclusion

Even though *Blastocystis* is considered as a widely prevalent protozoan parasite, conclusive information regarding its taxonomy, biology and virulence had been lacking. Recent insights into its genetic diversity and existence of subtypes that relate to virulence have thrown light on its epidemiology, lifecycle, molecular biology and prevalence. The introduction of the molecular screening methods has increased the sensitivity of laboratory diagnosis. More evidence is now in favor of its pathogenic potential and its etiological relationship with irritable bowel syndrome. Use of appropriate antimicrobial agents leads to its eradication and alleviation of symptoms thus favoring its pathogenic significance.

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