AN ABSTRACT OF THE THESIS OF

Yuri Alexander Lawrence for the degree of Master of Science in Veterinary Science presented on June 19, 2014.

Title: The Effect of Blastocystis sp. ST1 on the Expression of Serotonin Transporter, Tryptophan Hydroxylase, Toll-like Receptor 2, and Toll-like Receptor 4 in CACO-2 Cell Culture

Abstract approved:

Craig G Ruaux

Blastocystis spp. is a common intestinal parasite in humans and animals that has been associated with acute or chronic digestive disorders such as irritable bowel syndrome. Serotonin mediates intestinal motility, sensation, and secretory function in the normal intestinal tract. Serotonin (5-HT) signaling is decreased in animal models of colitis, as well as the colonic mucosa of humans with ulcerative colitis and irritable bowel syndrome. Altered 5-HT signaling may underlie the abnormal motility, secretion, and sensation seen in these inflammatory gut disorders. Molecular recognition of pathogens is facilitated through Toll-like receptors (TLR). The colonic epithelium expresses relatively high concentrations of mRNA for TLR2 and TLR4. Little is known about the expression patterns for these pattern recognition receptors in response to Blastocystis spp. infection in the human colon. The human colonic epithelial cell line, CACO-2 was used to examine whether enteric 5-HT signaling, TLR2 and TLR4 expression are altered in CACO – 2 intestinal cells in the presence of Blastocystis sp. ST1. Messenger RNA concentrations of components of serotonin signaling (Serotonin transporter, Tryptophan Hydroxylase – TPH1), TLR2, and TLR4 were quantified with a quantitative real-time reverse transcription polymerase chain reaction in CACO – 2 cells that had been co-cultured for
24 hours with live *Blastocystis* sp. ST1. Protein transcription was analyzed with a Western Blot technique. Live *Blastocystis* sp. ST1 parasites inhibited messenger RNA expression of serotonin transporter in CACO-2 cells and had no effect on TPH1 messenger RNA expression. Live *Blastocystis* sp. ST1 parasites alone did not activate TLR-2 or TLR-4. This study evaluated the effect of *Blastocystis* sp. ST1 on serotonergic signaling and TLR activation using an in vitro tissue culture model.
The Effect of *Blastocystis* sp. ST1 on the Expression of Serotonin Transporter, Tryptophan Hydroxylase, Toll-like Receptor 2, and Toll-like Receptor 4 in CACO-2 Cell Culture

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I understand that my thesis will become part of the permanent collection of the Oregon State University libraries. My signature below authorizes release of my thesis to any reader upon request.

Yuri Alexander Lawrence, Author
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Chapter 1 - Introduction

*Blastocystis* spp. is a common enteric parasite that colonizes the gastrointestinal tract of a diverse array of species, including humans. This pathogen has been shown to compromise the integrity of the gastrointestinal tract causing inflammation. The serotonin system is an important mediator of intestinal motility and is altered in the presence of inflammation. The intestinal epithelium performs an important surveillance role in the context of the innate immune response through the expression of pathogen recognition receptors, such as Toll-like receptors (TLRs). We used the human colonic epithelium cell line, CACO – 2 to investigate effects of *Blastocystis* sp. ST1 on serotonin signaling, specifically serotonin transporter (SERT) and tryptophan hydroxylase (TPH1) and TLR activation, specifically the activation of TLR-2, and TLR-4 in this study. We observed that live *Blastocystis* spp. parasites inhibit SERT and had no effect on TPH1, TLR-2 and TLR-4 activation using real time-qPCR analysis. The ability of *Blastocystis* sp. ST1 to inhibit SERT may contribute to abnormal intestinal motility that characterizes certain forms of inflammatory intestinal disease. Furthermore, *Blastocystis* sp. ST1 failed to activate TLR-2 and TLR-4, suggesting that these parasites are able to evade detection by these components of the innate immune system. Our study provides additional information about effect of *Blastocystis* sp. ST1 on the intestinal epithelium.
Chapter 2 – Literature Review

*Blastocystis* is a common intestinal protozoan parasite of humans and other mammals, birds, reptiles, amphibians, and insects. This parasite has a worldwide distribution and is often the most commonly isolated organism in human stool. *Blastocystis* spp. exhibit morphological diversity and regularly used diagnostic techniques such as light microscopy of fecal smears have low diagnostic sensitivity. Prevalence of this organism may be underestimated (Stensvold, Brillowska-Dabrowska et al. 2006). Culture of *Blastocystis* spp. is more sensitive for detection of the *Blastocystis* spp. in fecal samples but is not routinely done in all laboratories. The pathogenic role of *Blastocystis* spp. is also controversial, as it is commonly identified in apparently healthy and asymptomatic subjects in addition to patients with enteric symptoms (Tan, Singh et al. 2002).

*Pathogenic Potential of Blastocystis spp.*

In vivo endoscopy and examination of biopsy samples from patients exhibiting clinical signs of gastrointestinal disease indicated that *Blastocystis* spp. do not invade the colonic mucosa, but lead to disturbances of the enteric barrier function and permeability (Dagci, Ustun et al. 2002; Tan 2008). Experiments on immunocompetent BALB/c mice revealed intense inflammatory-cell infiltration in the mucosa of some infected with Blastocystis sp., but not all mice, suggesting that some host factors could also be involved (Tan 2008). Hussein et al (2008) investigated the pathogenic potential of human isolates obtained from both asymptomatic and symptomatic patients in rats. (Hussein, Hussein et al. 2008).
The intestinal tract of rats infected by *Blastocystis* isolates from symptomatic human patients showed moderate to severe degrees of pathological change and differences in severity were observed among the different strains of *Blastocystis*, indicating the existence of more virulent strains. A study using the rat epithelial cell line IEC-6 found that *Blastocystis* ST4 increases epithelial permeability through the induction of apoptosis, in a contact-independent manner (Puthia, Sio et al. 2006). A proinflammatory effect of *Blastocystis* ST1 culture filtrates has been demonstrated using HT-29 and T-84 human colonic epithelial cells, with production of interleukin 8 (IL-8) and granulocyte macrophage colony stimulating factor (GM-CSF) (Tan 2008). Cysteine proteases of *Blastocystis* ST4 have been shown to induce IL-8 production via an NF-k B pathway (Puthia, Lu et al. 2008). Proteases released in culture supernatants of both *Blastocystis* ST4 and ST7 were also shown to be able to cleave human-secreted immunoglobulin A, and modulate the immune response of the host (Tan 2008). A surface-located cysteine protease was recently shown to have a pro-survival role in *Blastocystis* ST7, and may activate other proteases (Wu, Yin et al. 2010). Collectively, the results of these studies suggest that *Blastocystis* mediates disease in a subtype specific manner, however it is unknown if all subtypes express the same virulence factors or pathophysiologic mechanisms.

*Blastocystis* spp. and Irritable Bowel Syndrome

Some studies have suggested an association of *Blastocystis* spp. with acute or chronic digestive disorders such as irritable bowel syndrome (IBS) (Tan 2008). IBS is a
functional gastrointestinal disorder characterized by abdominal discomfort and/or pain associated with changes in bowel habits, affecting 5%–24% of people in industrialized countries (Longstreth, Thompson et al. 2006). Hussain et al. noted that sera from IBS patients were characterized by higher IgG antibody concentrations against *Blastocystis* spp. when compared to healthy populations of people (Hussain, Jaferi et al. 1997). A possible link between *Blastocystis* spp. and IBS was first reported by Giacometti et al. (Giacometti, Cirioni et al. 1999) when describing the increased prevalence of *Blastocystis* spp. in individuals with gastrointestinal symptoms and IBS. Additional studies have also shown a higher prevalence of *Blastocystis* spp. among IBS patients when compared to healthy populations or to patients suffering from other gastrointestinal disorders (Dogruman-Al, Simsek et al. 2010; Yakoob, Jafri et al. 2010; Jimenez-Gonzalez, Martinez-Flores et al. 2012). Some other studies, however, have failed to demonstrate an association between *Blastocystis* spp. and IBS (Tungtrongchitr, Manatsathit et al. 2004; Ramirez-Miranda, Hernandez-Castellanos et al. 2010; Surangsrirat, Thamrongwittawatpong et al. 2010). The difference may result from low numbers of patients in IBS group studied, false positives, or low sensitivity of the diagnostic methods used. Two of these three studies were from Thailand and one from Mexico, whereas studies arguing for a link between *Blastocystis* spp. and IBS were from the Middle East and Europe. IBS is a functional disorder of multifactorial origin, and some genetic, environmental, and microbiological factors could also contribute to this discrepancy.

Recent studies suggest that visceral pain associated with IBS could be explained by alterations of the epithelial barrier, resulting in bowel motility and sensitivity disorders.
In vitro studies on colonic biopsies from patients with IBS showed an increase of paracellular permeability associated with perturbations of tight junctions (Piche, Barbara et al. 2009). Additionally it is now recognized that there is a low-grade inflammation of the mucosa in IBS patients (Chadwick, Chen et al. 2002). Protease-activated receptor type 2 (PAR-2) was proposed to be involved in both an increase of permeability and low-grade inflammation (Bueno and Fioramonti 2008). PAR-2 is activated by serine-proteases that cleave the N-terminal domain of the receptor. The released peptide may act as a ligand and turn on the receptor to enhance tight junction opening and trigger inflammation. This increase in paracellular permeability allows diffusion of both antigens and bacteria to the submucosa, precipitating inflammation. PARs are also present at the surface of intestinal neurons. The activation of some members of the PAR family could contribute to abdominal pain (Steck, Mueller et al. 2012). Stools from IBS patients show higher proteolytic activity than healthy controls (Gecse, Roka et al. 2008), whereas the presence of protease activity have been shown in supernatants from axenic cultures of both Blastocystis ST4 and ST7 (Tan 2008). This is supported by the prediction of 22 secreted proteases from genomic data of Blastocystis ST7 (Denoeud, Roussel et al. 2011). These experimental and genomic data suggest a possible involvement of parasite proteases in gastrointestinal disturbances. Thus, proteases from bacteria or Blastocystis spp., such as metalloproteases, cysteine, or serine proteases, could play a key role in IBS development (Steck, Mueller et al. 2012). This perturbation could be linked to a modification of lumen microbiota in IBS patients compared to healthy patients (Lee and Bak 2011). Dysbiosis may then mediate low-grade inflammation of the mucosa and IBS symptoms.
Toll-like receptors (TLRs) are a family of pathogen recognition receptors that play a pivotal role in innate immune-surveillance of microbial molecular patterns. All TLRs share three similar structural features: a divergent ligand-binding extracellular domain with leucine-rich repeats, a short transmembrane region, and a highly homologous cytoplasmic toll/interleukin (IL)-1 receptor domain that is similar to that of the IL-1 receptor family and is essential for initiation of downstream signaling cascades (Cario and Podolsky 2006). Thirteen TLRs have been identified in humans and mice, seven of which are expressed in the intestinal mucosa (Abreu 2010).

TLRs in the intestinal mucosa are able to rapidly recognize luminal pathogens and their associated molecular patterns, while still maintaining hyporesponsiveness to persistently present populations of harmless commensals (Cario and Podolsky 2006). Upon activation by pathogen-associated factors, TLRs activate downstream signaling cascades that mediate the activation of transcription factors such as NF-kB and culminate in the upregulation of immune response genes, such as those of pro-inflammatory cytokines and chemoattractant chemokines, to facilitate immune cell infiltration to the site of infection. Recent studies also suggest possible immune-regulatory roles of TLRs towards maintenance of intestinal homeostasis by regulating barrier function and modulating mucosal immune response; and management of intestinal injury through promoting the proliferation of intestinal epithelial cells (Abreu 2010).
Given the various effects of *Blastocystis* spp. on host intestinal epithelial cells, it is likely that the parasite could have a dysregulatory effect on TLR signaling, contributing towards a disruption of intestinal homeostasis and the manifestation of gastrointestinal disease states. A recent study has demonstrated the *Blastocystis* spp. does not induce expression of TLR2 or TLR4 in a human monocyte cell line, but it remains unknown if TLR2 or TLR4 expression is induced in human enterocytes (Teo, Macary et al. 2014).

*Serotonergic Signaling in the Gastrointestinal Tract*

The enteric nervous system requires sensors to monitor the conditions in the gastrointestinal lumen and mediate an appropriate response. There are no nerve fibers that terminate in either the intestinal epithelium or the basement membrane of the intestinal epithelium, therefore there are no intraluminal or intraepithelial nerve endings. One mechanism that mediates transepithelial sensation is via the use of enteroendocrine cells. The most well characterized enteroendocrine cell is the enterochromaffin cell. These cells store large quantities of serotonin, with over 95% of the body’s serotonin located in the gastrointestinal tract (Gershon 2004). Serotonin is primarily secreted into the lamina propria, where it is accessible to neuronal processes. The quantity of serotonin that enterochromaffin cells secrete constitutively is large, they secrete even larger quantities in response to stimulation, with these high concentrations serotonin may overflow into the portal circulation and intestinal lumen (Gershon 2004). This type of system is required because the intestinal epithelium is in constant motion with new
enterocytes being produced from intestinal crypts and lost to senescence continuously. Therefore a system to confer specificity is provided by specific serotonin receptors in addition to inactivation and termination of the action of serotonin. Serotonergic signaling is terminated by the intracellular reuptake of 5 – HT, in contrast to other neurotransmitters there are no extracellular enzymes that catabolize 5 – HT.

_Altered Serotonergic Signaling in Inflammatory Enteropathies_

The majority of the 5-HT within the gastrointestinal tract is present in the enterochromaffin (EC) cells of the mucosal epithelium, but other important sources of enteric 5-HT include the serotonergic neurons of the enteric nervous system and, in rats and mice at least, mast cells. Enterochromaffin cells function as sensory transducers. In response to increases in intraluminal pressure or chemical stimuli, EC cells secrete 5-HT, which stimulates intrinsic primary afferent neurons to initiate peristaltic and/or secretory reflexes. Enteric bacteria are another stimulator of 5-HT secretion because EC cells express Toll-like receptors (Bogunovic, Dave et al. 2007). Because dendritic cells, lymphocytes, macrophages, endothelial cells, and enteric epithelial cells all express 5-HT receptors, the secretion of 5-HT is potentially able to influence intestinal inflammation (Fiebich, Akundi et al. 2004; Idzko, Panther et al. 2004; Yin, Albert et al. 2006).

Once 5-HT acts on its receptors inactivation occurs through the plasmalemmal 5-HT transporter (SERT; SLC6A4) that mediates the reuptake of 5-HT. This transporter is expressed by serotonergic neurons in the brain and in the gut, and by enterocytes (Coates, Johnson et al. 2006; Camilleri, Andrews et al. 2007). Inhibition or deletion of SERT
potentiates the effects of endogenous 5-HT in the bowel (Chen, Li et al. 2001; Coates, Johnson et al. 2006). Mucosal SERT expression is decreased during 2,4,6-trinitrobenzene sulfonic acid (TNBS)-mediated colitis in guinea pigs and mice. SERT transcription also has been reported to be decreased in the rectum of human patients with ulcerative colitis (Linden, Chen et al. 2003; O'Hara, Ho et al. 2004; Linden, Foley et al. 2005). Deletion of SERT potentiates the inflammatory reactions induced by TNBS in mice and deletion of tryptophan hydroxylase-1 (TPH1) ameliorates intestinal inflammation supporting the concept that 5-HT is a potential modifier of inflammation. Thus, 5-HT could play a role in IBD, either in its pathogenesis or as a mediator of symptoms (Bischoff, Mailer et al. 2009; Ghia, Li et al. 2009).

Alterations in serotonergic signaling have been observed in association with various enteropathogens that include Vibrio cholera, Campylobacter jejuni, and Giardia lamblia (Flach, Qadri et al. 2007; Dizdar, Spiller et al. 2010). Given that alterations in serotonergic system activity in the gastrointestinal tract occur in association with infection by a variety of gastrointestinal pathogens, it seems reasonable to hypothesize that Blastocystis spp. may have a similar effect. At the time of writing, the effect of Blastocystis spp. on serotonergic signaling has not been characterized.

Post-infectious irritable bowel syndrome has been reported to occur in up to 30% of patients with bacterial gastrointestinal disease and this may account for the low degree of lymphoplasmacytic inflammation that characterizes irritable bowel syndrome (Spiller and Campbell 2006). Enteropathogens associated with post-infectious irritable bowel syndrome include Campylobacter jejuni, Salmonella enterica, and Shigella sp. (Spiller
These associations with enteropathogens make *Blastocystis* spp. an important research target due to its association with chronic gastrointestinal disease.

The objectives of this study were to characterize the effect of *Blastocystis* sp. ST1 on TLR2 and TLR4 activation and serotonergic signaling in a CACO-2 tissue culture model of the human intestinal epithelium. We hypothesized that serotonergic signaling would be decreased, Toll-like Receptor – 2 and Toll-like Receptor – 4 expression unaltered in *Blastocystis* sp. ST1 infection.
Chapter 3 - Materials and Methods

3.1 Culture of CACO–2 Human Colonic Epithelial Cell Line

The CACO–2 (ATCC® HTB-37™) human colonic epithelial cell line was used for all experiments investigating Blastocystis host interactions. CACO–2 cells were maintained in a 75cm² cell culture flask (Corning Inc., Corning, NY USA) in a humidified incubator at 37°C and 5% CO₂. The cells were passaged enzymatically (0.25% trypsin – 1 mM EDTA, Invitrogen, Grand Island, NY USA) and sub-cultured in 25cm² cell culture flasks (Corning Inc., Corning NY USA). CACO–2 cells were cultured in Eagle's Minimum Essential Medium (Invitrogen, Grand Island, NY USA) supplemented with 100 U/ml penicillin and 100 µg/ml streptomycin (Invitrogen, Grand Island, NY USA), HEPES (Invitrogen, Grand Island, NY USA), 2mM glutamine (Invitrogen, Grand Island, NY USA) and 20% heat-inactivated fetal bovine serum (Invitrogen, Grand Island, NY USA). Experiments were performed once all 25cm² cell culture flasks attained > 95% confluence and the cells were always used between passages 19 – 35. Cell culture viability was evaluated at >95% confluence using the trypan blue assay and only cultures with >95% viability were used for all experiments.

3.2 Culture of Blastocystis Parasites

Blastocystis hominis (Subtype I; American Type Culture Collection, Manassas, VA) were anaerobically cultured in a biphasic medium of inspissated whole egg slants overlaid with
4.5 ml Stone’s modification of Locke’s solution and 25% heat-inactivated horse serum (ATCC medium 1671) and incubated at 35°C.

3.3 Blastocystis Co-culture with Caco-2 Monolayer

Caco-2 monolayers were cultured in 25 cm² cell culture flasks to >95% confluence as described above. Experimental flasks were seeded with $1 \times 10^7$ live Blastocystis spp. parasites in 5 ml of the cell culture media and co-cultured for 24 hours. All flasks were cultured in triplicate and each experiment repeated in duplicate. Cells were harvested at >95% confluence (0 hours), 24 hours post >95% confluence (untreated control), and after 24 hours of co-culture with Blastocystis (treated).

3.4 Real-time Quantitative PCR

Total RNA was extracted from the Caco-2 cells using the RNeasy Mini Kit (Qiagen, Gaithersburg, MD USA). 1 µg total RNA used as a template for first strand cDNA synthesis using the QuantiTect Reverse Transcription Kit (Qiagen, Gaithersburg, MD USA). Reverse transcription reactions were incubated at 25°C for 5 min, 42°C for 30 min and lastly, 85°C for 5 min. Three candidate reference genes (Ribosomal Protein S14, 60S acidic ribosomal protein P0 (RPLP0) and ywhaz) were assessed for stability of expression using the method described by Vandesompele et al. (Hellemans and Vandesompele 2014). Of these three candidate reference genes, RPLP0 was found to show the most stable expression within our samples and this gene was used as the reference gene for subsequent analyses. The relative abundance of SERT, TPH1, TLR2, and TLR4 mRNA in control cells and cells co-cultured with Blastocystis spp. were
compared. cDNAs obtained by reverse transcription were used to determine respective mRNA expression levels. Real-time quantitative PCR reactions were run using the StepOne Plus Real-Time PCR System (Life Technologies). Messenger RNA (mRNA) levels were normalized against RPLP0, (Invitrogen, Grand Island, NY USA) using a ∆∆CT method. Test samples were added in triplicate at 2.5 µL per well in a 10 µL total reaction volume. Nuclease free water was used as a negative control.

3.5 Western Blot Analyses

Protein extracts of whole cells obtained using Radio Immunoprecipitation Assay Buffer (AMRESCO, Solon, OH USA) were subjected to Western blot analysis. A BCA protein assay kit (Thermo Fisher Scientific, Rockford, IL USA) was used to assess protein concentration. For each sample 20 µg of protein was separated by SDS-PAGE using 4 – 15% gradient polyacrylamide gels (Bio-Rad Laboratories, Hercules, CA USA) and transferred to polyvinylidene difluoride transfer membranes (GE Whatman, Piscataway, NJ USA). Membranes were blocked for 1 h at room temperature with 5% bovine albumin in TBS containing 0.05% Tween-20 and incubated overnight at 4°C with either anti-SERT (1:500; Millipore, Temecula, CA USA), anti-TPH1 (1:1000; Millipore, Temecula, CA USA), anti-TLR2 (1:500; Abcam, Cambridge, MA USA), or anti-TLR4 (1:500; Invitrogen, Grand Island, NY USA). After three washes of 5 min each in TBS containing 0.05% Tween-20, the membranes were incubated with the secondary antibodies for 45 min at room temperature. Protein bands were detected with GE Enhanced Chemiluminescence Substrate (VWR, Radnor PA, USA) and analyzed with an ImageQuant LAS 4000 (GE Healthcare Life Sciences, Pittsburg PA, USA).
3.6 Statistical analysis

Data were analyzed with a commercially available biological statistics program (GraphPad Prism 6.0). Data were tested for consistency with a normal distribution using the D’Agostino & Pearson omnibus normality test. As most data sets were found not to be consistent with a normal distribution, results are expressed as the median ± interquartile range. Non-parametric Mann–Whitney U-tests were used to compare untreated control group to Blastocystis sp. ST1 treated group. A calculated $P$ value <0.05 was considered significant.
Chapter 4 - Results and Discussion

4.1 Serotonin Transporter, Tryptophan Hydroxylase, Toll-like Receptor 2 and Toll-like Receptor 4 mRNA expression in CACO-2 cells after coculture with Blastocystis

Quantitative real-time PCR analysis demonstrated inhibition of SERT by 48.5% and no significant difference in TPH1 mRNA levels after co-culture of CACO-2 cells with Blastocystis spp. (P = 0.0159, P = 0.1111 Mann-Whitney U Test; Figures 1 – 2). Animal studies as well as studies in humans have revealed that SERT expression is decreased in the mucosa of the inflamed colon (Linden, Chen et al. 2003; Coates, Mahoney et al. 2004; Gershon 2004; Linden, Foley et al. 2005). Because of its physiological role in modulating peristalsis and visceral sensation in the gut, loss of the SERT could contribute to the altered motility and sensation seen in gastrointestinal disease. Our results suggest that Blastocystis sp. ST1 mediates decreases in SERT with no effect on TPH1 expression. This may contribute to the dysmotility and altered sensation described in infection with Blastocystis sp. ST1. The transcript for the rplp0, a highly conserved gene for a ribosomal phosphoprotein was evaluated to assess whether the observed decrease in SERT and TPH1 was the result of general transcriptional decreases in the cell (Tremblay, Auger et al. 2006). Transcription of rplp0 was not altered following co-culture with Blastocystis sp. ST1 suggesting that the change in transcriptional regulation of SERT is pathogen associated.
Figure 1 - Expression of the serotonin reuptake transporter (SERT) in CACO-2 cells co-cultured with *Blastocystis* sp. ST1 and untreated controls. Real-time quantitative PCR was used to quantify transcripts encoding SERT. Data are presented as box and whisker plots. *P = 0.0159 compared with untreated control (Mann-Whitney U test).
Figure 2 - Expression of the tryptophan hydroxylase 1 (TPH1) in CACO-2 cells co-cultured with *Blastocystis* sp. ST1 and untreated controls. Real-time quantitative PCR was used to quantify transcripts encoding TPH1. Data are presented as box and whisker plots, there was no significant difference between groups ($P = 0.1111$, Mann-Whitney U test).
There was no significant difference detected in TLR–2 or TLR–4 expression in CACO–2 cells after co-culture with *Blastocystis* sp. ST1 for 24h (P = 0.1255, P = 0.3889, Mann-Whitney U Test, Figures 3 and 4). There was no induction of NF-kB when THP1-Blue cells, a commercially available human monocytic TLR reporter cell line, were incubated with live *Blastocystis* spp. parasites or parasite components, with or without ligands for TLR-2, 4 and 5. This suggests that *Blastocystis* spp. is unable to activate these TLRs and downstream signaling cascades in a human monocytic cell line (Teo, Macary et al. 2014). Most known ligands of TLRs are either of bacterial or viral origin, with some protozoan parasites such as *Trypanosoma cruzi* and *Toxoplasma gondii* activating combinations of TLR’s -2, -4, -9, and -11 (Coates, Johnson et al. 2006). Bacterial pathogens have been observed to induce inflammatory responses via receptors that are not TLRs for example the *Serratia marcesens*-derived protease serralysin induces host inflammatory responses through protease-activated receptor 2 (PAR-2), activating NF-kB and upregulating IL-8 expression (Pallone and Monteleone 1998). Additionally, a novel secreted protease from *Pseudomonas aeruginosa* was found to be capable of activating NF-kB through PAR-1, -2 or -4 (Rojas-Cartagena, Flores et al. 2005). In addition to PAR-mediated mechanisms, cyclooxygenase-2-mediated prostaglandin E2-dependent modulation of IL-8 production and neutrophil infiltration has been observed in a human fetal intestinal xenograft mouse model used to study *Entamoeba histolytica* infection (Rojas-Cartagena, Flores et al. 2005). Given the complexity in intracellular interactions and signal transduction pathways that drive infection-associated host inflammation, further characterization is required to determine the specific mechanisms associated with the pathogenicity of
Figure 3 – Expression of the Toll-like receptor 2 (TLR-2) in CACO-2 cells co-cultured with *Blastocystis* sp. ST1 and controls. Real-time quantitative PCR was used to quantify transcripts encoding TLR-2. Data are presented as box and whisker plots. There was no significant difference between groups (P = 0.1255, Mann-Whitney U test).
Figure 4 - Expression of the Toll-like receptor 4 (TLR-4) in CACO-2 cells co-cultured with Blastocystis sp. ST1 and untreated controls. Real-time quantitative PCR was used to quantify transcripts encoding TLR-4. Data are presented as box and whisker plots. There was no significant difference between groups (P=0.3889, Mann-Whitney U test).
4.2 Serotonin Transporter, Tryptophan Hydroxylase, Toll-like Receptor 2 and Toll-like Receptor 4 protein production in Caco-2 cells after coculture with Blastocystis

Western blotting analyses reveal decreased protein expression of SERT and no change in protein expression of TPH1 in CACO – 2 cells after 24 hours of co-culture with Blastocystis sp. ST 1 (Figures 5 – 6). There was no detectable change in protein expression in TLR – 2 and TLR – 4 in CACO–2 cells after 24 hours of co-culture with Blastocystis sp. ST1 (Figures 7 – 8). These results agree with our observation that mRNA expression for SERT was decreased, while the TPH1, TLR-2, and TLR-4 expression was unchanged. The decreases in SERT and TPH1 mRNA likely lead to decreased expression of SERT and subsequent decreases in serotonergic function. Previous studies using immunohistochemistry to assess SERT expression have also shown measurable decreases with some chronic gastrointestinal diseases (Coates, Mahoney et al. 2004; Wheatcroft, Wakelin et al. 2005). Blastocystis spp. may decrease SERT uptake simply by decreasing SERT mRNA that leads to less translation of the SERT protein however, it is also possible that SERT function is additionally modulated via protein kinase C mediated phosphorylation, as has been shown in other systems (Jayanthi, Samuvel et al. 2005). This is the first study reporting Blastocystis spp. can regulate SERT expression in human colonic mucosal cells. Experiments to confirm unaltered expression in TPH1, TLR – 2, and TLR – 4 were performed and no change in expression of TPH1, TLR–2 or -4 was seen at 24 hr by Western blotting. This is consistent with the single previous report of the inability of Blastocystis spp. to induce TLR-2 and -4 (Teo, Macary et al. 2014).
Figure 5. Blastocystis sp. ST1 inhibits Serotonin Reuptake Transporter (SERT) protein expression in CACO-2 cell culture. Western blot analysis of SERT protein levels following co-culture with Blastocystis sp. ST1 shows reduced intensity of staining for SERT in protein extract from Blastocystis-treated cells. A – Time 0; B – Time 24hr; C – Blastocystis treated 24hr. All lanes were loaded with 20 µg of protein and the first unlabeled lane represents the pre-stained protein ladder.
Figure 6. Blastocystis sp. ST1 does not alter Tryptophan Hydroxylase (TPH1) protein expression in CACO-2 cell culture. Western blot analysis of TPH1 protein levels following co-culture with Blastocystis sp. ST1. A – Time 0; B – Time 24hr; C – Blastocystis treated 24hr. All lanes were loaded with 20 µg of protein.
Figure 7. Blastocystis sp. ST1 does not alter Toll-like Receptor 2 (TLR-2) protein expression in CACO-2 cell culture. Western blot analysis of TLR-2 protein levels following co-culture with Blastocystis sp. ST1. A – Time 0; B – Time 24hr; C – Blastocystis treated 24hr. All lanes were loaded with 20 µg of protein.
Figure 8. Blastocystis sp. ST1 does not alter Toll-like Receptor 4 (TLR-4) protein expression in CACO-2 cell culture. Western blot analysis of TPH1 protein levels following co-culture with Blastocystis sp. ST1. A – Time 0; B – Time 24hr; C – Blastocystis treated 24hr. All lanes were loaded with 20 µg of protein. The primary antibody utilized detects 2 – 3 bands between 70 – 100 kDa.
Figure 9. Amplification plots showing an example of the difference in serotonin reuptake transporter mRNA expression in CACO – 2 cells after co-culture with Blastocystis sp. ST1. A – Untreated Control; B – Blastocystis sp. ST1 Treated.
Figure 10. Amplification plots showing an example of the tryptophan hydroxylase 1 mRNA expression in CACO – 2 cells after co-culture with Blastocystis sp. ST1. A – Untreated Control; B – Blastocystis sp. ST1 Treated.
Figure 11. Amplification plots showing an example of the Toll-like Receptor – 2 mRNA expression in CACO – 2 cells after co-culture with Blastocystis sp. ST1. A – Untreated Control; B – Blastocystis sp. ST1 Treated.
Figure 12. Amplification plots showing an example of the Toll-like Receptor – 4 mRNA expression in CACO – 2 cells after co-culture with Blastocystis sp. ST1. A – Untreated Control; B – Blastocystis sp. ST1 Treated.
Chapter 5 - Conclusion

The study described here suggests that *Blastocystis* sp. ST1 inhibits SERT protein levels in CACO – 2 cells by suppressing mRNA levels of SERT. This may lead to increased levels of serotonin and result in altered intestinal sensation and increased intestinal motility that characterizes various gastrointestinal diseases. This provides insight into the mechanism that mediates gastrointestinal dysfunction in the presence of *Blastocystis sp.* ST1 and its association with irritable bowel syndrome. The lack of Toll-like receptor – 2, and – 4 activation suggests that *Blastocystis* sp. ST1 may interact with the innate immune system though other pathogen recognition receptor pathways or Toll-like receptors that were not assayed in this study.
Bibliography


