

# Relationship of Blastocystis subtypes and its co-infection with other parasites to the infection symptoms

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## Abstract

**Background:** One of the common gastro-intestinal parasites worldwide is *Blastocystis*. Diverse subtypes are employed to detect this microscopic parasite but there is not enough information on the issue if these subtypes may exhibit diverse clinical symptoms or not. This research aimed at the evaluation of the clinical symptoms of the disease like itching and gastro-intestinal symptoms in various subtypes as well as in *Blastocystis* co-infection with other parasites.

**Methods:** We extracted DNA from the fecal specimens of the cases that had been referred to the Center for Tropical Diseases in Verona-Italy. Moreover, real-time PCR (RT-PCR) was employed to identify the available parasitic infections and nested PCR to detect the subtypes. Then, SPSS was used to analyze data.

**Results:** Any significant relationship did not exist between the subtypes and clinical symptoms. In addition, co-infection did not significantly related to the disease symptoms and a number of parasites. The small statistical population may influence such results.

**Conclusion:** Further investigations must be conducted for determining the effects of *Blastocystis* sub-types and their co-infection on the disease symptoms.

**Keywords:** *Blastocystis* STs, co-infection, Gastrointestinal symptoms, itching

## Introduction

*Blastocystis* is a popular intestinal protists in humans. This parasite has been initially defined one decade ago but there is not enough information on the genetic diversity, host range, treatment, and pathogenicity.<sup>1</sup> The parasite has shown a global distribution with the greater numbers occurring in developing countries, which can be the result of poor sanitation.<sup>2</sup> It is also observed in diverse animals like birds, amphibians, and mammals. So far, researchers presented 17 sub-types with subtype (ST) 1–9 that has been observed in humans<sup>3</sup> and ST3 has been considered as the predominant ST in a majority of epidemiological investigations on humans.<sup>4-6</sup>

However, researchers have not reached an agreement on the *Blastocystis* pathogenicity in humans. While several researchers consider this parasite as a pathogen<sup>7,8</sup>, a lot of researchers are not sure of the contribution of *Blastocystis* in human-related diseases.<sup>9, 10</sup> They have referred to abdominal pain, vomiting, and diarrhea as the commonest symptoms of *Blastocystis* infection. Several studies have been done on the single cases, showing that any other cause of sickness has not been detected and *Blastocystis* has been the only infection diagnosed.<sup>11</sup> Moreover, a lot of case studies demonstrated the association of *Blastocystis* with urticarial and amoeboid forms of *Blastocystis* ST3 have been observed in a case of acute urticarial. Therefore, researchers assumed disruptions in the

immune homeostasis as the cause of cutaneous symptoms because the hosts produced inflammatory responses against the amoeboid forms.<sup>12</sup>

According to a study in the field, abdominal pain and diarrhea and other non-specific gastrointestinal symptoms like vomiting, nausea, dysentery, constipation, bloating, flatulence, weight loss as well as anorexia are the common characteristics of Blastocystosis. They found symptoms ranging between mild chronic diarrhea and acute enteritis.<sup>13</sup> Several documents have referred to the association between parasite density and severity of clinical symptoms due to *Blastocystis*.<sup>14, 15</sup> Other investigations have emphasized the correlation between sub-types and humans' pathologies but there are no data of the infection density.<sup>16, 17</sup> Therefore, since this information has been designed for eliminating any correlation between the parasite density and intestinal symptoms, additional investigations must be done on *the Blastocystis*-induced intestinal pathology, with an emphasis on the parasite density and relation to the subtypes.

According to the studies, urticaria or hives have been considered as one of the kinds of skin rash followed by red, raised, itchy bumps so that patches of rash frequently move around them. The symptoms persist some days and would not leave any scars or changes in the skin. In fact, <5% of patients experience urticaria for more than 6 weeks.<sup>18</sup> Researchers have shown the contribution of multiple causative parameters to the etiology of urticarial like stress, allergic reaction, antibiotics, infectious agents, insect bites, non-steroidal anti-inflammatory drugs (NSAIDs), physical stimuli, systemic dysfunctions as well as food additives.<sup>19</sup> Notably, a lot of studies showed *Blastocystis* infection is correlated to the cutaneous lesions, especially urticaria.<sup>20, 21</sup> Finally, a causal association of *Blastocystis* infection with urticaria has been reported by numerous cross-sectional or case studies.<sup>22</sup>

This retrospective research aimed at determining the correlation between *Blastocystis* STs and co-infection and gastro-intestinal symptoms for summarizing the major clinical manifestations, treatment, diagnosis, as well as outcomes of the related cases.

## Methods

**Sampling and participants:** We collected 1778 fecal samples from individuals referred to the Center for Tropical Diseases of SacroCuore-Don Calabria Hospital in Negrar (Verona) between January 2014 and December 2015. This center is a referral place for parasitic and tropical infections in Italy. Before processing, we kept the fecal samples in 95% ethanol.

**Ethics approvals and consents for participation:** Based on the requirement of the Declaration of Helsinki, we gathered the fecal samples. Each patient who participated in the research presented his/her written consent to the donation of the biological samples regarding to the objective of the study. Prior to the retrospective analyses, each datum was thoroughly anonymized. Moreover, we obtained the ethical clearance protocol from the local competent Ethics Committee (Comitato Etico per la Sperimentazione Clinica delle Province di Verona e Rovigo, protocol number 34680, 2017).

**Demographic data:** An electronic archive of the molecular parasitology laboratory was searched for each specimen to collect demographic data of cases like gender, nationality, and age with the use of the identification (ID) code.

**Extraction of DNA:** DNA was extracted according to a study in the field<sup>23</sup> and 200 mg of all the stool samples were kept at -20 °C in a solution of PBS 1X with 2% of polyvinyl-polypyrrolidone (PvPP) (Sigma-Aldrich; Milan: Italy) overnight. Then, Phocine Herpes Virus type-1 (PhHV-1, sincerely presented by Dr. Pas S., Erasmus MC in Department of Virology of Rotterdam) was poured into S.T.A.R. buffer (Roche, Milan, Italy), which was used as one of the internal controls for

amplification as well as isolation stages. In the next step, each sample was frozen and boiled at 100 °C for ten minutes. MagnaPure LC.2 instrument (Roche Diagnostic, Monza, Italy) was used to extract DNA with a DNA isolation kit I (Roche), and DNA elution was done in a final volume of 100 µl. Finally, we labeled the DNA samples and maintained them at -20 °C for other molecular experiments.

**RT PCR reaction:** As mentioned earlier, we collected 1778 fecal samples and used 3 individual multiplex Real time polymerase chain reaction (Rt-PCR) to screen them in order to identify *Entamoeba-histolytica*, *Entamoebadispar*, *Cryptosporidium spp*, *Giardia intestinalis*, *Dientamoebafragilis*, *Blastocystis*spp, *Strongy loidesstercoralis*, *Schistosoma spp*. and *Hymenolepis nana*. Moreover, RT PCR (CFX96-Biorad), which has been illustrated by Stensvold *et al.*'s<sup>24</sup> study, was employed to amplify all DNAs. In fact, RealTime has been proposed as one of the multiplex PCR, which is simultaneously capable of detecting the probable existence of 3 protozoa (*G. intestinalis*, *Blastocystis* & *D. fragilis* spp.). In addition, the above multiplex PCR detected PhHV DNA and thus it was added to the samples prior to the initiation of the extraction process. We followed directions provided in Verweij *et al.*'s<sup>25,26</sup> study to perform multiplex Rt-PCRs.

**Blastocystis subtype analysis:** Instructions reported in Scanlan *et al.*'s<sup>27</sup> study but with small changes were used to implement Nested-PCR. The first-step PCR was implemented for providing a *Blastocystis* specific 18S rDNA template for all ST-specific PCRs (ST1, ST2, ST3, & ST4). Table 1 gives the primer sequence. iTaq DNA polymerase (Bio-Rad; Milan: Italy) in 50 µL of the reaction volume was used to run PCR based on the company's directions. Here, the temperature profile of the first-step PCR is presented: initial denaturation at a temperature of 95 °C for three minutes, 30 cycles at a temperature of 94 °C for one minute, 59 °C for one minute, 72 °C for one minute, and the resulting elongation at a temperature of 72 °C for five minutes. Moreover, the implementation process of the ST-specific PCRs is presented: initial denaturation at a temperature of 95°C for three minutes, 35 cycles at 94 °C for 30 seconds, T<sub>annealing</sub> primers for 30 seconds, 72 °C for one minute, and the resulting elongation at a temperature of 72 °C for five minutes. Then, we utilized 1 µL of the first-step PCR product in each reaction. It should be mentioned that we constantly embedded a no-template control in each PCR run and used 2.5 % agarose gel electrophoresis for analyzing the PCR products for detecting certain DNA bands.

**Statistical analysis:** In this step, we categorized the samples studied for *Blastocystis* sub-types into 3 categories according to the existence of itching, absence of the symptoms as well as gastrointestinal symptoms. Moreover, we investigated geographical background of the participants (non-Italian Europeans, Italians, Asians, South Americans & Africans). After that, their features were examined in terms of correlation using the parametric and non-parametric statistical tests like Chi-Squared test and uni-variate logistic regression models. SPSS 16.0 and Chi square test were employed to assess data statistically. Finally, we considered p-values ≤ 0.05 to be significant.

## Results

**Characteristics of the patients:** Among 1778 samples selected to the molecular tests for exploring the intestinal parasite infections, we did experiments on 756 samples for *Blastocystis*. Then, 258 (34.1 %) positive samples with the *Blastocystis* spp. were detected which was the commonest parasite in the study population (Table 2). Moreover, a significantly greater frequency of the *Blastocystis* infection was found in the men (38.5%) than in the women (28.8%). Finally, we did not observe any correlation to age (t-test, p = 0.264).

**Characterization of *Blastocystis* subtypes:** According to the results, ST3 and ST1 are the commonest sub-types (single sub-type carriers), with the spread of 26.7% and 23.1%. Out of the mixed STs Co-infections, ST1-ST3 is the most widespread. Even though with the decreased frequency, triple STs infections are identified such as ST1-ST3-ST4 and ST1-ST3-ST2 (Figure 1).

**Analyzing the clinical phenotype:** With regard to the outputs, 60.2% of the cases with positive *Blastocystis* are symptomatic. Moreover, 39.4% of the cases exhibited the GI symptoms like abdominal pain, irritable bowel syndrome as well as diarrhea, 10.0% experienced itching, and finally, 10.8% exhibited both kinds of symptoms. In addition, symptomatic patients were shown to be significantly older than the asymptomatic cases with an average age of 38.7:31.7, t-test,  $p = 0.01$ ). Furthermore, we did not observe any correlation of the symptoms with gender ( $p = 0.291$ ). Consequently, symptoms related to the geographical background (Chi Square Test,  $p = 0.042$ ) so that Italians (72.0%) showed more symptoms.

In terms of symptoms, single and multiple subtype carriers did not significantly differ (Chi Square test,  $p = 0.63$ ) so that single ST carriers were 61.7% and multiple STs carrier cases were 57.5% with itching, GI or both. Even though we did not observe any statistically significant correlation of a certain subtype with the symptoms, most cases with ST1 and ST3 exhibited GI symptoms and most of them who carried ST2 were shown to be asymptomatic. However, ST3 or ST1-ST3 mixed subtypes infected the greatest percent of cases with itching manifestation.

**Characterizing the co-infecting parasites:** The presence of other co-infecting parasites such as *Entamoebahistolytica*, *Entamoebadispar*, *Giardia intestinalis*, *Dientamoebafragilis*, *Strongyloidesstercoralis*, *Cryptosporidium* spp., *Schistosomaspp*, *Hymenolepis nana* was analyzed in the cases carrying *Blastocystis*. Notably, co-infecting parasites were observed in 54% of our patients.

In addition, age inversely correlated to other parasite co-infections (Wilcoxon test,  $p=0.004$  with an average age: 31.7 versus 39.6 of the co-infected and non-coinfected cases). Moreover, a certain *Blastocystis* sub-type did not correlate to other parasites (Figure 2).

This step addressed the analysis of the probable correlation of the presence of symptoms to co-infection with diverse parasites; however, they did not significantly differ (Chi Square test,  $p = 0.43$ ) between the samples infected by just *Blastocystis* (63% showed the symptoms) and samples with numerous parasite co-infections (57% presented the symptoms). Figures 3 and 4 give the outputs on the correlation of the symptoms with *Blastocystis*, with and without other parasitic infections. Furthermore, a greater proportion of GI symptoms was used to characterize *Blastocystis-D. fragilis* co-infected samples whereas a greater proportion of asymptomatics was used to characterize the samples with other co-infections. The above 2 proportions were similar in the *Blastocystis*-only infected samples.

## Discussion

Based on the present retrospective research, *Blastocystis* was shown to be the commonest parasite in the samples experimented for intestinal parasite infections at our Centre for Tropical Diseases, which approves earlier investigations reflecting the greater spread of *Blastocystis* infection than other intestinal parasites.<sup>28</sup> As demonstrated, *Blastocystis* subtype was characterized for 260 positive samples and ST3 was shown to be the commonest subtype in the Italians of our regional area. Therefore, the obtained result showed an earlier study by the other Italian group.<sup>29</sup> Finally, we observed the presence of ST1 with a relatively lower frequency.

The results demonstrating the ST3 predominance are the same as a majority of earlier investigations conducted in Asia and Europe like the report presented by Jamtemtor in Thailand, which showed ST3 as the most predominant sub-type (57.1%) and then ST1 (21.4%).<sup>30</sup> In this regard, Wong et al. researched in Singapore and demonstrated ST3 as the most predominant sub-type (78%) and then ST1 (22%).<sup>31</sup> Moreover, Boondit et al. indicated ST3 as the most predominant subtype (76%), followed by ST1 (20%).<sup>32</sup> The same prevalence rate of subtype was shown by Ozyurt et al.'s study in Turkey and other countries like Germany, China, Denmark, and Japan.<sup>33</sup> However, Awatif et al.'s finding was different in Libya wherein ST1 is the most dominant sub-type in outpatients (51.1%), followed by ST2 (24.4%) and ST3 (17.8%).<sup>34</sup> In addition, Dominguez et al.'s study in Spain was different with ST4 as the most dominant sub-type (94.1%), followed by ST1 (2%) and ST2 (3.9%).<sup>35</sup> Furthermore, Souppart et al.'s study did not show any correlation of *Blastocystis* infection with the specific subtypes; however, there was not any correlation with the risk factors in infection transmission such as environmental factors; for example, source of contamination and transmission route, parasite factors like pathogenic potential and zoonosis, as well as host factors like immunity, age, and genotype.<sup>36</sup>

Current studies have considered the presence of the mixed subtype infections as one of the prominent characteristics, which must be investigated for exploring the distribution and diversity of the parasite in the humans' gut. We applied a sophisticated subtype-specific procedure<sup>27</sup> for detecting the mixed infection in 37.3 % of patients so that ST1-ST3 was shown to be the commonest mixed sub-type combination; a result that has been reported by other investigations (review in<sup>37</sup>). It was found that the reciprocal existence of diverse sub-types may be one of the substantial cooperation strategies for host colonization through *Blastocystis spp.* However, this concept may be employed for the presence of numerous parasite co-infections. In addition, the correlation of *Blastocystis* with *D. fragilis* could indicate a cooperative interaction of both protozoa. Other studies have also referred to the correlation of *Blastocystis spp.* and other parasites; for example, with *G. intestinalis*<sup>38</sup> and particularly with *D. fragilis*<sup>39</sup>); however, researchers have not illustrated any indefinite correlation with the symptoms.

## Conclusion

Even though this research showed no statistically significant correlation of the STs with the symptoms (possibly because of the lower number of samples, or with inherent genetic variability and or immunological variables), in the presence of the mixed sub-types or other parasite co-infections, a relatively greater proportion (48.1 %) of the symptomatic cases versus asymptomatic cases was seen in the cases with positive *Blastocystis*, with ST1 and ST3 pervasiveness in cases with the GI symptoms whereas ST2 showed a low effect. In addition, the presence of *D. fragilis* and both *Blastocystis* enhanced the percent of cases with GI symptoms with regard to just *Blastocystis* or in co-infection with other experimented parasites. Finally, further research must be performed for confirming the above indications due to the lack of statistically significant clues.

The present research suffers from multiple limitations that are largely associated with the retrospective study design. Particularly, it is possible that reports on the symptom had not enough accuracy, which can be an additional reason for the absence of a statistically significant association of molecular findings and clinical features. We proved the presence of strongly variable symptomatology through cases infected by *Blastocystis* infected, which cannot be thoroughly understood via the presence of various sub-types or other parasite co-infection, though the samples' variability may need a greater number of observations for reaching the statistically significant

indications. Therefore, the obtained data supported the hypothesis: the host condition is a major dimension with the possible effect on the pathogenicity of *Blastocystis* spp. colonization. Additional investigations of the patients' immunological properties, as well as gut microbiota related to the *Blastocystis* infection, can enhance our understanding of the cause of the observed variable pathogenic penetrance of the mentioned parasite.

### Conflict of interest

There is no conflict of interest.

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## Tables:

Table 1. Primers sequences in the nested PCR.

Primer s	Sequences	Target <i>Blastocystis</i> ST	Annealing tempratur e (°C)	Produc t size (bp)	Referenc e
<b>RD5</b>	ATCTGGTTGATCCTGCCAGT	All	59	≈ 600	40
<b>BhRDr</b>	GAGCTTTTAACTGCAACAAC	All	59	≈ 600	40
G					
<b>ST1-F</b>	AGTCCTCTGGTGAGGTGTGT	ST1	56	433	27
<b>ST2-F</b>	AGTAAAGTCCCGTAGGGATG	ST2	56	459	27
<b>ST3-F</b>	GTCTTGTA GACTGCAT	ST3	48	427	27
<b>ST4-F</b>	CCA AKAGACTTCGGTCT	ST4	48	399	27

Table 2. Baseline Demographic and Clinical Characteristics of the *Blastocystis* positive Cohort, Stratified by Area of Origin.

Characteristic	Entire Cohort	Area of Origin <sup>a</sup>				
	N, %	Italy	Europe	Africa	South America	Asia
Total	221	93 (42.1)	7 (3.2)	76 (34.4)	25 (11.3)	20 (9.0)
Mean age, years (SD)	35.9 (19.6)	48.6 (17.3)	22.4 (13.5)	25.8 (13.7)	37.3 (20.3)	18.9 (10.6)
Female. N (%)	86 (38.9)	46 (49.5)	3 (42.9)	17 (22.4)	15 (60.0)	5 (25.0)
Symptoms, N (%)	133 (60.2)	67 (72.0)	4 (57.1)	40 (52.6)	13 (52.0)	9 (45.0)
Itch	22 (10.0)	9 (9.7)	0 (0.0)	6 (7.9)	5 (20.0)	2 (10.0)
GI	87 (39.4)	42 (45.2)	3 (42.9)	28 (36.8)	7 (28.0)	7 (35.0)
Both	24 (10.8)	16 (17.2)	1 (14.3)	6 (7.9)	1 (4.0)	0 (0.0)
<i>Blastocystis</i> Subtype <sup>b</sup> , N (%)						
Subtype 1	51 (23.1)	20 (21.5)	1 (14.3)	20 (26.3)	4 (16.0)	6 (30.0)
Subtype 2	17 (7.7)	10 (10.8)	2 (28.6)	4 (5.3)	1 (4.0)	0 (0.0)
Subtype 3	59 (26.7)	22 (23.7)	2 (28.6)	20 (26.3)	9 (36.0)	6 (30.0)
Subtype 4	14 (6.3)	9 (9.7)	0 (0.0)	4 (5.3)	1 (4.0)	0 (0.0)
Combination	80 (36.1)	32 (34.4)	2 (28.6)	28 (36.8)	10 (40.0)	8 (40.0)

Abbreviations: SD, standard deviation; GI, gastrointestinal.

<sup>a</sup> European cases were from Europe, outside Italy; that is, from Romania, Germany, European Russia and Switzerland and<sup>b</sup> Sub-types show the presence of a single subtype and combination involves all probable combinations of the above.

Figures:

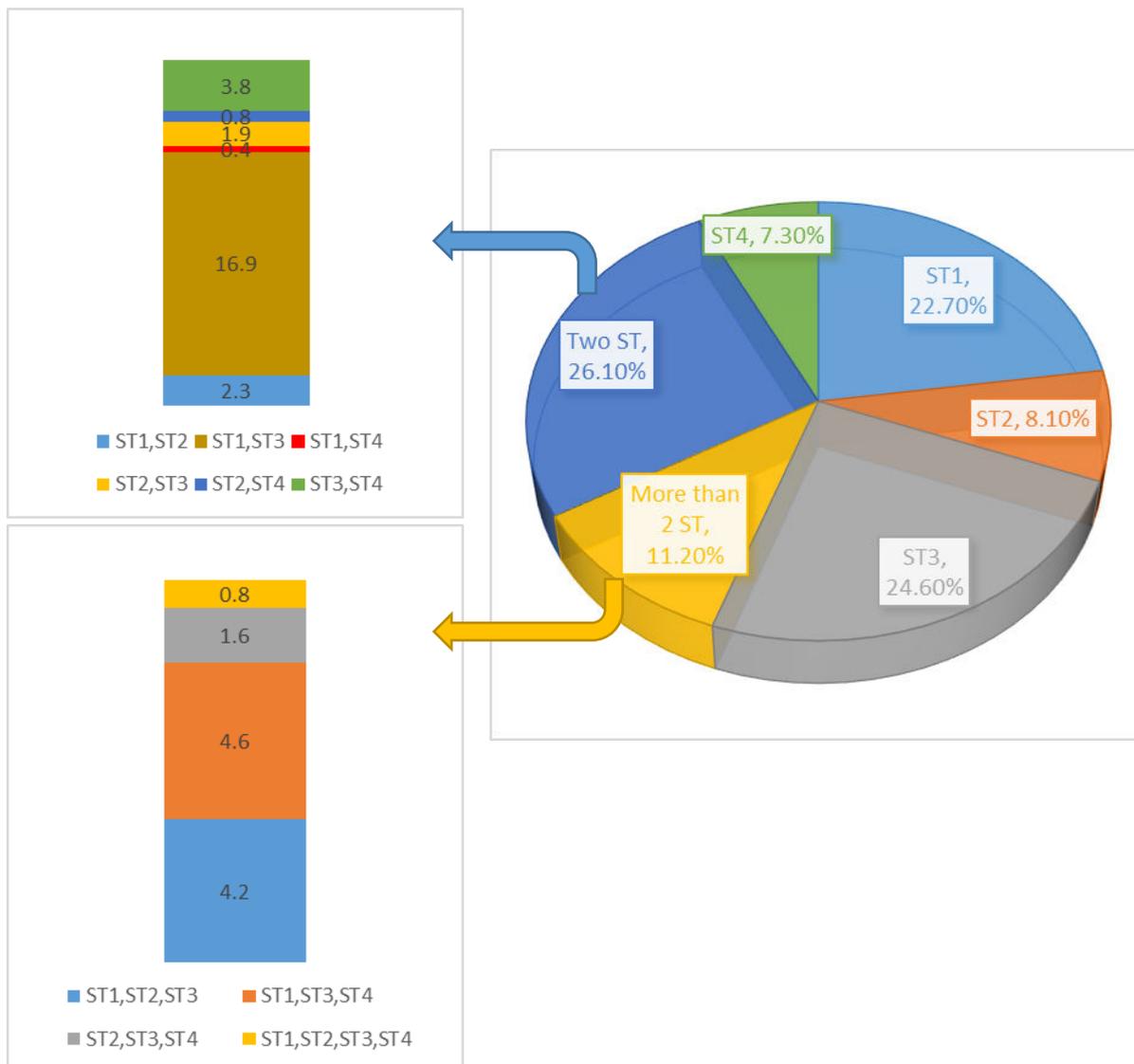


Figure 1. *Blastocystis* sub-type and distribution of the mixed sub-types.

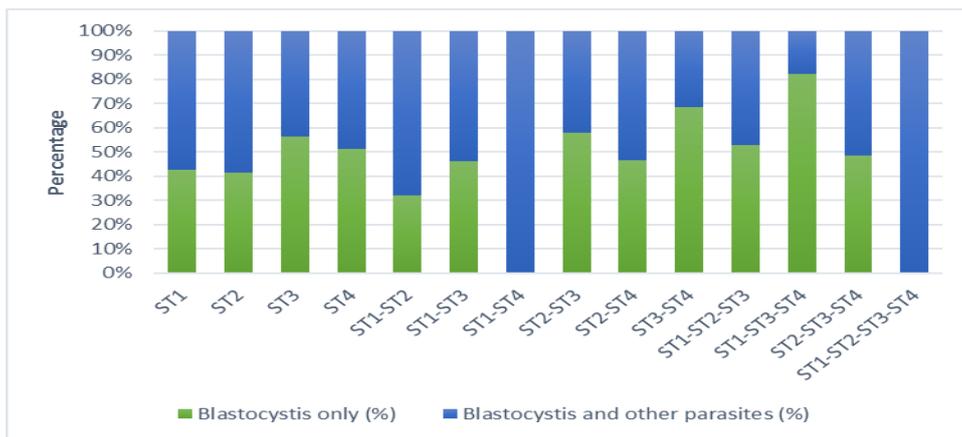


Figure 2. Distribution of the *Blastocystis* STs between cases infected with *Blastocystis* sp. only and coinfected with other parasites.

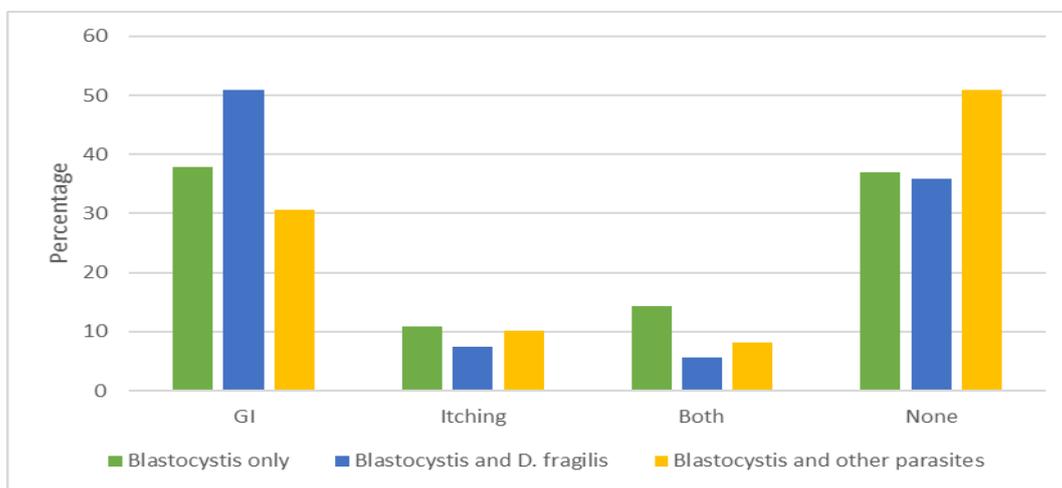


Figure 3. *Blastocystis* and other parasite co-infections distribution based on the clinical data.

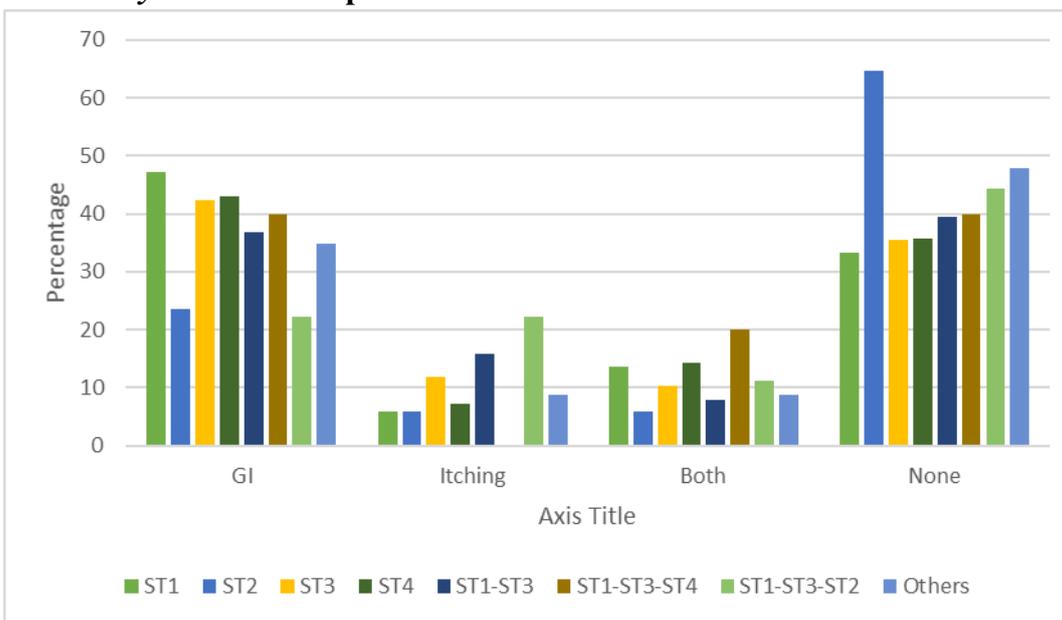


Figure 4. Distribution of *Blastocystis* STs based on the clinical data