



Pathogenic mechanisms in *Blastocystis* spp. – Interpreting results from *in vitro* and *in vivo* studies



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ABSTRACT

Blastocystis spp. are commonly reported intestinal protists but whose clinical significance remains controversial. Infections have ranged from asymptomatic carriage to non-specific gastrointestinal symptoms and have also been linked to irritable bowel syndrome and urticaria in some patient populations. *In vitro* studies showed that both parasite and parasite lysates have damaging effects on intestinal epithelial cells causing apoptosis and degradation of tight junction proteins occludin and ZO1, resulting in increased intestinal permeability. Adhesion of trophic forms to the intestinal epithelium and release of cysteine proteases appear to be the major triggers leading to pathogenesis. Two putative virulence factors identified are cysteine proteases legumain and cathepsin B. *Blastocystis* spp. also have immuno-modulatory effects including degradation of IgA, inhibition of iNOS and up-regulation of proinflammatory cytokines, IL8 and GM-CSF in intestinal epithelial cells and IL1 β , IL6 and TNF α in murine macrophages. *Blastocystis* spp. have also been reported to dampen response to LPS in intestinal epithelial cells and monocytes. Studies in rodent models and naturally infected pigs have shown that the parasite localizes to the lumen and mucosal surface of the large intestine mostly in the caecum and colon. The parasite has been found to cause mucosal sloughing, increase in goblet cell mucin, increased intestinal permeability and to induce a pro-inflammatory cytokine response with upregulation of TNF α , IFN γ and IL12. In this review, we summarize findings from *in vitro* and *in vivo* studies that demonstrate pathogenic potential but also show considerable inter and intra subtype variation, which provides a plausible explanation on the conflicting reports on clinical significance.

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1. Overview

Blastocystis spp. are extra cellular, non-motile protists that inhabit the gastrointestinal tract, are widely prevalent and have been associated with non-specific gastrointestinal symptoms of diarrhea, nausea, vomiting and abdominal pain [1], irritable bowel syndrome [2,3] and urticaria [4]. The clinical significance of this parasite has been controversial [5,6] but increasingly, findings from *in vitro* and *in vivo* studies have demonstrated pathogenic potential leading to plausible models of pathogenesis put forward by multiple research groups [7,8].

Although originally described over a century ago and seen in a wide range of animal hosts, this protist has only been relatively recently classified as a stramenopile, a highly diverse group of organisms that include brown algae and diatoms based on phylogenetic analysis of small subunit rRNA gene (SSU rDNA) [9]. *Blastocystis* spp. are a notable

exception among stramenopiles due to their anaerobic nature and lack of flagella and are the only human pathogens within this subgroup [10]. The classical spherical “vacuolar” form of this parasite comprises of a large central vacuole occupying >90% of the cell volume with the cytoplasm containing multiple nuclei and mitochondria like organelles pushed to the periphery [1,11]. Vacuolar and the granular forms are seen more commonly in fecal samples and cultures while the amoeboid form and the cyst stage are seen less frequently. *Blastocystis* spp. have been further classified into 17 different subtypes (ST) based on the SSU rDNA and subtypes 1–9 have been reported in humans [12]. Subtypes 1–4 are more prevalent in humans comprising over 90% of reports with wide geographic variation in prevalence rates [13]. Among these subtypes, ST1, 2 and 4 have been described to have low host specificity and are probably zoonotic infections [14]. ST3 has been described as more anthroponotic and although this subtype has also been found in non-human primates, cattle and pigs [15,16], phylogenetic analysis has revealed that human isolates form a separate subgroup within this subtype distinct from the animal isolates [17].

Reports on prevalence rates of *Blastocystis* spp. in diarrhea vary widely with higher burdens exceeding 30–40% reported from multiple studies in developing countries [18,19]. High community-wide

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prevalence rates and increasing risk with age in developing countries point towards more water borne transmission in addition to possible human-to-human or zoonotic transmission. However some studies in both developing countries and developed countries have reported similar prevalence rates in asymptomatic individuals [20,21]. *Blastocystis* spp. have also been associated with irritable bowel syndrome (IBS) and urticaria in some patient populations [2,3,22] but other workers did not find an association [23,24]. Some studies have also found an increased prevalence in immunosuppressed individuals (HIV and renal transplants) [25–28] and in inflammatory bowel disease (IBD) [29]. This variation in reported prevalence rates and association with disease are possibly influenced by epidemiological factors including geographic variations in parasite distribution [13] and transmission patterns in the community as well as inter and intra subtype variability in virulence [30,31], undocumented co-infections with other bacterial or viral diarrheal agents and host genetic susceptibility [32]. Technical parameters including sensitivity of the detection method and expertise in microscopic detection of non-classical forms of this highly polymorphic parasite could also play a significant role in reported prevalence rates [33,34]. Recently, a study in Senegalese school children with gastrointestinal symptoms demonstrated a prevalence rate of 100% when PCR based screening was applied [35]. This report illustrates how blastocystosis is probably under diagnosed with conventional diagnostic tools as well as its ubiquitous nature in underdeveloped communities.

In this review, we have summarized findings using *in vitro* and *in vivo* approaches to study the pathogenesis of *Blastocystis* spp. Most of the *in vitro* studies have been carried out with well-characterized axenic strains that have been subtyped. *In vivo* studies have been carried out with axenic strains of known subtypes [36], purified cysts [37] as well as isolates obtained by culture of fecal samples (these may have contained other undocumented bacterial or viral agents as well as altered microflora) from symptomatic and asymptomatic individuals [38]. Both series of studies show considerable inter and intra subtype variability and could explain, in part, the uncertainty of pathogenic potential and the wide range of symptomatology associated with blastocystosis.

2. *In vitro* studies

Studies involving co-culture of intestinal epithelial cells with *Blastocystis* spp. as well as treatment of intestinal epithelial cells with parasite lysate or excretory/secretory products in culture filtrates have led to the identification of several pathogenic mechanisms (Fig. 1). These include apoptosis [39], degradation of tight junction proteins resulting in increased intestinal permeability [8,39], induction of pro-inflammatory cytokines [40] and downregulation of iNOS [41]. However, it is important to note that studies using cell culture based models may be hampered by the strict anaerobic nature of the parasite that could result in rapid transition to granular forms and cell death [42]. Additionally, current limitations include a lack of axenic isolates of the different subtypes, especially the most common anthroponotic subtype ST3 and non-completion of the life cycle *in vitro*. It is also important to note that axenic cultures used in these studies are predominantly in the vacuolar form and may not reflect the morphological forms associated with adhesion [43,44] and pathogenesis *in vivo* [36,45]. Cell culture based studies have also used varying ratios of infection and these may not reflect the actual parasitic load in the intestine. Never the less, these studies have provided valuable insights on potential pathogenic mechanisms in blastocystosis that can be validated in further studies using appropriate animal models.

In work carried out by 2 independent groups, the central role of cysteine proteases in virulence of *Blastocystis* spp. have been demonstrated. Legumain, a 31 kDa asparaginyl cysteine protease has been found to be localized on the cell surface and also plays a pro-survival role as treatment of *Blastocystis* spp. with monoclonal antibody to legumain (1D5) [46] results in apoptosis of parasites [47]. Cathepsin B, an 18 kDa cysteine protease has also been identified as a potential virulence factor [48]. Cysteine proteases have also been found to localize to the central vacuole in a ST4 isolate [40] and could explain why in some studies, parasite lysate has been found to have more severe effects than live parasites [39].

2.1. Apoptosis

When non-transformed rat intestinal epithelial cells were co-cultured with an axenic ST4 isolate WR1, obtained from rat fecal

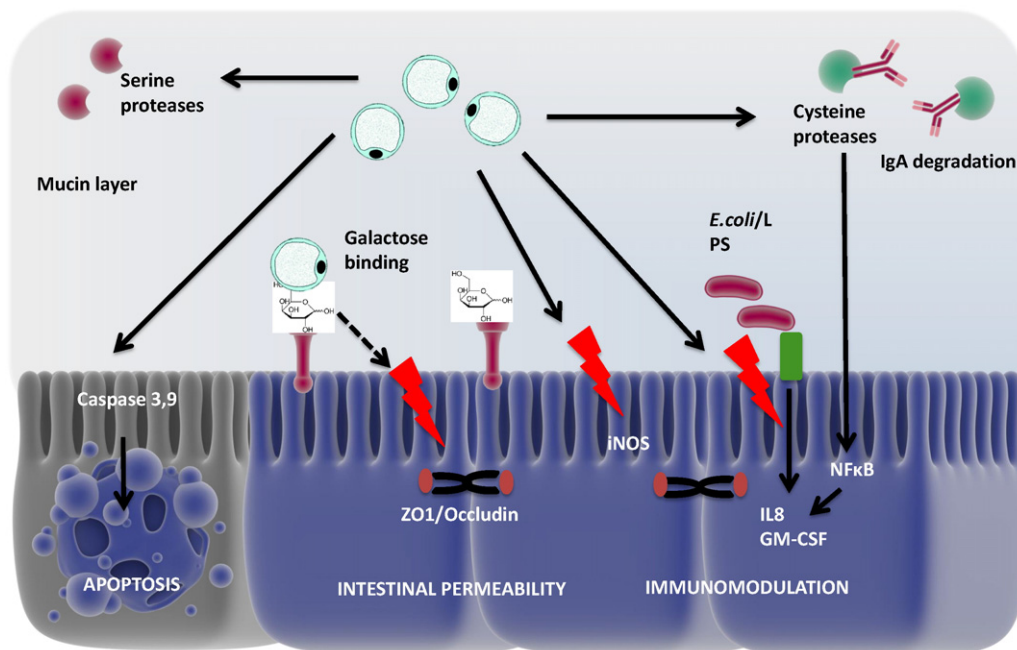


Fig. 1. Current model for pathogenic effect of *Blastocystis* spp. on intestinal epithelial cells.

samples in trans-well inserts, contact independent apoptosis was induced while treatment of cells with parasite lysate resulted in increased necrosis [39]. However in Caco-2 cells infected with the ST4 isolate, WR1 and ST7 isolate B, obtained from a symptomatic patient, apoptosis was induced by ST7 but not ST4. This absence of effect of a rodent isolate on human epithelial cells could indicate host specificity. Apoptosis induced by ST7 isolate B was found to be mediated by caspase 3 and caspase 9 [31].

2.2. Intestinal permeability

Earlier studies on non-transformed rat intestinal epithelial cells showed that both live parasites and lysate of ST4 increased intestinal permeability in a time dependent manner [39]. This study also showed that treatment with lysate and live cells resulted in F-actin disruption and formation of actin stress fibers. In Caco-2 cells ST7 isolate B (both live cells and lysate) was also found to mediate increase in intestinal permeability in a time and dose dependent manner [8]. Rho kinase/myosin light chain phosphorylation induced by parasite cysteine proteases resulted in loss of ZO1 protein and F-actin reorganization [8]. Loss of ZO1 and occludin protein was found to correlate to subtype with ST7 resulting in increased permeability compared to negligible effects of ST4 in human intestinal epithelium. Considerable intra-subtype variability was also seen among isolates of ST7 obtained from symptomatic patients [30,31].

Interestingly, this hierarchical gradation in effect on intestinal permeability correlated to adhesiveness and isolates were found to bind significantly more often to the apical junction in intestinal epithelial cells [30]. This ability to adhere and degrade tight junctions was also inversely correlated to metronidazole resistance indicating a possible loss of fitness in drug resistant isolates. Adhesion was prevented by galactose in a dose dependent manner and this also resulted in prevention of ZO1 and occludin degradation, thereby indicating that binding to galactose residues on the epithelial surface was an important early event resulting in compromise of the intestinal barrier [30]. Further studies are required to identify the receptor/ligand involved in galactose dependent attachment of *Blastocystis* spp. to intestinal epithelial cells. Studies are also required to determine whether this binding mechanism is also exploited to bind to galactose residues on the intestinal mucin layer enabling colonization as seen in *Entamoeba histolytica* [49].

2.3. Immunomodulatory effects

In addition to direct effects on the host intestinal epithelium, *Blastocystis* spp. also have immunomodulatory properties. Parasite cysteine proteases in lysates as well as conditioned medium containing excretory/secretory products degraded IgA in a pH and time dependent manner [50]. Protease activity was found to peak at 24–48 h following culture and also showed inter subtype variation with higher levels seen in ST7 than ST4 [51]. As a pro-survival mechanism, a metronidazole resistant isolate that was highly susceptible to nitrosative stress was able to avoid cell death by downregulating the expression of iNOS in intestinal epithelial cells even in the presence of pro-inflammatory cytokines and also by increasing the uptake of arginine [41]. Cysteine proteases from a ST4 isolate have been found to induce NFκB-mediated upregulation of IL8 in human intestinal epithelial cells [40]. In murine macrophages, both live parasites and lysates induced MAP kinases mediated upregulation of IL1β, IL6 and TNFα. Serine proteases in ST7 lysate were found to act through ERK and JNK pathways while cysteine proteases were able to induce these pro-inflammatory cytokines in a MAP kinase independent pathway [52]. In an earlier study, *Blastocystis* spp. ST1 isolate *NandII* induced both IL8 and GM-CSF production in human intestinal epithelial cells T84 and HT-29 after 24 h co-culture. However, at 6 h there was no upregulation of cytokines and instead, *Blastocystis* spp. live cells and culture filtrates dampened the effect of *Escherichia coli* and LPS on intestinal epithelial cells [53].

In a more recent study, when co-cultured with THP1-Blue monocytic cells, *Blastocystis* spp. ST7 and ST4 live cells were observed to dampen LPS mediated NFκB activation (determined by levels of alkaline phosphatase expressed controlled by NFκB inducible promoter) while parasite lysate was found to augment the effect of LPS. *Blastocystis* spp. were also found to dampen the effect of zymosan, a TLR2 agonist, but had no effect on flagellin [54]. However, these results need to be interpreted with caution as *Blastocystis* spp. were found to have a direct effect on the secreted phosphatase. Further studies on downstream effects of NFκB eg: upregulation of IL8, may provide a clearer picture in future.

These immunomodulatory effects could be both a pro-survival mechanism for this extra cellular, lumen dwelling parasite and could also facilitate other enteric pathogens either directly or by the resultant changes in microflora. A recent study found that male patients with IBS-C (a symptom based subgroup of IBS with a predominant bowel habit of constipation) and *Blastocystis* spp. harbored fewer *Bifidobacterium* spp. compared to IBS-C patients not infected with *Blastocystis* spp. while healthy male controls with *Blastocystis* spp. had lower *Bifidobacterium* spp. and *Faecalibacterium prausnitzii* than healthy uninfected controls [55]. *Bifidobacterium* spp. and *F. prausnitzii* have been reported to be protective and shown to have anti-inflammatory effects [56,57]. While previous studies have linked *Blastocystis* spp. to IBS, this is the first study to show that infections with *Blastocystis* spp. could result in alterations in microbiota leading to dysbiosis.

3. In vivo studies

Till date there have been numerous studies carried out using various animal models in *Blastocystis* spp. including different breeds of rats (most common), mice, guinea pigs and chicken using well characterized isolates or isolates from fecal culture (summarized in Table 1). These have been introduced either by intra-caecal inoculation of trophic forms or by oral gavage with cyst stages. While these studies have helped establish some of the basic tenets of infectivity and provided significant evidence of pathogenic potential, none have successfully proven Kochs' postulates. In some studies, *Blastocystis* spp. trophic forms given orally have also been associated with pathological changes but these findings need greater scrutiny. In addition, studies using non-axenized cultures of human or animal fecal samples require a 'xenic culture associated microflora' control to delineate the specific effects of *Blastocystis* spp.

3.1. Transmission

Feco-oral transmissibility has been shown using uninfected naïve rats co-housed with infected animals [58] and the requirement for a cyst stage in order to establish infections through the oral route [59]. Encystation and recovery of cysts in fecal samples have been found to be influenced by strain, subtype and host species. Iguchi et al. have shown that ST4 and 7 encysted in both chickens and rats while ST2 encysted in chickens. The human associated subtype ST3 did not encyst in either of these animal hosts [37]. This rate of encystment in turn probably affects the rate of transmission, as less time for animal to animal transmission is taken by isolates that encyst more efficiently [58].

The duration of infection is influenced by strain, subtype, dosage of infection and also the animal model. In an early study, porcine strains of *Blastocystis* spp., probably ST5 successfully infected outbred laboratory mice but not inbred BALB/c mice and gerbils [60]. Rats are usually infected stably for longer periods of several weeks and can shed cysts while mice have acute, self-limiting course of infection of 1–2 weeks and usually do not shed cysts. Older mice are refractory to infection but recent studies using immunosuppressant drugs have shown a demonstrable increase in susceptibility and severity and could be exploited to develop a susceptible mouse model [61,62]. Mice remain an attractive option as although rats are stably infected, they are also natural hosts for *Blastocystis* spp. ST4 and could acquire infections or

Table 1
Animal models on the pathogenicity and transmissibility of *Blastocystis* spp.: relevant findings and limitations.

Year	Author	Animal model	Parasite description	Route	Detection methods	Read outs	Relevant findings	Limitations
1993	Suresh [59]	4 week old Wistar rats pre-screened for <i>Blastocystis</i>	Isolate C, axenic with cysts/Isolate MS, non-axenic, trophic forms (Human source)	Oral; C: 10^4 ; Isolate MS: 10^{4-6}	Stool; Histology	Infection seen by day 2 for isolate C, no infection with isolate MS; On day 7, infection in caecum with isolate C not MS	<i>Infection by oral route requires cyst stage</i>	Absence of xenic culture associated microflora controls
1997	Moe [36]	2 to 8 week old BALB/c screened to be <i>Blastocystis</i> negative	Human sample/? ST/Concentrated cysts Human Diarrhea/ST7/Axenic culture	Oral: 10^{2-6} Intra-caecal: $4-8 \times 10^6$	Fecal microscopy and culture; Histology	Cyst: 2–6 week old mice were positive for <i>Blastocystis</i> infection up to 2 weeks while 8 week old mice were resistant; Intra-caecal - all 4 week old mice infected up to 3 weeks followed by recovery; Macroscopically caecal tissue bloated <i>Vacuolar and granular forms in caecum and cyst forms in colon - seen mainly in luminal content and in mucosa; No invasive lesions; Intense inflammatory infiltrates, edematous lamina propria with mucosal sloughing in proximal and distal colon</i>	<i>Age related susceptibility in mice with acute, self-limiting course of infection</i> <i>Luminal and mucosal localization of the parasite</i>	Goblet cells not documented
1998	Moe [73]	6–8 week old BALB/c mice	Human Diarrhea/ST7/Isolate B/axenic	I/M: live and fixed 5×10^5	Histology	Inflammation within 1 h with increase in PML, necrosis and vasodilatation over time; Recovery and regeneration from 48 h. Fixed cells caused mild to no inflammation	<i>Inflammatory potential and chemoattractant properties of Blastocystis spp.</i>	No serum cytokine or general markers of tissue inflammation tested
2001	Abou El Naga [69]	4 week old Swiss albino mice	Human diarrhea/?ST/Cyst from fecal samples	Oral: 10^4	Histology	All mice infected by Day 5, granular, vacuolar and cysts stages seen in the luminal contents and mucosal edge of large intestine with intense inflammation, sloughing of mucosa and edema. Moderate inflammation and hemorrhage in small intestine	Similar to previous findings by Moe et al.	Absence of xenic culture associated microflora controls; No mention of prior screening for <i>Blastocystis</i> spp.; No details on subtype
2004	Yoshikawa [58]	3 week old SPF Wistar male rats	RN 94–9/Brown Norway Rat/ST4/- Xenic culture	Oral: $10^5-4 \times 10^7$	Fecal and intestinal content culture; Co-housing with uninfected rat; Infectivity of cysts from feces;	All RN 94-9 rats culture positive after oral inoculation due to presence of cysts; NIH 1295:1 rats positive only after intra caecal inoculation; Feco-oral transmission in 9 and 15 days respectively; Infectivity of up to 100% demonstrated with viable cyst concentrations ranging from 10 to 1000; Cyst forms seen in caecum and large intestine but not in small intestinal contents	<i>Feco-oral transmissibility of cysts in animal model; Strain dependent variability in infectivity probably influenced by presence of cysts in culture; Predominant morphological forms in different gut segments</i>	Absence of xenic culture associated microflora controls; No mention of prior screening for <i>Blastocystis</i> spp.
2005	Yao [62]	4 week old female ICT mice screened to be <i>Blastocystis</i> negative	NIH 1295:1/Guinea Pig/ST4/Xenic culture Human diarrhea/ST7/Xenic culture	Intra-caecal: 5×10^6 Oral: 10^{4-6}	Intestinal contents; Histology	<i>Blastocystis</i> spp. seen in all segments of the intestinal tract up to the stomach; Edema in mucosa of normal mice (recovery in 7–14 days) and inflammatory	<i>Used immunosuppressed animal model with demonstrable increase in susceptibility, severity of disease and delay in recovery</i>	No details on subtype

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Table 1 (continued)

Year	Author	Animal model	Parasite description	Route	Detection methods	Read outs	Relevant findings	Limitations
2006	Zhang [61]	Kunming mice	Cyst	Rectal: 10^6 Oral: 2×10^4	TEM/SEM	infiltrates and epithelial desquamation in mice immunosuppressed with cyclophosphamide with longer recovery of 4–6 weeks <i>Blastocystis</i> localized to lumen and iliocaecal mucosa; destruction of microvilli, mitochondrial edema, rough endoplasmic reticulum dilatation and degranulation in absorptive and goblet cells; lymphocyte and eosinophilic infiltrates in mucosa <i>Mice immunosuppressed with dexamethasone had more severe disease</i>	Descriptive EM images of <i>Blastocystis</i> spp. in the intestinal tract	No details on subtype
2007	Iguchi [37]*	3 week old SPF Wistar male rats screened to be <i>Blastocystis</i> negative	Human samples/ST2,3,4 and 7/Xenic culture	Intra-caecal: 5×10^6	Fecal culture; genotyping; cyst infectivity	ST2-infected chickens with cyst formation; ST4,7 - chickens and rats with cyst formation; ST3 - lower infection rates and no cysts found; Cysts and trophic forms obtained were confirmed as identical to that inoculated; ST2, 4 and 7 cysts resulted in infection in naïve rats and chickens with least dose required for ST2	<i>Subtype and strain dependent variation in infectivity, variation in host susceptibility and in host encystation</i>	Absence of xenic bacterial culture controls
2007	Hussein [38]	1 week old Hy-line Laura male chicks 4 week old Wistar male rats	Human Diarrhea (ST1, ST3, ST4)/Xenic culture	Oral: 4×10^7	Histological score; Ussing chamber	Infected up to 6 weeks later; ST1 and 3 symptomatic strain associated severe pathology, <i>ST1 associated precancerous polyps in mucosa</i> , ST2 and 4 - mild to moderate; <i>Increased intestinal permeability based on change in potential difference in ST1, 3 and 4 symptomatic strains</i>	Subtype dependent variation in severity of intestinal damage and permeability - ST1 and 3 more severe than ST2 and 4	Absence of xenic culture associated microflora controls; No mention of prior screening for <i>Blastocystis</i> spp.
2008	Eida [74]	4 week old albino mice	Asymptomatic (ST2, ST3, ST4)/Xenic culture Human diarrhea/?ST/culture	Oral: 4×10^7	NO estimation	Concentrations of Nitric Oxide in infected mice tissue, blood and stool higher than uninfected controls with highest levels seen in caecal tissue.	<i>Induction of NO in tissue with greater response in caecal tissue than ileum</i>	No specific detection methods for <i>Blastocystis</i> spp. in histology Absence of xenic culture associated microflora controls; No details on subtype
2008	Helmy [75]	4 week old albino mice	Human diarrhea/?/culture	Oral: 5×10^6	Histology	Pretreatment with antioxidants resulted in decreased pathology	Possible role of free radical damage in <i>Blastocystis</i> infection	Absence of xenic culture associated microflora controls; No details on subtype
2009	Santos [70]	6–8 week old BALB/c mice	ATCC50613/Mixed ST/Xenic	Oral: 5×10^6 ; Trophic stage	Blood and intestinal secretions from 1 to 8 weeks	Elevated serum IgM from week 1–5; In intestinal secretions IgA predominated with peak at 5 weeks.	Kinetics of Immunoglobulin response determined	Absence of xenic culture associated microflora controls
2009	Iguchi [45]	3 week old SPF Wistar male rats	RN 94–9/Brown Norway Rat/ST4/-Cyst concentrated	Oral: 10^5	Caecal feces culture; histology;	All infected rats positive from 1 to 4 weeks later; Intact mucosa,	<i>First in vivo study demonstrating cytokine response with</i>	Absence of xenic culture associated microflora controls

Table 1 (continued)

Year	Author	Animal model	Parasite description	Route	Detection methods	Read outs	Relevant findings	Limitations
		screened to be <i>Blastocystis</i> negative	from rat feces		cytokine analysis; tight junctions	non-significant inflammatory infiltrates; increase in neutral mucin containing goblet cells 2 weeks post infection; upregulation of IFN γ , IL-12 and TNF α at 2–3 weeks post infection; no change in expression of occludin or ZO	upregulation of IFN γ , IL-12 and TNF α	
2010	Zhou [76]	6 week old female BALB/c mice	Human diarrhea/ST1/Xenic culture	Intra-peritoneal · 10 ^{4–6}	Abdominal contents	Granular and vacuolar forms seen in abdominal cavity and passaged to healthy mice		
2010	Elwakil [65]	4 week old albino mice	Human diarrhea/?ST/culture	Oral: 10 ^{2–7}	Fecal microscopy; Histology	Increased infectivity with inoculum, with 10 ⁷ , lethargy, weight loss and mucus feces seen; Infiltrates into lamina propria and submucosal muscle layer; inflammatory infiltrates	Invasive potential	Absence of xenic culture associated microflora controls; No specific detection methods for <i>Blastocystis</i> spp. in histology
2010	Chandramathi [77]	4 week old Sprague Dawley Rats prescreened for <i>Blastocystis</i>	Human sample/cysts/?ST	Oral: 10 ⁵	Feces microscopy, culture; Urine Hyaluronidase activity; Cytokines in serum	Positive from day 5 to Day 30; HA activity increased on day 28; IL6 and IL8 elevated compared to uninfected controls on day 30	Invasive and inflammatory potential	Absence of xenic culture associated microflora controls; No details on subtype
2010	Chandramathi [78]	4 week old Sprague Dawley Rats prescreened for <i>Blastocystis</i>	Human sample/purified cysts/?ST	Oral: 10 ⁵	Feces	Positive from day 5 coincident with increase in oxidative indices to day 30	Potential oxidative damage induced by <i>Blastocystis</i>	No details on subtype
2013	Li [66]	6 week old SPF Sprague Dawley	Human Diarrhea/ST1/Xenic culture/Cyst concentration	Oral: 10 ^{2–4}	Fecal culture; Intestinal contents; Histology	All infected rats positive by day 10; Granular and vacuolar forms in caecum, cyst forms more common in distal colon; Mucosal adhesion, inflammatory infiltrates, mucosal sloughing and in high doses invasion to lamina propria	ST1 animal model with inflammation and invasive potential	Absence of xenic culture associated microflora controls; No mention of prior screening for <i>Blastocystis</i> spp.; No specific detection methods for <i>Blastocystis</i> spp. in histology
2014	Chandramathi [79]	3 week old Wistar albino Rats prescreened for <i>Blastocystis</i>	Human sample/purified cysts/?	Oral: 10 ⁴	Oxidative damage	Increased cyst shedding, serum IgE and oxidative damage increased in mice with additional stress than mice with <i>Blastocystis</i> infection alone. Decreased PBMC proliferation.	Stress associated increase in pathogenicity of <i>Blastocystis</i>	No details on subtype, No other pathogen control

have pre-existing immunity due to prior exposure [63]. Few studies using rats as animal models have documented pre-screening of the litter for *Blastocystis* spp. and hence findings have to be interpreted with caution.

3.2. Localization

Once infected, the segments of the gastrointestinal tract affected most often have been identified as caecum and colon with predominant morphological forms seen - vacuolar and granular forms predominating in the caecum and cyst stages seen more often in the colon. Some rodent studies have also identified *Blastocystis* spp. trophic forms in the ileum, jejunum and stomach [62]. Lending credence to this, recently in naturally infected pigs, *Blastocystis* spp. trophic forms were also identified in the jejunum in addition to the large intestine, indicating that *Blastocystis* spp. could potentially colonize much larger segments of the gut than

previously described [43]. The localization of this protist in the intestinal tissue has mostly been described to be in the lumen or on the mucosal edge along with deposits of mucin both in experimentally infected rodents [36,61] as well as naturally infected pigs [43,44]. Iguchi et al. also reported increase in neutral mucin containing goblet cells following infection in rats [45]. Further studies on interaction of *Blastocystis* spp. with mucin will shed light on how this non-motile protist is able to traverse the thick double layer of colonic mucin in order to access the host intestinal epithelium. It will be interesting to note whether the more 'pathogenic' strains are able to degrade mucin compared to infections with 'non-pathogenic' or commensal strains that only colonize the host [64].

Some reports of invasion to lamina propria and muscular layer by isolates from symptomatic patients have been seen using histology sections but need more appropriate methods to identify the invasive forms for example, immunohistochemistry or immunofluorescence with

Blastocystis spp. specific antibodies as well as 'xenic culture associated microflora' controls [65,66]. Invasion of the intestinal barrier, however, is unlikely as based on clinical symptoms and reports from human endoscopic studies, *Blastocystis* spp. are lumen dwelling parasites [67]. Occasional invasive and extra-intestinal lesions could possibly arise from other co or pre-existing conditions including ulcers and perforated bowels [68].

3.3. Effects on intestinal tissue

During the course of infection, studies have reported presence of inflammatory infiltrates in the sub-mucosa (lymphocytic and eosinophilic), mucosal sloughing and edematous lamina propria [36,61,62,69]. Severity of inflammation has been associated with dosage of infection and subtype. Rats infected with ST4 isolates can remain asymptomatic while Hussein et al. showed that ST2 and 4 resulted in mild to moderate infections but ST3 and ST1 were associated with more severe pathology, including presence of intestinal polyps [38]. The same report also used Ussing chambers to demonstrate increased intestinal permeability. However in another study, unlike *in vitro* studies, no change in expression of ZO or occludin mRNA was seen in rat intestinal tissue following infection with ST4 [45]. However, since these rats remained asymptomatic with ST4 and *in vitro* findings were found in ST7 and additionally, parasitic effects could be post-transcriptional, more subtype-controlled studies are required on the *in vivo* effect of *Blastocystis* spp. on intestinal permeability. In the same study, upregulation of pro-inflammatory cytokines in intestinal tissue was reported, namely IFN γ , IL-12 and TNF α [45].

Studies on serological response to blastocystosis in animal models have been limited with one study describing elevation of anti-IgM and IgG in serum and anti-IgA in intestinal secretions [70]. Using western blots of parasite lysate, a range of potentially immunodominant proteins were identified but remain to be further characterized. In pigs, fecal IgA response was demonstrated in ~80% of naturally infected pigs. In addition, an increased response to a high molecular weight antigen (>250 kDa) was seen among piglets and pigs that had been immunosuppressed [71].

4. Conclusions and future research

In order for *Blastocystis* spp., lumen/mucosal surface dwelling parasites to colonize the intestine and at the same time evade the host response, the parasite has been found to have mechanisms to attach to the host epithelium [30] and avoid the damaging effects of host response [41,50,54]. Furthermore, multiple *in vitro* studies have elucidated the pathogenic potential of this parasite by direct damage to the host intestinal epithelium [8,31,39], identification of potential virulence factors [47,48] and by immune modulation [53,54] but there continues to remain uncertainty regarding its clinical importance. Further studies are required to examine these effects in broader range of axenized isolates among human subtypes. Development of suitable genetic tools including a transfection system for *Blastocystis* spp. to carry out knock out studies will also help in delineating the relative importance of virulence factors identified till date. These *in vitro* findings also need to be validated in appropriate animal models. This may help identify or differentiate the potentially 'pathogenic' subtypes from the non-pathogenic, although studies have also shown considerable genetic diversity and pathogenic variability within subtypes [30,72]. A suitable animal model will also help carry out studies for the fulfillment of Koch's postulates and also help identify the effects of *Blastocystis* spp. on the host microbiota. What is also lacking at present is the identification of immunodominant antigens and markers of a protective host response. Although smaller scale studies on antibody response in animal models and naturally infected animals have been carried out [70,71], there is still no clear understanding of what constitutes a protective response. To avoid the conflicting reports on the pathogenicity of this parasite,

standardized techniques for the axenic culture of trophic forms of *Blastocystis* spp. need to be applied. Using suitable animal models rendered susceptible to *Blastocystis* spp. by immunosuppressive agents or genetic modifications in which parasites have been introduced by appropriate techniques may bring us closer to a better understanding of host-parasite interactions of this poorly understood but widespread parasite.

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