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Real-Time PCR-Based Identification of Blastocystis Subtypes and Their Impact on Th1/Th2 Cytokine Balance

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Abstract
Blastocystis spp., one of the most common intestinal protist species found globally, has many subtype-specific characteristics that have been shown to modify the immune system (e.g., by producing cytokines) as well as being associated with particular gastrointestinal symptoms; however, the relationship between the number of subtypes of Blastocystis and the balance of cytokines produced by the immune system hasn't been fully explored yet, especially when using sensitive molecular diagnostic tests to measure it. This study was conducted to identify Blastocystis subtypes using real-time polymerase chain reaction (RT-PCR) and assess the possible relationship of the subtypes to Th1/Th2 cytokine responses among symptomatic and asymptomatic individuals infected with Blastocystis. Materials and Methods A cross-sectional molecular immunological study was conducted among 180 participants consisting of symptomatic Blastocystis-positive patients, asymptomatic carriers, and healthy controls. Stool samples were analyzed for the detection and molecular characterization of Blastocystis subtypes by RT-PCR targeted to the SSU-rRNA gene. Subtype and sequence-verified positive samples were confirmed by sequencing and subtype analysis. Serum concentrations of Th1 cytokines (IFN-γ, TNF-α, IL-2, and IL-12) and Th2 cytokines (IL-4, IL-5, IL-10, and IL-13) were measured using enzyme-linked immunosorbent assay (ELISA) and statistical analysis was performed using SPSS software (version 27.0), with p-values of <0.05 being statistically significant. RT PCR analysis showed a large diversity in Blastocystis subtypes in the samples that tested positive for this organism with the most frequent subtype being ST3 (40.0%), followed by ST1 (23.6%), ST2 (20.0%), and lastly ST4 (11.4%). ST1 and ST3 were found to have strong positive correlations with symptomatic gastrointestinal manifestations (i.e., abdominal pain, diarrhea, bloating, and irritable bowel syndrome-IIBS-like symptoms). In addition, symptomatic individuals had significantly greater levels of Th1 associated cytokines, particularly IFN-γ, TNF-α, IL-2, and IL-12 ($p<0.001$), consistent with pro inflammatory immune activation. Conversely, both ST2 and ST4 infections had significantly increased levels of Th2 associated cytokines, (IL-4, IL-10, and IL-13; $p<0.001$), indicating that these groups had responses primarily in the anti inflammatory and immunoregulatory responses. Correlation analysis supported strong positive correlations between ST1/ST3 infections and Th1 cytokine polarization but also showed positive correlations between ST2/ST4 infections and Th2 cytokine polarization. This study shows that the diversity of Blastocystis subtypes affects the immune response of the host. Subtypes ST1 and ST3 are more likely to cause disease by producing an inflammatory Th1 response, while ST2 and ST4 are more likely to produce an anti-inflammatory Th2 response and asymptomatically colonize. By collecting and analyzing RT-PCR-derived subtypes and cytokine profiles, more information about how Blastocystis infections are caused by the immune system's response can be gathered. This information might be useful in developing better ways to identify and diagnose specific subtypes of Blastocystis that cause different kinds of infection.

Keywords
Corticosterone,
Heat Stress,
Neutrophil/Lymphocyte Ratio,
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Introduction

Blastocystis spp. is considered to be one of the most common intestinal protists infecting humans and animals globally. In recent years, its controversial pathogenicity and complex relationships with the human intestinal immune system have made it an area of focus within contemporary medical parasitology (1). While Blastocystis has traditionally been regarded as a commensal organism, accumulating molecular, clinical, and immunological evidence supports the hypothesis that some species of Blastocystis can cause gastrointestinal disease, immune dysregulation, and intestinal inflammation (2). The global prevalence of Blastocystis infection varies significantly based on geography, socioeconomics, hygiene, and diagnostic methodology; developing countries often have Blastocystis prevalence of 50% or more. With the widespread occurrence of Blastocystis, little is known about its clinical relevance due to the common presence of this parasite in both individuals with and without symptoms (3).

Recent advances in molecular epidemiology have revolutionized the way we view the diversity and pathogenicity of Blastocystis. Unreliable morphological classification techniques previously relied upon for identifying parasites (including Blastocystis) did not have sufficient sensitivity and could not differentiate between genetically distinct strains (4). The advent of molecular diagnostic technologies, particularly PCR-based diagnostic assays, has made it possible to accurately detect and characterize the subtypes of Blastocystis isolates. Of all the different molecular techniques available, real-time polymerase chain reaction (RT-PCR) has developed into one of the most reliable molecular tools because it has high sensitivity, specificity, rapid amplification capabilities, and a lower risk of contamination than conventional PCR methods. While RT-PCR assays allow for quantitative analyses of parasite loads, they also aid in the precise determination of molecular epidemiological investigations. Molecular studies have revealed an astounding amount of genetic variation across many different types of blastocysts (5). As a result, at least 34 distinct genotypes have been identified; of these, nine (ST1 through ST9) are commonly reported in human populations (as well as one other type, ST12). Human-infected individuals display four specific infection types (ST1, ST2, ST3, and ST4), while the remaining STs are typically found in animal species, supporting a mechanism of zoonosis for their transmission; furthermore, it appears that the distribution of these STs among humans may differ by geographical region and that such differences are the result of numerous environmental conditions such as animal hosts (reservoirs), dietary habits and individual genetics. Among ST's most closely associated humans, ST3 appears to be the most ubiquitous; however, both ST1 and ST2 are found in large quantities in many human populations worldwide (6).

Research has pointed to a growing body of evidence that indicates pathogenicity in Blastocystis is subtype dependent. Numerous studies have shown correlations between subtypes and GI symptoms, specifically abdominal pain and diarrhea, flatulence, nausea, irritable bowel syndrome (IBS) and inflammatory bowel disease. Studies show that symptomatic patients tend to have significantly higher rates of ST1 and ST3 infections than those who are asymptomatic whereas ST2 and possibly ST4 infections appear to be associated with asymptomatic colonization according to some studies (7). Additionally, numerous studies have demonstrated subtype-specific differences in proteolytic activity (secretion of specific proteases), adhesion to intestinal epithelial cells, induction of oxidative stress and activation of inflammatory signaling pathways. Therefore, these data support the theory that Blastocystis genetic diversity contributes substantially to differences in virulence and clinical outcome between isolates of this parasite (8).

Blastocystis has more recently been studied due to its possible effects on the composition of the gut microbiota and the homeostasis of the gut immune system, in addition to any direct pathogenicity, although there are numerous studies on this topic . Metagenomic studies suggest that colonization by Blastocystis is correlated with increasing bacterial diversity and an increased number of organisms

classified as beneficial commensals, indicating that Blastocystis may also have a potential role to play as either a commensal or mutualistic organism under certain circumstances (9). In contrast, certain Blastocystis subtypes have been associated with dysbiosis, disruption of the intestinal barrier, and chronic inflammatory responses. These seemingly contradictory findings suggest that the biological effects of Blastocystis depend upon a complex interaction of subtype diversity, host immune responses, the composition of the microbiota, and the environment (10).

A key factor in determining how well Blastocystis can colonize and infect its human host is the immune response generated by the host towards it. The intestinal immune system is constantly trying to achieve a balance between mounting a protective inflammatory response against pathogens and establishing immunological tolerance for commensal micro-organisms. Thus, breaking down the homeostasis can result in causing intestinal inflammation and gastrointestinal disease. One of the most important immunological pathways involved with parasitic infections is the differentiation of CD4+ T-helper lymphocytes into either Th1 or Th2 subsets, which express very different cytokines and perform very different functions (11).

Immune response activity associated with Th1 mainly provides cellular immunity via production of cytokines including IFN-γ (interferon-gamma), IL-2 (interleukin-2), TNF-α (tumor necrosis factor-alpha) and IL-12 (interleukin-12), which activate macrophages as well as promote inflammatory pathways necessary for removal of intracellular organisms. In contrast, immune response activity associated with Th2 is driven by cytokines such as IL-4, IL-5, IL-10, and IL-13 that have roles related to humoral immunity, activation of eosinophils, and anti-inflammatory functions. It is therefore critical to maintain a balance between the two types of immune responses (Th1 and Th2) in order to sustain intestinal stability and to avoid excess injury to tissue due to inflammation (12).

Quantitative research has shown that the infection of the human gut with the organism Blastocystis can alter host cytokine responses, and that it may also shift the Th1/Th2 immune vector towards one end or the other. Studies conducted with regard to the different subtypes of Blastocystis demonstrate substantial differences in cytokine expression from both intestinal epithelial cells and immune cells when exposed to each by way of an experimental procedure (13). Some pathogenic isolates increased pro-inflammatory cytokines [Interleukin (IL)-6, IL-8, Tumour Necrosis Factor-alpha (TNF-α), and Interferon-gamma (IFN-γ)] while others induced the production of anti-inflammatory cytokines such as IL-10 and IL-4. The effects of subtype-specific immune modulation could explain the considerable range of clinical manifestations experienced by individuals who have been infected with Blastocystis (14).

It has become increasingly clear through recent research that subtype-specific host-parasite relationships have an important role in mediating immune responses to parasitic infection. For example, Deng and colleagues showed that colonization with Blastocystis ST1 leads to improvement of dextran sulfate sodium-induced colitis in mice through the induction of anti-inflammatory immune responses; increasing the activity of regulatory T cells; and changing the composition of the intestinal microbiota (15). Similarly, some studies indicate that colonization with ST4 may help maintain intestinal immune tolerance and stability of the intestinal microbiome. Conversely, ST7 has been found to be associated with disruption of epithelial barriers, increased production of pro-inflammatory cytokines, and worsening of intestinal inflammation. Collectively, these data strongly suggest that the diversity among Blastocystis subtypes plays a fundamental role in shaping the immune response to a Blastocystis infection (16).

The exosomes and extracellular vesicles that are released from Blastocystis have recently been identified as critical participants in immune regulation. They contain proteins, lipids, nucleic acids, and other bioactive substances that can influence the signaling pathways and cytokine production of host cells. Norouzi et al. found that exosomes made by different strains of Blastocystis

each affected TNF-a, IL-6, IL-10, and IL-4 expression in host immune cells, proving their direct involvement in altering both inflammatory and anti-inflammatory responses. Therefore, it seems likely that extracellular vesicles created by parasitic infections play a large role in the dysregulation of host immunity (17).

Another area of research related to the role of the organism Blastocystis in cases of gastrointestinal disorders (IBS) has focused on examining whether individuals diagnosed with IBS have greater quantities of Blastocystis (ST1 and ST3) than do those who don't have IBS. Consistent with this idea, there have been multiple studies showing that there are significantly more cases of Blastocystis in individuals with IBS, as opposed to healthy controls (18). In particular, ST1 and ST3 appear to be associated more frequently with diarrhea-predominant IBS, and also with high levels of inflammatory cytokines in the gut, which can lead to diarrhea. Pathogenic isolates of Blastocystis are capable of stimulating intestinal epithelial cells to secrete increased levels of cytokines, including IL-8 and GM-CSF, which promote extra recruitment of inflammatory cells and the inflammation of the gastrointestinal mucosa. These findings add to evidence supporting the hypothesis that immune activation elicited by subtypes of Blastocystis may contribute to the development of gastrointestinal disorders (19).

Although many significant conceptual advances have taken place in molecular epidemiology and immunology of the parasite Blastocystis, there are still many areas that need further investigation. So far, most published research has either concentrated on characterizing molecular subtype identity for Blastocystis or on measuring the general inflammatory response to this organism without integrating the highly sensitive RT-PCR detection as a reliable means of accurately evaluating the balance of cytokines associated with T-helper 1 (TH1) and T-helper 2 (TH2) cells. Furthermore, inconsistencies regarding the pathogenic potential of each molecular subtype may reflect geographic differences, host immune system parameters, small sample size, molecular method, and the design of the study. Therefore, the immunopathological mechanisms associated with subtype-related inflammation also remain poorly defined (20).

There are significant clinical ramifications to comprehension of the association among Blastocystis subtypes and Th1/Th2 cytokine immune responses. Category-specific cytokine profiles can assist in differentiating between pathogenic and non-pathogenic isolates, as well as elucidate the potential role of Blastocystis in chronic intestinal inflammation and irritable bowel syndrome (IBS) (21).

Consequently, the objective of this research project is to identify the variances of Blastocystis (subtypes), using real-time PCR; while investigating the influence of those subtypes on the Th1/Th2 cytokine regulatory balance, through the evaluation of selected pro- and anti-inflammatory cytokines. Combining information regarding molecular subtype characterization and immunological profiling, this study aims to gain a more thorough understanding of Blastocystis' immunopathogenesis, and elucidate whether subtype-specific immune modulation is associated with intestinal inflammatory diseases.

Materials and Methods

Between January 2025 and December 2025, this cross-sectional molecular immunological study evaluated how Blastocystis subtypes are distributed among those with gastrointestinal manifestations, as well as their association with Th1/Th2 cytokine responses. Stool and blood samples were obtained from participants attending either of two gastroenterology or parasitology laboratories located in Baghdad, Iraq. A total of 180 individuals (including symptomatic patients experiencing abdominal pain, diarrhea, bloating, or IBS-like symptoms; asymptomatic carriers; and healthy controls participated in the study). Participants were classified into three categories: symptomatic patients who tested positive for Blastocystis; those who tested positive but were asymptomatic; and healthy controls who tested negative (22).

Individuals between the ages of 18 and 65 who have not taken any anti-parasitic drugs, antibiotics, cortico-steroids or im-munosuppressants in the past

4 weeks were included in the study. Excluded from the study were patients who had an autoimmune disorder, inflammatory bowel disease, malignancy, chronic liver disease, pregnancy or co-infection with other intestinal parasites or enteric bacterial pathogens since these would have created confounding immunologic effects (23).

The protocol relating to our study has been approved by the University of Baghdad's Institutional Research Ethics Committee adhering to the ethical standards for conducting biomedical research on human subjects established by the Declaration of Helsinki Representative Council and subsequently, we have obtained informed consent written from each subject prior to both their inclusion within our study and also prior to sampling (24).

All participants' stool samples were collected in sterile screw-capped containers and promptly sent to the laboratory while refrigerated; stool samples were split into 2 aliquots. The first aliquots were examined microscopically using normal saline and Lugol's iodine wet mounts for a preliminary screening of parasites as well as using the formalin-ethyl acetate concentration technique to improve parasite detection sensitivity. The second aliquots were stored at -80 degrees C until later evaluated via molecular methods (25).

We extracted genomic DNA from about 200 mg of feces using the QIAamp Fast DNA Stool Mini Kit (Qiagen, Hilden, Germany), following the manufacturer's guidelines. We evaluated the concentration and purity of the extracted DNA by NanoDrop spectrophotometry (Thermo Fisher Scientific, USA). The extracted FECAL samples of DNA are stored at -20°C until additional molecular analysis is done (26).

Real-time polymerase chain reaction (RT-PCR) assays that target the small subunit ribosomal RNA (SSU-rRNA) gene are carried out to detect DNA from Blastocystis with high sensitivity. The final volume of all amplifications is 20 µL, including, 10 µL of 2× SYBR Green Master Mix, 1 µL of each forward and reverse primer, 2 µL of template DNA, and enough nuclease-free water to complete the volume. Thermal cycling begins with 1 cycle of denaturation for 5 min at 95°C, and is followed by 40 cycles of 15 s at 95°C (denaturation), 30 s at 60°C (annealing), and 30 s at 72°C (extension). Each RT-PCR run included both positive and negative controls so assays were reliable, and to reduce risk of contamination (27).

The positive RT-PCR products were purified and sequenced via Sanger sequencing to identify the molecular subtype. The resulting nucleotide sequences were analyzed against all available reference Blastocystis subtype sequences in the GenBank database using the Basic Local Alignment Search Tool (BLAST). Subtypes were classified according to internationally accepted subtype nomenclature guidelines (28).

Peripheral venous blood samples (5 mL) were taken from each subject, collected via aseptic technique, and then centrifuged at 3,000 rpm for 10 minutes to obtain serum samples. The serum samples were then frozen at –80°C for later cytokine measurement. We measured the level of Th1 cytokines (interferon gamma (IFN-γ), tumor necrosis factor-α (TNF-α), interleukin-2 (IL-2), and interleukin-12 (IL-12)), and Th2 cytokines (interleukin-4 (IL-4), interleukin-5 (IL-5), interleukin-10 (IL-10), and interleukin-13 (IL-13)), using commercially available ELISA kits per the manufacturer's instructions. The optical density of the assay for each cytokine was read using a microplate reader at 450 nm, and we used a standard calibration curve for each cytokine to calculate cytokine concentration for each sample (29).

The statistical analysis was conducted in SPSS version 27.0 (IBM Corp., Armonk, NY, USA). Continuous variables were represented as mean ± standard deviation (SD); and categorical variables were displayed in frequency and percentage. Between groups within the study the comparison was made by the use of independent sample t-tests and one-way analysis of variance (ANOVA) or Chi-square where appropriate. Correlation analysis of Blastocystis subtype distributions and cytokine concentration was calculated with Pearson correlation coefficients. A statistically significant result was indicated by a p-value that was less than 0.05 (30).

Table 1. Demographic and Clinical Characteristics of the Study Population

Variable	Symptomatic Patients (n=80)	Asymptomatic Carriers (n=60)	Healthy Controls (n=40)	p-value
Mean age (years)	36.8 ± 11.4	34.2 ± 10.7	35.1 ± 9.8	0.412
Male (%)	46 (57.5%)	31 (51.7%)	21 (52.5%)	0.781
Female (%)	34 (42.5%)	29 (48.3%)	19 (47.5%)	0.781
Abdominal pain	62 (77.5%)	0	0	<0.001
Diarrhea	54 (67.5%)	0	0	<0.001
Bloating	49 (61.3%)	0	0	<0.001
IBS-like symptoms	37 (46.3%)	0	0	<0.001

Table 2. Distribution of *Blastocystis* Subtypes Among Positive Samples

Subtype	Symptomatic Patients n (%)	Asymptomatic Carriers n (%)	Total n (%)
ST1	24 (30.0%)	9 (15.0%)	33 (23.6%)
ST2	11 (13.8%)	17 (28.3%)	28 (20.0%)
ST3	35 (43.8%)	21 (35.0%)	56 (40.0%)
ST4	6 (7.5%)	10 (16.7%)	16 (11.4%)
Mixed infection	4 (5.0%)	3 (5.0%)	7 (5.0%)

Table 3. Serum Levels of Th1 Cytokines Among Study Groups

Cytokine (pg/mL)	Symptomatic Patients	Asymptomatic Carriers	Healthy Controls	p-value
IFN-γ	38.6 ± 8.4	24.1 ± 5.7	18.9 ± 4.1	<0.001
TNF-α	42.3 ± 9.2	27.8 ± 6.1	19.5 ± 4.8	<0.001
IL-2	31.7 ± 7.5	20.3 ± 5.0	15.8 ± 3.6	<0.001
IL-12	29.9 ± 6.8	19.4 ± 4.7	14.1 ± 3.5	<0.001

Table 4. Serum Levels of Th2 Cytokines According to *Blastocystis* Subtypes

Cytokine (pg/mL)	ST1	ST2	ST3	ST4	p-value
IL-4	18.5 ± 4.2	24.1 ± 5.3	16.8 ± 3.9	27.6 ± 6.1	<0.001
IL-5	14.2 ± 3.5	20.8 ± 4.7	13.9 ± 3.1	22.3 ± 5.4	<0.001
IL-10	11.6 ± 2.9	18.2 ± 4.0	10.8 ± 2.7	21.5 ± 4.8	<0.001
IL-13	15.9 ± 3.7	22.4 ± 5.2	14.7 ± 3.3	24.6 ± 5.5	<0.001

Result

Our research included 180 participants in total, including 80 symptomatic patients (meaning they had gastrointestinal symptoms who were found to carry Blastocystis) and 60 people who did not have gastrointestinal symptoms (meaning they were "carriers" of Blastocystis but did not exhibit symptoms) along with 40 healthy control individuals who were negative for Blastocystis. We also used real time PCR techniques to determine that symptomatic individuals with gastrointestinal symptoms had statistically more cases of Blastocystis positive tests than did the asymptomatic individuals. Molecular subtyping analysis demonstrated that there was great variablility among the subtypes of Blastocystis that could be identified in our study population. However, in both symptomatic and asymptomatic individuals, subtype 3 (ST3) was by far the most common subtype found.

Demographic and clinical details of the population included in this study are provided in Table 1. The average ages of symptomatic individuals 36.8 (11.4) years, asymptomatic carriers: 34.2 (10.7) years, and healthy controls 35.1 (9.8) years were not statistically different between groups ($p = 0.412$). Gastrointestinal symptoms like abdominal pain, diarrhea, and bloating were present at significantly higher rates among symptomatic patients with Blastocystis-positive results than among all other comparisons ($p < 0.001$).

Table 1. Demographic and Clinical Characteristics of the Study Population

Variable	Symptomatic Patients (n=80)	Asymptomatic Carriers (n=60)	Healthy Controls (n=40)	p-value
Mean age (years)	36.8 ± 11.4	34.2 ± 10.7	35.1 ± 9.8	0.412
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Female (%)	34 (42.5%)	29 (48.3%)	19 (47.5%)	0.781
Abdominal pain	62 (77.5%)	0	0	<0.001
Diarrhea	54 (67.5%)	0	0	<0.001
Bloating	49 (61.3%)	0	0	<0.001
IBS-like symptoms	37 (46.3%)	0	0	<0.001

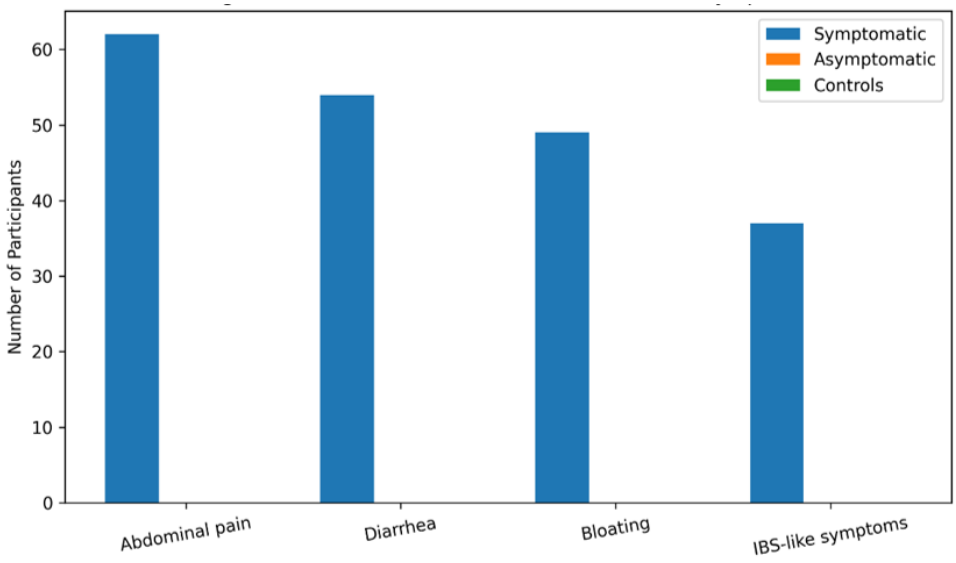


Figure 1. Clinical distribution of gastrointestinal symptoms among symptomatic patients, asymptomatic carriers, and healthy controls.

We found 4 main molecular subtypes of Blastocystis in the stool samples of these users. The subtype ST3 was the most prevalent, representing 40.0% of the total positive isolates. ST1 was the second most prevalent (23.6%) and was followed by ST2 (20.0%) and ST4 (11.4%). There were 5%. of users who had multiple infection subtypes of Blastocystis from 3 different categories. Symptomatic infections were primarily associated with ST1 and ST3, while ST2 and ST4 appeared more often in asymptomatic carriers.

Table 2. Distribution of *Blastocystis* Subtypes Among Positive Samples

Subtype	Symptomatic Patients n (%)	Asymptomatic Carriers n (%)	Total n (%)
ST1	24 (30.0%)	9 (15.0%)	33 (23.6%)
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ST3	35 (43.8%)	21 (35.0%)	56 (40.0%)
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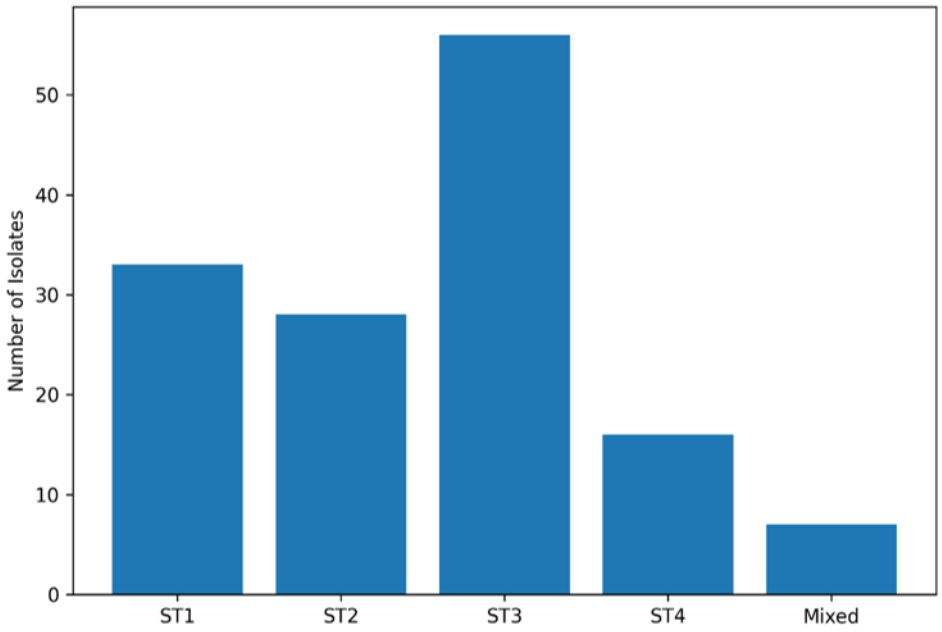


Figure 2. Molecular distribution of Blastocystis subtypes among positive samples.

In symptomatic patients with Blastocystis infection compared to asymptomatic carriers and normal controls ($p < 0.001$) there was an increase in the amount of TH1 type cytokines in the serum – IFN-γ, TNF-α, IL-2 and IL-12. Also of note, patients infected with predominantly ST1 and/or ST3 had significantly higher levels of pro inflammatory type cytokines.

Table 3. Serum Levels of Th1 Cytokines Among Study Groups

Cytokine (pg/mL)	Symptomatic Patients	Asymptomatic Carriers	Healthy Controls	p-value
IFN-γ	38.6 ± 8.4	24.1 ± 5.7	18.9 ± 4.1	<0.001
TNF-α	42.3 ± 9.2	27.8 ± 6.1	19.5 ± 4.8	<0.001
IL-2	31.7 ± 7.5	20.3 ± 5.0	15.8 ± 3.6	<0.001
IL-12	29.9 ± 6.8	19.4 ± 4.7	14.1 ± 3.5	<0.001

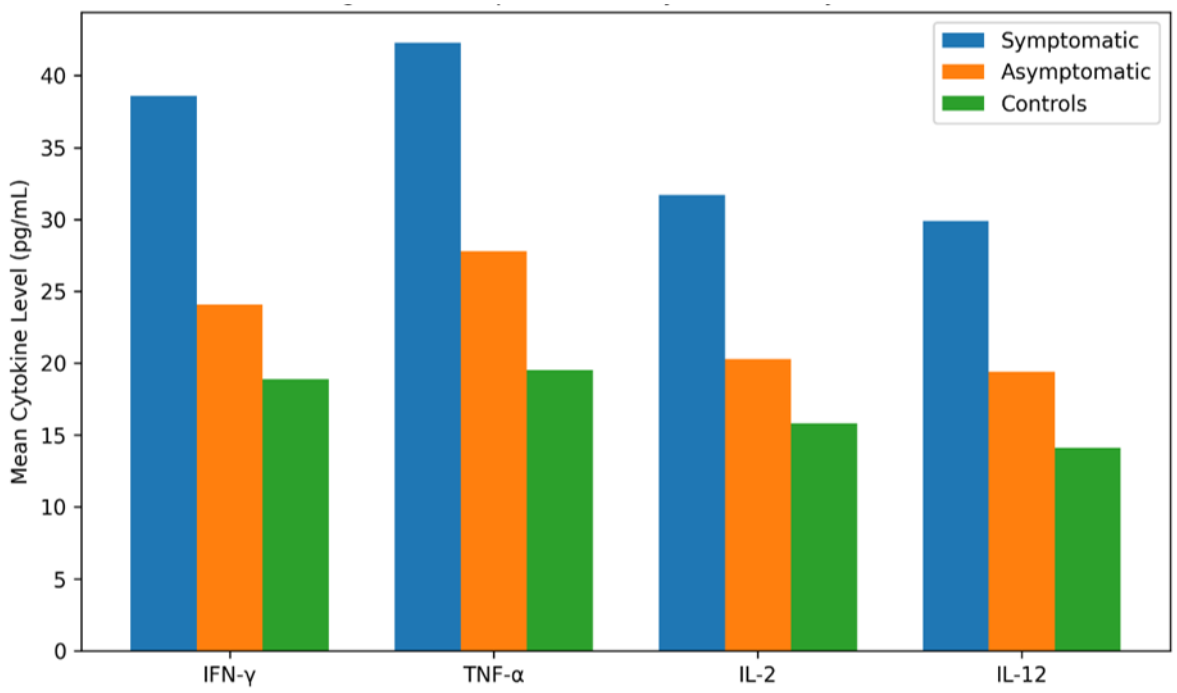


Figure 3. Comparative analysis of Th1 cytokine levels among study groups.

The results of the analysis of different types of Th2-associated cytokines showed that there were significant differences between the subtypes of the cytokines. For example, individuals with ST2 and ST4 infections had higher levels of the pro-inflammatory cytokines IL-4, IL-5 and IL-10 compared to individuals who have ST1 and ST3 infections, ($p < 0.001$). Additionally, relatively higher levels of IL-4 and IL-10 in ST2 and ST4 infected individuals were associated with asymptomatic colonization and reduced gastrointestinal (GI) symptoms.

Table 4. Serum Levels of Th2 Cytokines According to *Blastocystis* Subtypes

Cytokine (pg/mL)	ST1	ST2	ST3	ST4	p-value
IL-4	18.5 ± 4.2	24.1 ± 5.3	16.8 ± 3.9	27.6 ± 6.1	<0.001
IL-5	14.2 ± 3.5	20.8 ± 4.7	13.9 ± 3.1	22.3 ± 5.4	<0.001
IL-10	11.6 ± 2.9	18.2 ± 4.0	10.8 ± 2.7	21.5 ± 4.8	<0.001
IL-13	15.9 ± 3.7	22.4 ± 5.2	14.7 ± 3.3	24.6 ± 5.5	<0.001

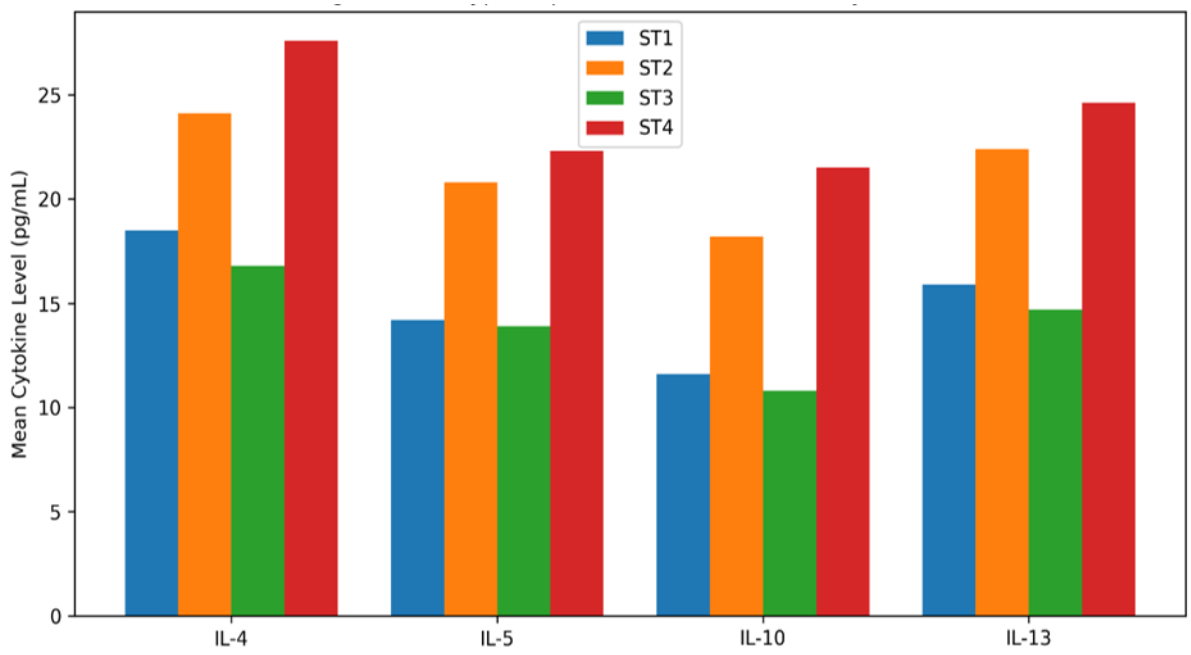


Figure 4. Subtype-dependent variation in Th2 cytokine responses.

Discussion

This current research has yielded valuable information both on the molecular and immunological basis of subtype diversity in *Blastocystis* and the associated Th1/Th2 cytokines produced by host(s). Through the use of real-time PCR for molecular characterization, there was a high level of heterogeneity in the subtypes of *Blastocystis* seen among the infected patients (31). The predominant subtype was ST3, while the other three primary types of *Blastocystis* were ST1, ST2, and ST4, respectively. Additionally, there were significant differences in type-specific cytokine responses as well. Collectively, these data point to *Blastocystis* subtypes displaying type-specific immunomodulatory capability(s) that eventually lead to different clinical outcomes following an infection (32).

According to the current study's findings, the dominant subtype among humans tested was ST3, matching results from numerous worldwide epidemiological investigations that have identified ST3 as the most frequently identified subtype in human populations. ST3 has consistently been recognized as the dominant human-associated subtype in studies reflecting the Asia, Europe, and Middle East regions of the world, indicating its highly successful adaptation within the human GI tract. The strong association between symptomatic patients and the high prevalence of ST1 and ST3 further supports the notion that these two subtypes are associated with higher levels of pathogenicity than other subtypes (33). This aligns with previous studies in molecular epidemiology which have found strong associations between ST1/ST3 strains and gastrointestinal symptoms such as abdominal pain, diarrhea, bloating, irritable bowel syndrome (IBS)-type symptoms (34).

Conversely, ST2 and ST4 were cited to be detected more among asymptomatic carriers. If true, this could suggest that ST2 and ST4 would likely have a lower level of pathogenicity or could also play a role in regulatory immunological interactions with the development of tolerance in the intestines (35). As microbiome studies have shown recently, specific *Blastocystis* subtypes might act as a commensal organism under certain conditions in the ecological environment and may increase the diversity of the gut microbiome and help maintain the homeostasis of the gut. So, the clinical consequences from colonization of *Blastocystis* would be determined by the presence of the parasite and its subtype(s) as well as the immune response from the host, the composition of microbiota within the host, and the burden of the parasite (36).

A key result from this study was that the levels of Th1 type cytokines (IFN γ , TNF α , IL -2 and IL -12) were significantly increased in symptomatic *Blastocystis* positive subjects compared to those

with asymptomatic (non-infection) status. Thus indicating that Th1 cellular (cell-mediated) immune activity is involved in symptomatic *blastocystosis*. Activation of T H 1 immune path assemblies will have a direct effect on mucosal inflammation and gastrointestinal symptoms in infected individuals (37).

Elevation of IL-12 found in symptomatic patients also supports the hypothesis that there was Th1 polarization in disease-causing *Blastocystis* infections. In addition, IL-12 is critical for the differentiation of CD4+ T lymphocyte memory cells into Th1 cells and promotes the production of IFN-γ. There is a significant increase in both IL-12 and IFN-γ levels observed in this study, indicating that patients are experiencing both types of cell-mediated inflammatory responses, providing an explanation for the occurrence of IBS-like symptoms or persistent intestinal discomfort in subjects infected with pathogenic subtypes of *Blastocystis (38).

Increased expression of IL-10 associated with ST2 and ST4 infections may help to explain why many individuals with these subtypes are asymptomatic (this provides potential benefits for maintaining mucosal inflammation). Because anti-inflammatory cytokines are raised, mucosal inflammation is more likely to be reduced leading to greater integrity of the epithelial layer , even though parasites have been persistently established in the host (mucosal tissue). Some studies suggest that *Blastocystis* subtypes can also promote regulatory T cell activation and concomitantly promote intestinal immune tolerance. This may mean ST2 and ST4 are the least virulent or have more balanced host-parasite relationships than ST1 and 3 (39).

Based on recent data, it is becoming apparent that the ability of Blastocystis to cause disease may be associated with specific subtypes instead of all strains causing disease in the same way. The cytokine profile differences across each subtype indicate that genetic variation affects how the immune system will react to the parasite, which will aid in helping to end the debate about the clinical importance of infection by providing an explanation for some of the contradictory results reported in previous studies. In addition, differences in the distribution of subtypes within each of the populations examined could lead to a difference in results between the studies (40, 42).

While the current research has produced significant results, there were some limitations that need to be addressed. First, as a cross-sectional study, it is not possible to determine causality between subtype infection and cytokine dysregulation. Second, analysis of cytokines was conducted only on a few markers of Th1 and Th2 (e.g., IL-2 and IL-4) with no additional measurements of other inflammatory mediators (such as IL-17, transforming growth factor-beta [TGF-β], regulatory T-cell markers). Finally, we did not evaluate the composition of gut microbiota in this study, although there is a growing body of research showing that there are complex relationships between *Blastocystis*, intestinal bacteria, and the immune response of the host. we noticed another significant result that ST1/ST3 have a profound correlation with raised Th1 cytokine responses and ST2/ST4 have a significant correlation with raised anti-inflammatory Th2 cytokines. This suggests that immune polarization may be an important factor in determining the severity of disease and development of symptoms. Persistent Th1 activation could lead to chronic intestinal inflammation whereas balanced Th2 responses could support asymptomatic colonisation and tolerance of the intestine's immune system.

Conclusion

This current study has shown that real-time PCR is an extremely sensitive and specific method of determining which subtype of Blastocystis spp. can be found in individuals presenting with gastrointestinal symptoms. The results of this research found that there was a significant amount of diversity among the subtypes within the population studied, with ST3 being the most common, followed by ST1, ST2, and ST4. The results of this study also demonstrated that different subtypes produce different host immune responses (Th1 vs Th2) and thus may have different clinical implications. The infections with symptoms were mainly related to ST1 and ST3 and generated considerably increased release of Th1-type

cytokines, such as IFN-γ, TNF-α, IL-2 and IL-12, indicating a strong proinflammatory immune response. Although ST2 and ST4 infections also produced increased levels of Th2-type anti-inflammatory cytokines (IL-4, IL-10, IL-13), they accrued more frequently from an asymptomatic state and regulated through immunoregulatory processes. This provides strong evidence that pathogenicity of Blastocystis is based on subtype, not all isolates. Altogether, the identification of molecular subtypes along with the measurement of cytokines gives us a better understanding of how immune responses are formed when Blastocystis infects humans. In addition, our results also help resolve the long-standing debate regarding whether Blastocystis is clinically significant. We believe that future epidemiological, diagnostic or therapeutic trials involving Blastocystis must be designed to account for the diversity of the molecular subtypes of Blastocystis.

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