Molecular epidemiology of *Blastocystis* and association with intestinal parasites among patients in Negrar hospital, Italy

By

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Dedicated with love to my wife

‘Shima’

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Abbreviations

DNA	Deoxyribonucleic acid
IBS	Irritable bowel syndrome
ST	Subtype
GI	Gastrointestinal
SSU-rDNA	Small-subunit ribosomal DNA
PCR	Polymerase chain reaction
Rt-PCR	Realtime PCR

Abstract in English

Blastocystis is a common intestinal protozoan, but its clinical significance and its role in the human gut microbiome is still not completely understood. Clinical manifestations among symptomatic subjects are mainly gastrointestinal (GI) or cutaneous symptoms, but also several asymptomatic cases have been described. The aim of the present study was to characterize the presence of *Blastocystis* in fecal specimens from patients attending to our center for tropical diseases, in order to explore the prevalence and the diversity of *Blastocysti*s infections in our study population. We characterized the presence of the 4 most common subtypes in the selected cohort, composed by subjects with different geographical origins (mainly Italians and Africans). Analysis of co-infections with other parasites has been also performed and description of symptoms found in different subtypes or co-infections combinations has been reported.

Blastocystis resulted to be the most prevalent parasite in our population and we found that 48.1 % of *Blastocystis* positive subjects presented GI symptoms. ST3 was the most prevalent subtype in Italians, while in Africans ST1 and ST3 were found with the same frequency. Interestingly, in all the analyzed geographical areas, the most prevalent group was composed by subjects infected by more than one *Blastocystis* ST. No association between a particular subtype or STs-combination with symptoms has been detected. We observed the presence of co-infecting parasites in the 48.5 % of our cases. An association between Nationality and GI symptoms has been highlighted (P=0.031).

Our study confirms that *Blastocystis* infection symptomatology cannot be completely explained neither by the different subtypes presence nor by other parasite co-infections, thus supporting the hypothesis that the host condition is the key aspect that can influence the pathogenicity of *Blastocystis spp* colonization. Future studies on the association between *Blastocystis* infection and patients' gut microbiota and/or immunological conditions, could elucidate the cause of the observed variable pathogenic penetrance of this parasite.

Riassunto in Italiano

La *Blastocisti* è un protozoo intestinale comune, ma il suo significato clinico e il suo ruolo nel microbioma intestinale umano non sono ancora completamente compresi . Le manifestazioni cliniche tra i soggetti sintomatici sono principalmente sintomi gastrointestinali (GI) o cutanei, ma sono stati descritti anche diversi casi asintomatici . Lo scopo del presente studio era di caratterizzare la presenza di *Blastocisti* in campioni fecali da pazienti che frequentano il nostro centro per malattie tropicali, al fine di esplorare la prevalenza e la diversità delle infezioni da *Blastocisti* nella nostra popolazione di studio. Abbiamo caratterizzato la presenza dei 4 sottotipi più comuni nella coorte selezionata, composta da soggetti con diverse origini geografiche (principalmente italiani e africani). E 'stata anche eseguita un'analisi delle coinfezioni con altri parassiti e sono stati riportati i sintomi trovati in diversi sottotipi o combinazioni di coinfezioni.

La *Blastocisti* è risultata essere il parassita più diffuso nella nostra popolazione e abbiamo scoperto che il 48,1% dei soggetti positivi a *Blastocisti* presentava sintomi GI. ST3 era il sottotipo più diffuso negli italiani, mentre in Africani ST1 e ST3 erano stati trovati con la stessa frequenza. È interessante notare che, in tutte le aree geografiche analizzate, il gruppo più prevalente era composto da soggetti infettati da più di un *Blastocisti* ST. Non è stata rilevata alcuna associazione tra un sottotipo particolare o una combinazione ST con sintomi. Abbiamo osservato la presenza di parassiti co-infettanti nel 48,5% dei nostri casi. È stata evidenziata un'associazione tra nazionalità e sintomi gastrointestinali (P = 0,031).

Il nostro studio conferma che la sintomatologia dell'infezione da *Blastocisti* non può essere completamente spiegata né dalla diversa presenza di sottotipi né da altre parassiti parassitarie, supportando quindi l'ipotesi che la condizione ospite sia l'aspetto chiave che può influenzare la patogenicità della colonizzazione di *Blastocisti* spp. Studi futuri sull'associazione tra l'infezione da blastocisti e il microbiota intestinale dei pazienti e / o condizioni immunologiche potrebbero chiarire la causa della penetranza patogena variabile osservata di questo parassita.

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CHAPTER 1:

INTRODUCTION

1.1 Introduction

Blastocystis is an unusual protistan enteric parasite classified under a highly diverse group of organisms called stramenopiles, and is the only known member of this group associated with human pathology (1).

Blastocystis is commonly identified in stool specimens and it is one of the most common parasites that reside in the human intestinal tract. The disease it causes is called blastocystosis but most publications refer it to as *Blastocystis* infections. Clinical symptoms attributed to *Blastocystis* infections include recurrent watery diarrhea, mucous diarrhea, vomiting, abdominal cramps and flatulence. *Blastocystis* can infect both children and adults and its geographical distribution appears to be global with prevalence ranging from 30 to 50% in developing countries (2).

At first, the name B. enterocola was proposed by Alexeieff (3) and later it was isolated from human feces and the name *B. hominis* was coined (4). Initially, it was described as harmless intestinal yeast. Its association with human disease was suggested by a number of reports and eventually work by Zierdt increased the awareness of *Blastocystis* infections in humans. In spite of its description about a century ago, the exact pathogenesis mechanisms of *Blastocystis* infections are uncertain (5). A number of clinical and epidemiological studies implicate the parasite as a potential pathogen, while others exonerate it as an etiology of intestinal disease (6,7). Significant progress has been achieved on descriptions of the morphology and genetic diversity of *Blastocystis* but most aspects of its life cycle, molecular biology, and pathogenicity remain unresolved (2,6).

1.2 Taxonomy

The taxonomic classification of *Blastocystis* is a controversial subject and there are many disagreements among researchers. *Blastocystis* was earlier described to be a yeast or a fungus (3,8), a cyst of another protozoa (9), or a degenerating cell (10). *Blastocystis* was described as a protist on the basis of morphological and physiological features (11). These protistan features included presence of one or more nuclei, smooth and rough endoplasmic reticulum, Golgi complex, mitochondria-like organelles, inabilty to grow on fungal medium, ineffectiveness of antifungal drugs, and susceptibility to some

antiprotozoal drugs. Later, *Blastocystis* was classified as a sporozoan (5) and finally reclassified as a sarcodine. Molecular sequencing studies of *Blastocystis* partial small-subunit rRNA (ssrRNA) showed that *Blastocystis* is not monophyletic with the yeasts, fungi, sarcodines, or sporozoans (12) and it was concluded that *Blastocystis* is not related to yeasts. In another study, the complete *Blastocystis* ssrRNA gene was sequenced and phylogenetic analysis suggested that *Blastocystis* should be classified within the Stramenopiles (also known as Heterokonta) (13).

Molecular phylogenetic analysis showed that *Blastocystis* is closely related to the Stramenopile Proteromonas lacerate (14). Another study involving molecular analysis of *Blastocystis* ssrRNA, cytosolic-type 70-kDa heat shock protein, translation elongation factor 2, and the non-catalytic 'B' subunit of vacuolar ATPase confirmed that *Blastocystis* is a Stramenopile (14). Stramenopiles characteristically possess flagella with mastigonemes. Interestingly, since *Blastocystis* does not have flagella and is non-motile, it was therefore placed in a newly formed Class Blastocystea in the Subphylum Opalinata, Infrakingdom Heterokonta, Subkingdom Chromobiota, and Kingdom Chromista (15). In addition, elongation factor- 1α (EF- 1α) sequencing for phylogenetic analysis also showed that *Blastocystis* is not a fungus and suggested that it diverged before Trypanosoma, Euglena, Dictyostelium and other eukaryotes. Most studies in the past named *Blastocystis* species according to host origin and this may have resulted in confusion regarding specificity, cell biology and pathogenicity of the parasite. A consensus report on the terminology for *Blastocystis* genotypes was published (16). Based on this report humans can be host to *Blastocystis* from a variety of animals including mammals (subtype 1), primates (subtype 2), rodents (subtype 4), cattle and pigs (subtype 5), and birds (subtype 6 and 7) (17,18).

1.3 Morphology

The morphological forms exhibited by species are many. Cysts develop into vegetative forms. The well characterized forms are the vacuolar, granular and the amoeboid forms. Other vegetative forms such as avacuolar or multi-vacuolar also have been identified. Other rareforms observed include medusa head and chest nut burr cells especially in aging cultures and on exposure to oxygen. All these above forms can be viewed by phase contrast microcopy and bright field microscopy of wet mounts, stained smears and electron microcopy (2,5,19).

The vacuolar form has a large central vacuole filling the entire cell space and limiting the cytoplasm and its intra cellular components to a thin peripheral rim. There is size variation from 3 µm to 120 µm, though most human isolates measure 5-15 µm. Subsequently, the central vacuoles were actually found to be the membrane bound bodies containing carbohydrate and lipid dispersed as flocculent or granular material. These central bodies are probably storage organelles and take part in apoptosis (1,20). The cytoplasmic rim contains one or two nuclei and the mitochondria are observed as rosettes around the nuclei. These structures may bulge inwardly into the central body and appear as filaments. Rarely, in fresh clinical isolates a surface coat or capsule has been observed, which presumably protects the organism from osmotic shock and trap bacteria for nutrition (1,5).

The granular form resembles the vacuolar form except for the presence of granules both in the central body and cytoplasm. The granules may be metabolic, reproductive or lipid containing. The reproductive granules have a possible role in schizogony. The granular forms exhibit lesser degree of pleomorphism and range in size from 15-80 µm (19).The amoeboid form is less frequently encountered. As its name implies, they are irregular in shape, possess one or two pseudopodia but are non-motile. The cytoplasm contains a single large vacuole and this form converts to cyst. This form is more frequently observed in symptomatic patients suggesting its pathogenic potential (21). Because they resemble neutrophils and macrophages, they can be easily missed in conventional stool examination. To identify them, Zierdt suggested simultaneous gram staining of an unfixed smear where these forms lyse on exposure to air, while the leucocytes remain intact (1,5). The cyst form is spherical or ovoid and is smaller in size (3-6 µm). Some cysts encountered in animals are larger (22). Each cyst has a thick multilayered wall with or without a surface coat. The condensed cytoplasm has several mitochondria and storage vacuoles.

The number of intracystic nuclei may vary from one to four. The cyst can remain viable for up to one month at 25 °C and on exposure to air. These cysts serve as transmissible infective forms(1). Vegetative forms transform into other vegetative forms of various morphology and hence these can be easily overlooked in fecal samples (20). Though the vacuolar and granular forms appear irregularly stained under the microscope, Vdovenco was able to demonstrate uniformly stained live organisms in fresh cultures. So the vacuolar and granular forms may represent degenerative changes in the organism. The avacuolar forms and the multivaculoar forms have recently been recognized to be the most predominant forms in vivo and also the forms which are frequently missed during microscopic examination (23).

1.4 Life cycle and Transmission

Many life cycles have been proposed for *Blastocystis* (2,3,6) owing to a lack of controlled experimental studies and the pleomorphic nature of the organism. The first life cycle was proposed by Alexeieff and it described the involvement of binary fission and autogamy (3). Some of the reports suggest modes of division like plasmotomy and schizogony (24). Most of these observations were based on microscopic analysis. Although *Blastocystis* had been isolated from laboratory animals, the lack of a suitable animal model was considered to be a major reason for the disagreement on its life cycle (6). Recent studies have shown successful experimental infection of *Blastocystis* in chickens (25) and rats (25,26,27). Rats appear to be good animal models for *Blastocystis* infection but reproducibility of animal infection needs to be ascertained.

A life cycle proposed by Tan states that infection is initiated when cysts of *Blastocystis* are orally ingested by humans or animals. Ingested cysts develop into vacuolar forms in the large intestine and later reproduce by binary fission. Some of the vacuolar forms encyst and are passed through the feces and the cycle is repeated. The role of the amoeboid and granular form in the life cycle of *Blastocystis* is not understood and remains to be elucidated (6). More recently, Tan revised the life cycle and included findings from molecular typing suggesting that *Blastocystis* isolated from humans actually comprise human and zoonotic genotypes of varying host specificities (28). A modified life cycle of *Blastocystis* must take into consideration the large reservoir of this parasite in a range of animal populations with humans as potential hosts(28).

1.5 Pathogenesis

There is still much debate about the pathogenicity of *Blastocystis* in humans. Though many authors have given credit to it as a pathogen (29,30), there are still many that doubt the role of *Blastocystis* in human disease (7,31). The most common symptoms associated with *Blastocystis* infection include diarrhoea, abdominal pain and vomiting. There are many reports of single patients that show there was no other cause of sickness identified in patients, with *Blastocystis* being the only infection detected. There have been several case reports suggesting that *Blastocystis* is related to urticaria (28). The amoeboid forms of *Blastocystis* ST3 were found in a case of acute urticaria and the authors suggested that cutaneous symptoms may be caused by disruptions to

the immune homeostasis as the host produces an inflammatory response against the amoeboid forms (32).
Another case showed the presence of *Blastocystis* ST2 in a severe case of gastrointestinal symptoms and chronic urticaria in the absence of any other infectious agent. Symptoms persisted after initial antibiotic therapy but were finally eradicated after combined metronidazole and paromomycin treatment (33). A retrospective study reported 8/8 (11%) *Blastocystis* infected patients to have skin manifestations as well as gastrointestinal symptoms (34). Unfortunately this study relied solely on microscopy, so no information on ST related to cutaneous lesions can be gathered; however all of these studies do show the potential for *Blastocystis* to cause cutaneous symptoms. It was recently suggested that gastrointestinal symptoms related to *Blastocystis* might be ST related but results remain inconclusive (35,36,37). It was suggested that ST1 may be related to pathogenicity with a higher subtype-symptom relationship being noted (38).
There have been conflicting reports on the pathogenicity of ST2 with some studies showing high symptom- infection rates (33,34) whereas others have seen no link (39,40). A study in Colombia showed that 100% of patients with diarrhoea had ST2 where asymptomatic people all had ST1 (41). There have been two previous studies that have suggested ST4 to be a pathogenic strain due to the high incidence of this ST in patients with severe diarrhoea (42,43). It was also suggested that ST8 could be a pathogenic strain. ST8 is a rare subtype found in humans and in two studies has been related to severe symptoms (35,44). Even though ST3 is the most common ST found in humans, there is a low association between ST and symptoms shown by patients (35). An animal study in rats showed that ST1 was statistically related to pathogenicity and that there may be pathogenic and non- pathogenic strains within ST3 and ST4 (27).

1.5.1 Intestinal symptoms

Blastocystosis frequently presents with diarrhea and abdominal pain (28), along with other nonspecific gastrointestinal symptoms including nausea, vomiting, constipation, dysentery, flatulence, bloating, anorexia and weight loss (45). Symptoms range from mild chronic diarrhea to acute enteritis (46). There is some evidence associating parasite density with severity of clinical symptoms caused by *Blastocystis* (47,48). Greater than five parasites per high-power field (× 400) for wet mounts or oil immersion (× 1000) in permanent stained smears are associated with higher frequency of acute intestinal symptoms (47,48). Recent studies however focus on association of subtypes with human pathology, without any information on infection density (45,49).

Since that data is limited to rule out the association of parasite density with intestinal symptoms, future studies might benefit by investigating *Blastocystis* induced intestinal pathology, focusing on parasite density along with subtype association.

1.5.2 Extraintestinal Symptoms

A correlation between *Blastocystis* and cutaneous lesions, particularly urticaria, has been reported (32,50). Multiple case studies suggest a causal link between acute or chronic urticaria and *Blastocystis*. There are reports on association between delayed-pressure urticaria, angioedema, and palmoplantar pruritus with *Blastocystis* as well (28,33). Resolution of cutaneous symptoms after chemotherapeutic treatment observed in these studies, re-enforce the role of *Blastocystis* with skin disorders (51).

1.5.3 *Blastocystis* and Irritable bowel syndrome

Irritable bowel syndrome (IBS) is "a functional disorder of the gastrointestinal tract characterized by regular occurrence of abdominal pain or discomfort along with alteration in frequency or consistency of the stool in the absence of organic etiology" (52,53,54). It is a very common disorder with a worldwide prevalence of 10-20% (55). Young adult patients are more frequently diagnosed with IBS than people over the age of 50 years (56) and most studies find a female predominance (57,58) although only a few people see their family doctor. The disease results in a reduced quality of life and is a multi-billion pound health-care problem (59). In the UK, only 50% of IBS cases are thought to be diagnosed (60). The symptoms of IBS differ from one person to another and may include irregular bowel movements, abdominal pain or discomfort, flatulence, and diarrhoea or constipation (61). Stress worsens IBS rather than being causative in any way (52).

The cause and pathophysiology of IBS are complicated and not well explained, and the main significant abnormalities include visceral hypersensitivity, irregular. Gut motility and autonomous nervous system dysfunction (61). Barbara et al. (62) demonstrated that there is reliable evidence showing that IBS may be the adverse result of an acute episode of infectious gastroenteritis, the so-called postinfectious (PI) IBS, and the infectious agents involved in the development of IBS include pathogenic bacteria, parasites, and viruses. Studies have illustrated that genetic factors, chronic stress and enteric infections can predispose persons to developing IBS (63). Most drug therapies to date are unable to make a major impact on the quality of life for sufferers (63).

Hence, an important problem is to understand what lies behind the development of symptoms in IBS (64).
An understanding of the role of *Blastocystis* in IBS is restricted by the ambiguity surrounding its pathogenicity (65). Nevertheless, symptoms that have been attributed to infection with *Blastocystis* are non-specific, IBS-like and include diarrhoea, abdominal pain, cramps or discomfort, and nausea (66,67). Furthermore, chronic excretion of *Blastocystis* with persistent symptoms has been reported (67).
Hussain et al. showed that IgG antibody levels to *Blastocystis* in patients with IBS were significantly higher compared with asymptomatic controls, demonstrating immune activation, and suggesting some association between *Blastocystis* and IBS (68). In a study by Yakoob et al. *Blastocystis* was more frequently demonstrated in the faecal samples of IBS patients (46 %) than the control group (7%) (69).
Giacometti et al. evaluated a possible link between *Blastocystis* infection and IBS and their findings support a link between the two(70) . In contrast, Tungtrongch et al. found no relationship between presence of *Blastocystis* in faeces and IBS diagnosis (31).
Some authors have suggested that an intestinal tract that is abnormal for any reason may provide conditions suitable for proliferation of *Blastocystis* (71,72). It is possible that *Blastocystis* infection is an indicator of intestinal dysfunction or resident intestinal flora disorders rather than a cause of IBS. Whenever *Blastocystis* is detected in stool samples of patients with IBS it does not necessary mean that the symptoms are due to this organism and other infective and non-infectious causes should be investigated (65). In summary, accumulating reports suggest an association between *Blastocystis* infection and IBS. However, it is unclear whether *Blastocystis* is a primary etiological agent in IBS, and it has been suggested that an abnormal intestinal situation like IBS may give an environment in which parasite numbers can increase (71).

1.6 Treatment of *Blastocystis*

Since pathogenesis of the parasite is controversial, antibiotics are seldom prescribed for *Blastocystis* infections. The mild nature of the disease and self-limitation of symptoms also make clinicians skeptical about prescription of chemotherapy against the parasite (73). Several drug trials and clinical studies suggest the efficacy of metronidazole against *Blastocystis* (73), but frequent reports of treatment failure make antiparasitic therapy against it even more

controversial (46). Chronic infections in which all other etiologies have been excluded, metronidazole is the treatment of choice (46). Several factors might influence treatment outcomes, including infection density, acquisition of mutations, resistance of certain developmental stages to metronidazole and, most importantly, subtype or strain-to-strain variation in drug susceptibility (46). In fact, in-vitro studies have suggested a varying response of different *Blastocystis* isolates to metronidazole (74).

The most commonly prescribed alternatives to metronidazole for *Blastocystis* infections are co-trimoxazole and paramomycin (73). Unfortunately, resistance to these drugs has also been reported in the parasite (73). There is a pressing need to identify alternative treatment option of *Blastocystis* infections. Major roadblocks in development of new treatments against the parasite are the absence of efficient drug resistance and susceptibility screening tools. A lack of knowledge concerning molecular mechanisms of *Blastocystis* antibiotic resistance as well as pathogenesis further complicates the situation. Since *Blastocystis* infections are predominantly reported in developing countries, interest and resources required to develop anti-*Blastocystis* treatment options are also limited.

1.7 Epidemiology and Prevalence

Blastocystis is reported to be one of the most common protozoans found in fecal samples of both symptomatic patients and asymptomatic individuals (75,76,77). There is a significant increase in prevalence reports which has helped us to better understand the distribution of genotypes, mode of transmission and pathogenicity aspects. *Blastocystis* has a worldwide distribution and findings of many surveys reported it to be most frequently isolated protozoan parasite (76,78-80).

Prevalence of *Blastocystis* infection is higher in developing countries than in developed countries (81) and occurrence as high as 60% were reported from some developing countries (78). Occurrence of *Blastocystis* varies from country to country. A low prevalence of 0.5% has been reported among asymptomatic healthy individuals in Japan (82). A moderate prevalence of 14-21% and 23% was reported in Thailand (83) and United States (84) respectively.

A high prevalence of 40.7% and 60% was reported in Philippines (80) and Indonesia (78) respectively. High incidences (36.9-44%) of *Blastocystis* were also observed in Thai military personnel (76,85). Prevalence of *Blastocystis* may vary widely within various geographical regions of the same country. In

Thailand, a prevalence of 0.8% and 45.2% was reported from Nan province (86) and Pathum Thani province (87) respectively.

Variations in the same geographical region may represent true differences between communities or living conditions. Nevertheless, these reported variations might be due to lack of a standardized diagnostic methodology and difficulty in identifying parasitic forms other than the common vacuolar form. Recent studies have used PCR-based approaches to further elucidate genotype information which has shed light on the distribution of *Blastocystis* genotypes in humans and animals. Studies have found that *Blastocystis* subtype 3 was the most common subtype among isolates from countries including Turkey (37), Greece (88), Singapore, Japan, Pakistan, Bangladesh, and Germany (39). In summary, studies suggest that there is no association between specific genotype and geographic origin; and due to its predominance in urbanized countries, subtype 3 is probably the subtype of human origin. It has been observed that humans with compromised health and poor hygiene are more susceptible to *Blastocystis* infections.

Blastocystis infections are also of special clinical interest to developed countries as millions of travelers going to developing countries are at risk of acquiring infection (90). *Blastocystis* infections are more common during hot weather and during the pre-monsoonal months (2). Based on current knowledge, it is generally accepted that *Blastocystis* is transmitted by the fecal-oral route. This assumption is strengthened by animal infection studies (26,91) and reports showing high prevalence of *Blastocystis* in population living in poor hygiene (92). Therefore, control measures should consist of good hygiene practices and community sanitary facilities. Because *Blastocystis* is generally regarded as a zoonotic parasite, animals and their fecal material represent a risk for human infection. Contamination of food, water, and environment by animal fecal material should be prevented. High prevalence of *Blastocystis* has been shown in pets particularly dogs and cats and it was suggested that these domestic animals could be an important source of infection to humans (93).

Routine antiparasitic treatment practice for pet animals may be useful to eliminate the parasite. Animal handlers must take additional precautions for their personal hygiene and may go for stool examination especially if experiencing any gastrointestinal symptoms. In unhygienic and high *Blastocystis* prevalence areas, sterilization of water is recommended. Currently, the best sterilization method is to boil water as chemical methods of water sterilization have not been extensively studied for *Blastocystis*. Travelers to high prevalence areas should ensure that they consume clean water and cooked food. *Blastocystis* has been found in sewage (94) and there is growing

evidence for waterborne transmissions (84) which makes it necessary to develop preventive measures to ensure water sanitation.

1.8 Diagnosis

Because of its uncertain pathogenesis, reasonable clinical significance is seldom given to *Blastocystis* infections. Generally, diagnosis and other important aspects of *Blastocystis* infections are not included in the curriculum of medical studies and thus diagnosis of *Blastocystis* remains a challenging task for a diagnostic laboratory. Although an experienced laboratory technician can perform diagnosis in direct fecal smears, most diagnostic laboratories do not have expertise on identification of this parasite and there is a need for training to enable identification of all forms of *Blastocystis* in fecal samples. Identification of *Blastocystis* in direct fecal smears is relatively difficult as the parasite can be confused with yeast, cyclospora, or fat globules. In the past, laboratory diagnosis of *Blastocystis* was based on the identification of vacuolar and granular forms in direct fecal specimens (95).

Direct microscopy of fecal specimens is performed by wet mounts with Lugol's iodine or permanent fixed smears with Giemsa, acid-fast, trichrome and Field's staining. Rather than the characteristic vacuolar form, the cyst form may predominate fecal samples. Cyst forms might be difficult to identify by direct microscopy because of their small size (3-5 µm) but these can be effectively concentrated by density-gradient methods (74).

Diagnostic labs should therefore include the fecal cyst form as an indicator of *Blastocystis* infection. Many researchers suggest that when all other known bacterial, viral or parasitic causes of symptoms are absent and *Blastocystis* is present in large numbers it should be treated as a pathogen. More than five organisms per high power field (×400 magnification) should be considered as a heavy infection. For confirmative diagnosis in stool samples, in vitro culture in Jones' medium is a method of choice (96). It was reported that in vitro culture of fecal samples was six times and twice more sensitive than direct fecal smears and trichrome staining methods respectively (97). However, it was also reported in this study that the in vitro culture method failed to detect some parasites suggesting that not all *Blastocystis* isolates can be readily cultured in laboratory. *Blastocystis* can be cultured in various mediums including Jone's medium, Boeck and Drbohlav's inspissated medium or diphasic agar slant medium with Jone's as a medium of choice for patient samples. Diphasic agar

slant medium was reported to be good for the culture of *Blastocystis* from pigs, cattle and chickens (98,99). In axenized cultures, cell densities of up to 2.5 ×107 can be achieved (100) and doubling time may vary from 6 to 23 h, depending on type of medium and isolate (101). Colony growth of *Blastocystis* has been shown on solid medium and cultures were viable for up to 2 weeks (102). Molecular approaches, particularly PCR-based diagnosis have been described for *Blastocystis* (103). PCR amplification using subtype specific primers is suggested to be useful for identifying and genotyping *Blastocystis* from patient samples. Knowledge of the genotype can be extremely valuable if certain *Blastocystis* genotypes are found to be more virulent than others.

A study has demonstrated that PCR-based detection of *Blastocystis* from fecal specimens is more sensitive than in vitro propagation (104). A sensitive and specific real-time light cycler PCR assay was developed to detect a 152 bp sequence in an uncharacterized region of the *Blastocystis* genome and 11 strains of *Blastocystis* from subtypes 1, 3, and 4 were with this method (105). Using this method, *Blastocystis* was detected in stool samples that were found *Blastocystis* negative during microscopy and conventional PCR. In addition, this method showed no cross-reactivity with other common gastrointestinal pathogens.

Other methods like enzyme-linked immunosorbent assay (ELISA) and immunofluorescence detection have not been comprehensively investigated for *Blastocystis*. Although development of monoclonal antibodies against *Blastocystis* has been reported (106), antigenic diversity of *Blastocystis* seems to be a limiting factor in the use of immunological methods.

Blastocystis infections have been reported to induce IgG and IgA responses in patients and detected by indirect fluorescent antibody test (IFA) and ELISA (68,107-110). ELISA titers ranged from 1:50-1:1,600 (108) and it was observed that high titers were associated with symptomatic infections of *Blastocystis* (68,107,108,110). In a recent study using ELISA, secretory IgA, serum IgA and serum IgG levels were detected in *Blastocystis* infected patients with and without clinical symptoms (110). It was found that serum from only symptomatic patients had significantly higher antibody levels. On the other hand, Kaneda et al. (109) reported asymptomatic patients with serum antibodies to *Blastocystis* and high levels were observed in chronic cases. Overall, it may be desirable to develop specific monoclonal antibodies against different genotypes and evaluate different serological assays for the diagnosis of *Blastocystis* infections.

Diagnosis of blastocystosis has been reported with the help of invasive diagnostic techniques like endoscopy but it has not been evaluated. *Blastocystis* colonization in the lower ileum and cecum of a patient was

detected in the microscopic examinations of the lumen fluids aspirated during endoscopy (111). As *Blastocystis* can be detected in feces and no characteristic intestinal lesions are associated with infection, invasive diagnostic techniques are not recommended for routine examinations. In brief, a number of methods have been described for the diagnosis of *Blastocystis*. Direct microscopy of stained fecal smears is useful and it should be supplemented with numbers of parasites observed per high power field to help clinicians ascertain parasitic load. For confirmatory diagnosis, microscopic examination should be supplemented by in vitro culture and/or PCR-based methods.

1.9 Aims and Objectives

In the present study, we analyzed all fecal specimens for which, in previously molecular diagnosis, we observed the presence of *Blastocystis* DNA. The stool specimens were collected from patients suspected of harboring intestinal parasites and attending to our center for tropical disease in two years (2014 – 2015). The aim of the study was to characterize *Blastocystis* genotype distribution in our patients, evaluating the influence of different geographical origins, the dynamics of mixed STs and the association with other parasite co-infections, in order to explore the prevalence and the diversity of *Blastocystis* infections in our cohort population. A description of symptoms found in patients carrying different subtypes or co-infection combinations has been reported.

CHAPTER 2:

MATERIALS AND METHODS

2. Materials and Methods

2.1 Samples selection

This retrospective study was performed at the Center for Tropical Diseases of Sacro Cuore-Don Calabria Hospital in Negrar (Verona), a referral center for tropical and parasitic infections in Italy. In this center receives patients coming from all Italian regions, people who need medical assistance and immigrants lived in temporary accommodation centers.
The sample identification code was retrieved from the electronic archive of the molecular parasitology laboratory searching among all specimens collected from January 2014 to December 2015. In this period, a total of 1778 fecal samples were screened by three separate multiplex Real time polymerase chain reaction (Rt-PCR) for detecting *Entamoeba histolytica - Entamoeba dispar - Cryptosporidium spp, Giardia intestinalis - Dientamoeba fragilis - Blastocystis spp, Strongyloides stercoralis - Schistosoma spp - Hymenolepis nana*. Among these, 756 samples were subjected to the molecular test for the presence of *Blastocystis spp*.
260 Samples positive for *Blastocystis spp*. were further analyzed for the 4 most frequent human subtypes (ST1, ST2, ST3 and ST4) molecular characterization, as described in the following paragraphs. The samples investigated for *Blastocystis* subtypes were divided into three groups based on the presence of gastrointestinal symptoms, itching or absence of symptoms. The geographical origin of the patients was also considered (Italians, non-Italian Europeans, Africans, Asians and South Americans).

2.2 Fecal samples collection and DNA extraction

According to the routine procedure of our laboratory, fecal samples collected for molecular test were stored in 95% ethanol prior to be processed. DNA extraction was performed as previously described (112). 200 mg of each stool sample was stored at –20 °C overnight in a solution of PBS 1X with 2% of polyvinylpolypyrrolidone (PvPP) (Sigma-Aldrich, Milan, Italy). In each sample, Phocine Herpes Virus type-1 (PhHV-1, kindly provided by Dr. Pas S., Erasmus MC, Department of Virology , Rotterdam) was added within the S .T.A.R. buffer (Roche, Milan, Italy), serving as an internal control for the isolation and amplification steps. All the samples were then frozen and boiled for 10 min at 100° C. The DNA was extracted by MagnaPure LC.2 instrument (Roche

Diagnostic, Monza, Italy), using the DNA isolation kit I (Roche). The DNA was eluted in a final volume of 100 µl. DNA samples were appropriately labelled and stored at -20°C for subsequent molecular tests.

2.3 Real-Time PCR

Each DNA was amplified by Real-Time PCR (CFX96-Biorad) as described by Stensvold et al. (113). The realtime is a multiplex PCR able to detect at the same time the possible presence of 3 protozoa (*D. fragilis*-*G. intestinalis*-*Blastocystis spp*). Moreover, this multiplex PCR detect the PhHV DNA; it is an exogenous DNA added to the samples before to start the extraction. It is necessary to verify the good performance of extraction and the presence of inhibitory for the polymerase enzyme. Also, in each PCR run are presents 2 positive controls (high and low DNA quantity) and a negative control.

2.4 *Blastocystis* subtype analysis

Nested-PCR was performed according to the protocol described by Scanlan et al (114). with minor modifications. First step PCR was performed to provide a *Blastocystis* specific 18S rDNA template for each of the subsequent ST-specific PCRs (ST1, ST2, ST3, and ST4). RD5, BhRDr, ST1-F, ST2-F, ST3-F, ST4-F primers sequences were retrieved from (114) and PCR was performed using iTaq DNA polymerase (Bio-Rad, Milan, Italy) in 50 µL of reaction volume, according to the manufacturer's instructions. The following cycling conditions were used for the first step PCR: initial denaturation 95°C for 3 min, 30 cycles at 94°C for 1 min, 59°C for 1 min, 72°C for 1 min, final elongation 72°C for 5 min. 5 µL of DNA sample was used (Table 1). The ST-specific PCRs were performed as follows: initial denaturation 95°C for 3 min, 35 cycles at 94°C for 30 sec, Tannealing primers for 30 sec, 72°C for 1 min, final elongation 72°C for 5 min. The following Tannealing were used: 56° C for ST1 and ST2, 48° C for ST3 and ST4.
1 µL the initial PCR product was used per each reaction (Table 2). A no-template control was always included in each PCR run. PCR products were analyzed by 2.5 % agarose gel electrophoresis, to detect the specific DNA bands. 2 examples of gel images are reported in figure1 and figure 2.

2.5 Statistical analysis

Descriptive statistics were used to analyze the characteristics of the entire cohort and separately for each continent of origin of patients. The statistical analysis was performed on data collected in our electronic database. Data entry of each specimens cover: ID, age, sex, nationality, symptoms, *Blastocystis ST*, other parasites (*Entamoeba histolytica, Entamoeba dispar, Giardia intestinalis, Dientamoeba fragilis, Strongyloides stercoralis, Schistosoma spp, Hymenolepis nana*). We then investigated on associations between all patients' characteristics through univariate logistic regression models and parametric and non-parametric statistical test such as Chi-Squared test. The data were statistically assessed using SPSS 16.0. Chi square test and percentages were used for data analysis, the *P* values below or equal to 0.05 were regarded as significant.

CHAPTER 3:

RESULTS

3. Results

3.1 Patients' features

In a group of 1778 samples subjected to molecular tests for the investigation of intestinal parasite infection, 756 were tested for *Blastocystis*. Among these, we identified 260 (34.4 %) positive samples for *Blastocystis* spp. From a total of 260 persons infected with *Blastocystis sp*. 159 (61.2%) were male and 101 (38.8%) were female. Our study population was composed by people of different geographical origins, with Italians 115 (44.2%) and Africans 86 (33.1%) being the predominant ones; other patiens were from South Americans 26 (10%), Asians 21 (8.1%) and non-Italian Europeans 12 (4.6%) (Table 3).

According to table 4, 29.2% of subjects were more than 50 years old. Prevalence in other age groups were 10.8% at below 10 years old, 12.7% at 11-20, 19.2% at 21-30, 14.2% at 31-40 and 13.9% at 41-50 years old.

3.2 Characterization of *Blastocystis* subtypes

We further analyzed the *Blastocystis* positive subjects by a specific Nested-PCR assay (114), in order to evaluate the presence of the 4 most prevalent *Blastocystis* STs and we were able to characterize a total of 260 samples. Obtained results are reported in Table 5. In particular, we found that ST3 and ST1 were the most frequent subtypes (single subtype carriers), with a prevalence of (24.6%) and (22.7%) respectively, also ST2 and ST4 were detected in the (8.1%) and (7.3%) of samples. The most prevalent group was composed by subjects infected by more than one *Blastocystis* ST (37.3%). Among mixed STs co-infections, the most frequent was clearly the ST1-ST3 (16.9%) (Table 5). We found multiple double infection, ST3-ST4 (3.8%), ST1-ST2 (2.3%), ST2-ST3 (1.9%), ST1-ST4 (0.4%) and ST2-ST4 (0.8%). Although with a lower frequency, triple STs infections were also detected, like ST1-ST3-ST4 (4.6%) and ST1-ST2-ST3 (4.2%) and ST2-ST3-ST4 (1.6%), and finally (0.8%) of quadruple infection by ST1-ST2-ST3-ST4 (Table 5).

Table 6 shows the prevalence of subtypes of *Blastocystis* according to gender that 26.4% of male were infected with ST1 also 28.7% of female were infected

with ST3. No significant correlation was found between patients' gender and *Blastocystis* subtypes infection (*P*= 0.381).

Analyzing the clinical phenotype of our study, we found that 48.1% % of *Blastocystis* positive subjects presented GI symptoms (abdominal pain, diarrhea, irritable bowel syndrome) (Table 7), 18.8 % reported itching (*P*= 0.074) (Table 8). No association between GI symptoms and gender has been observed (*p* = 0.310) (Table 7).

A slightly correlation (*P*=0.038) was observed between the gender and infected with *Blastocystis* and other parasites (Table 9).

Table 10 shows distribution of *Blastocystis* subtypes among age groups.

25.4% of individuals that were infected with ST1, had more than 50 years old. Also in the individuals infected with ST2, 33.3% were observed in the 21-30 years old group (Table 10). There was correlation between age groups and *Blastocystis* subtypes (*P*= 0.044).

Among age groups 32.8% of more than 50 years old presented GI symptoms. 9.6% of Subjects less than 10 years old, also 10.4% of 11-20, 20.8% of 21-30, 14.4% of 31-40 years old have GI symptoms. (*P*= 0.660) (Table 11). 40.8% of more than 50 years old reported Itching (*P*=0.080) (Table12).

According to the Table 13, 78.6% of subjects less than 10 years old that, in addition to *Blastocystis* were infected with other parasites. Between the age groups and infected with other parasites were correlation (*P*= 0.008).

Table 14 shows Frequency of individuals infected with *Blastocystis* subtypes According to Nationality (*P*= 0.125).

According to the correlation between Nationality and GI Symptoms, 58.3% of the Italian subjects presented GI symptoms. There was significant between GI sypmtoms and Nationality (*P*=0.031) (Table 15).

Frequency of individuals infected with *Blastocystis* sp. according to Nationality and Itching shows 53.1% of subjects were Italian (*P*=0.508) (Table 16).

Frequency of individuals infected with *Blastocystis* sp. only and *Blastocystis* with other parasites According to Nationality shows there was significant

between Nationality and infected with other parasites (*P*= 0.009). 76.2% of Asian subjects infected with *Blastocystis* sp. and other parasites (Table 17).

Among single subtype carriers, 59.3% of individuals infected with ST1 presented GI symptoms. 33.3% of ST2, 51.6% of ST3 and 47.4% of ST4 subjects presented GI symptoms (*P*= 0.353) (Table 18).

According to the table 19, 50.7% of subjects infetcted with *Blastocystis* only have GI symptoms also 45.2% of subjects infected with *Blastocystis* and other parasites were presented GI symptoms (*P*= 0.222) (Table 19).

In patients carrying *Blastocystis*, we analyzed also the presence of other co-infecting parasites: *Entamoeba histolytica, Entamoeba dispar, Giardia intestinalis, Dientamoeba fragilis, Strongyloides stercoralis, Cryptosporidium spp, Schistosoma spp, Hymenolepis nana.* We analyzed the possible association between the presence of GI symptoms and co-infection with different parasites, but no statistically significant difference has been detected (*P* = 0.754) between samples infected only by *Blastocystis* (54.4 % presenting symptoms, 68/134) and samples with multiple parasite coinfections (45.2 % presenting symptoms, 57/126). Table 20 reports the results on the association between symptoms and *Blastocystis*, both with and without other parasitic infections (*P*= 0.754).

Table 21 shows distribution of subtypes of *Blastocystis* according to infected with *Blastocystis* only or infected with other parasites (*P*= 0.401). 27.6% of subjects infected with *Blastocystis* only reported ST3 and 26.2% of subjects infected *Blastocystis* and other parasites reported ST1.

CHAPTER 4

DISCUSSION AND CONCLUSIONS

4. DISCUSSION AND CONCLUSION

4.1 Discussion

As a result of our retrospective study, *Blastocystis* emerged as the most frequent parasite in samples tested for intestinal parasite infections at our Centre for Tropical Diseases, confirming previous reports indicating that the prevalence of *Blastocystis* infection is higher than that of other intestinal parasites (115). We could characterize the *Blastocystis* subtype for 260 positive samples. Our analysis highlighted that ST3 was the most prevalent subtype in Italians of our regional area, and this result reflected previous report by another Italian group (116). ST1 was also present with a slightly lower frequency.

The findings showing the dominance of ST3 are similar to most previous studies in Europe and Asia, such as Jamtemtor's study in Thailand that reported ST3 as the most dominant subtype (57.1%), followed by ST1 (21.4%), ST7 (17.9%) and ST6 (3.6%) (117) Similarly, Wong et al. study in Singapore found ST3 to be the most dominant subtype (78%), followed by ST1 (22%) (89). Boondit et al. study also reported ST3 as the most dominant subtype (76%), followed by ST1 (20%) (118). Meloni et al. in Italy found the following ST distribution: ST3 (47.1%), ST2 (20.6%), ST4 (17.7%), ST1 (8.8%), and ST7 and ST8 (2.9%) (119). A study by Dogruman et al. reported similar results, with ST3 being the most dominant in the symptomatic (59.3%) and asymptomatic groups (48.5%), followed by ST2 with 15.3% and 33.3% and ST1 with 20.3% and 15.2%. Similar subtype prevalence rates were also reported by Ozyurt et al. in Turkey and other countries such as China, Germany, Japan, and Denmark (40).

A different result was reported in a study by Awatif et al. in Libya where ST1 was the most dominant subtype in outpatients (51.1%), followed by ST2 (24.4%) and ST3 (17.8%) (120). An alternate result was also reported in a study by Dominguez et al. in Spain where ST4 was the most dominant subtype at 94.1%, followed by ST1 (2%) and ST2 (3.9%) (42). Souppart et al. stated that *Blastocystis* infection may not link to certain subtypes but to risk factors in infection transmission, including environmental factors (transmission route and source of contamination), parasite factors (pathogenic potential and zoonosis), and host factors (genotype, immunity, and age) (121).

In our study, we had the opportunity to compare subjects coming from different geographical areas. In Africans, the second predominant group, ST1

and ST3 were the most prevalent subtypes, present with equal frequencies. Forsell and colleagues reviewed the subtype prevalence in Africa and, depending on the considered country, they reported ST1 and ST3 as actually the most prevalent subtypes (122). For sake of clarity, we have to consider that STs distribution in our population could be also influenced by the time of permanence in Italy of the foreign subjects, but this variable was not retrievable in our retrospective study. This aspect could also explain the presence of ST4 in few African individuals, since ST4 has been reported as being absent in African populations by several studies (reviewed in (122,123)).

Anyway, globally, our cohort confirmed that ST3 and ST1 were the most prevalent subtypes. An intriguing result of our analysis was the frequent presence of mixed subtype infections, across all the geographical areas. This observation confirms how a subtype-specific PCR assay could highlight a frequent presence of mixed subtype co-infections (114). This aspect has received little attention in the past, since it was often underestimated by methodological limits and considered just an incidental finding. Recently, the presence of mixed subtype infections has been highlighted as an important characteristic to be studied, in order to explore the diversity and distribution of this parasite in the human gut (114). Applying the state-of-the-art subtype-specific method (114) we were able to detect mixed infection in the 37.3 % of our cases, with ST1-ST3 being the most common mixed subtype combination, as already observed in other studies (reviewed in (124)). This data could indicate that the mutual presence of different subtypes could be a successful cooperation strategy for host colonization by *Blastocystis spp.* The same concept could also be applied for the presence of multiple parasite co-infections: the association between *Blastocystis* and *D. fragilis* could also indicate a cooperative interaction between the two protozoa. Association between *Blastocystis spp* and other parasites has already been observed in literature (e.g. with *G. intestinalis* (122). and in particular with *D. fragilis* (125)) but no conclusive association with symptoms has been highlighted.

Although we did not detect any statistically significant correlation between any STs and symptoms (probably due to the limited number of samples in our study, or to the intrinsic genetic variability and/or immunological factors), neither in mixed subtypes presence or other parasite co-infections, we observed a slightly higher proportion (48.1 %) of symptomatic versus asymptomatic subjects in *Blastocystis* positive subjects, with a prevalence of ST1 and ST3 in patients with GI symptoms, while ST2 seems to have a lower impact; moreover, the presence of both *Blastocystis* and *D. fragilis,* in our

study, seems to increase the percentage of patients with GI symptoms, respect to *Blastocystis* alone or in co-infection with other tested parasites. Anyway, since no statistically significant clue has been obtained, additional data are needed to confirm these indications.
This study has several limitations, mainly related to the retrospective study design. In particular, symptom reporting may not be sufficiently accurate and this might be a further reason for the lack of statistically significant correlation between the molecular findings and the clinical characteristics.

4.2 Conclusions

Our study confirms that *Blastocystis* infected subjects present a highly variable symptomatology, that is not completely explicable by the different subtypes presence or by other parasite co-infections; although the variability of the samples could require a higher number of observations to reach a statistically significant indication, our data support the hypothesis that the host condition is the key aspect that can influence the pathogenicity of *Blastocystis spp* colonization. Future studies on patients' immunological conditions and gut microbiota, associated to *Blastocystis* infection, could fill the gap of information to explain the cause of the observed variable pathogenic penetrance of this parasite.

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List of Tables

Table 1: Reagents used in the first PCR

Reagent	Final concentration	1 Sample(µl)
Buffer PCR 10X	1X	5
Mg++ 50 mM	2mM	2
dNTPs 10mM	200mM	1
Primer RD5 5µM	0.5µm	5
Primer BhRDr 5µM	0.5µM	5
Taq pol 5U/50µl	1.25 U/50 µl	0.25
H2o	_	26.75
DNA	_	5
Total volume	_	50

Table 2: Reagents used in the second PCR

Reagent	Final concentration	1 Sample(µl)
Buffer PCR 10X	1X	2.5
Mg++ 50 mM	2mM	1
dNTPs 10mM	200mM	0.5
Primer ST1,ST2,ST3,ST4 5µM	0.5µm	2.5
Primer BhRDr 5µM	0.5µM	2.5
Taq pol 5U/50µl	1.25 U/50 µl	0.125
H2o	_	14.875
Firs PCR Product	1/50 l' PCR	1
Total volume	_	25

Table 3: Frequency of individuals infected with *Blastocystis sp.* According to Nationality and Gender.

Nationality	Gender	*Blastocystis sp.*		Total	
		N	%	n	%
Italians	Male	56	48. 7	115	44.2
	Female	59	51.3		
Africans	Male	69	80.2	86	33.1
	Female	17	19.8		
Asians	Male	15	71.4	21	8.1
	Female	6	28.6		
south Americans	Male	10	38.5	26	10
	Female	16	61.5		
[a] non-Italian Europeans	Male	9	75	12	4.6
	Female	3	25		
Total	Male	159	61.2	260	100
	Female	101	38.8		

[a] European patients are intended coming from Europe, outside Italy. Namely, from Germany, Romania, Switzerland and European Russia

Table 4: Frequency of individuals infected with *Blastocystis sp.* According to Age groupes and Gender.

Age Groups	Gender	*Blastocystis sp.*		Total	
		n	%	n	%
<10	Male	14	50	28	10.8
	Female	14	50		
11-20	Male	29	87.9	33	12.7
	Female	4	12.1		
21-30	Male	36	72	50	19.2
	Female	14	28		
31-40	Male	16	43.2	37	14.2
	Female	21	56.8		
41-50	Male	18	50	36	13.9
	Female	18	50		
>50	Male	46	60.5	76	29.2
	Female	30	39.5		
Total	Male	159	61.2	260	100
	Female	101	38.8		

Table 5: The distribution of subtypes of *Blastocystis* among 260 subjects.

Subtypes	n	%
ST1	59	22.7
ST2	21	8.1
ST3	64	24.6
ST4	19	7.3
ST1,ST2	6	2.3
ST1,ST3	44	16.9
ST1,ST4	1	0.4
ST2,ST3	5	1.9
ST2,ST4	2	0.8
ST3,ST4	10	3.8
ST1,ST2,ST3	11	4.2
ST1,ST3,ST4	12	4.6
ST2,ST3,ST4	4	1.6
ST1,ST2,ST3,ST4	2	0.8

Table 6: Prevalence of subtypes of *Blastocystis* according to Gender.

Subtypes / Gender	ST1 n(%)	ST2 n(%)	ST3 n(%)	ST4 n(%)	ST1-ST2 n(%)	ST1-ST3 n(%)	ST1-ST4 n(%)	ST2-ST3 n(%)	ST2-ST4 n(%)	ST3-ST4 n(%)	ST1-ST2-ST3 n(%)	ST1-ST3-ST4 n(%)	ST2-ST3-ST4 n(%)	ST1-ST2-ST3-ST4 n(%)	Total
Male	42 (26.4%)	12 (7.6%)	35 (22%)	9 (5.7%)	4 (2.5%)	23 (14.5%)	1 (0.6%)	5 (3.1%)	1 (0.6 %)	8 (5%)	7 (4.4%)	7 (4.4 %)	3 (1.9%)	2 (1.3%)	159 (100%)
Female	17 (16.8%)	9 (8.9%)	29 (28.7%)	10 (9.9%)	2 (2%)	21 (20.8%)	0 (0%)	0 (0%)	1 (1%)	2 (2%)	4 (4%)	5 (4.9 %)	1 (1%)	0 (0%)	101 (100%)
Totale	59 (22.7%)	21 (8.1%)	64 (24.6%)	19 (7.3%)	6 (2.3%)	44 (16.9%)	1 (0.4%)	5 (2%)	2 (0.8 %)	10 (3.8%)	11 (4.2%)	12 (4.6 %)	4 (1.5%)	2 (0.8%)	260 (100%)

P = 0.381

Table 7: Frequency of individuals infected with *Blastocystis sp.* According to Gender and Gastrointestinal Symptoms.

Gender	GI n(%)		Total
	No	Yes	
Male	85(53.5%)	74(46.5%)	159(100%)
Female	50(49.5%)	51(50.5%)	101(100%)
Total	135(51.9%)	125(48.1%)	260(100%)

P = 0.310

Table 8: Frequency of individuals infected with *Blastocystis sp.* According to Gender and Itching.

Gender	Itching(n)		Total
	No	Yes	
Male	134(84.3%)	25(15.7%)	159
Female	77(76.2%)	24(23.8%)	101
Total	211(81.2%)	49(18.8%)	260(100%)

P = 0.074

Table 9: Frequency of individuals infected with *Blastocystis sp.* only and *Blastocystis* with other parasites According to Gender.

Gender	*Blastocystis* only n(%)	*Blastocystis* and other parasites n(%)	Total
Male	73 (45.9%)	86 (54.1%)	159 (100%)
Female	61 (60.4%)	40 (39.6%)	101 (100%)
Total	134(51.5%)	126(48.5%)	260(100%)

P = 0.038

Table 10: Distribution of *Blastocystis* subtypes among Age groups

Subtypes / Age Groups	ST1 n(%)	ST2 n(%)	ST3 n(%)	ST4 n(%)	ST1-ST2 n(%)	ST1-ST3 n(%)	ST1-ST4 n(%)	ST2-ST3 n(%)	ST2-ST4 n(%)	ST3-ST4 n(%)	ST1-ST2-ST3 n(%)	ST1-ST3-ST4 n(%)	ST2-ST3-ST4 n(%)	ST1-ST2-ST3-ST4 n(%)	Total
<10	9 (15.3 %)	1 (4.8%)	5 (7.8%)	3 (15.8%)	0 (0%)	6 (13.6 %)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (9.1%)	3 (25%)	0 (0%)	0 (0%)	28
11-20	12 (20.3 %)	1 (4.8%)	3 (4.7%)	3 (15.8%)	0 (0%)	6 (13.6 %)	1 (100 %)	0 (0%)	0 (0%)	1 (10%)	1 (9.1%)	2 (16.7%)	1 (25%)	2 (100%)	33
21-30	11 (18.6 %)	7 (33.3 %)	9 (14.1 %)	1 (5.3%)	4 (66.7 %)	8 (18.2 %)	0 (0%)	0 (0%)	0 (0%)	3 (30%)	3 (27.3%)	3 (25%)	1 (25%)	0 (0%)	50
31-40	8 (13.6 %)	1 (4.8%)	14 (21.9 %)	2 (10.5%)	0 (0%)	4((9.1%)	0 (0%)	1 (20%)	2 (100 %)	1 (10%)	2 (18.2%)	1 (8.3%)	1 (25%)	0 (0%)	37
41-50	4 (6.8%)	5 (23.8 %)	12 (28.8 %)	3 (15.8%)	0 (0%)	7 (15.9 %)	0 (0%)	1 (20%)	0 (0%)	0 (0%)	2 (18.2%)	1 (8.3%)	1 (25%)	0 (0%)	36
>50	15 (25.4 %)	6 (28.6 %)	21 (32.8 %)	7 (36.8%)	2 (33.3 %)	13 (29.5 %)	0 (0%)	3 (60%)	0 (0%)	5 (50%)	2 (18.2%)	2 (16.7%0	1 (25%)	0 (0%)	76
Total	59 (100%)	21 (100%)	64 (100%)	19 (100%)	6 (100 %)	44 (100 %)	1 (100 %)	5 (100 %)	2 (100 %)	10 (100 %)	11 (100%)	12 (100%)	4 (100%)	2 (100%)	260

P = 0.044

Table 11: Distribution of *Blastocystis sp* among Age groups according to Gastrointestinal Symptoms.

Age Groups	GI n(%)		Total
	No	Yes	
<10	16(11.8%)	12(9.6%)	28
11-20	20(14.8%)	13(10.4%)	33
30-21	24(17.8%)	26(20.8%)	50
31-40	19(14.1%)	18(14.4%)	37
41-50	21(15.6%)	15(12%)	36
>50	35(25.9%)	41(32.8%)	76
Total	135(100%)	125(100%)	260

P= 0.660

Table 12: Distribution of *Blastocystis sp* among Age groups according to Itching.

Age Groups	Itching n(%)		Total
	No	Yes	
<10	27(12.8%)	1(2.1%)	28
11-20	28(13.3%)	5(10.2%)	33
30-21	43(20.4%)	7(14.3%)	50
31-40	27(12.8%)	10(20.4%)	37
41-50	30(14.2%)	6(12.2%)	36
>50	56(26.5%)	20(40,8%)	76
Total	211(100%)	49(100%)	260

P= 0.080

Table 13: Frequency of individuals infected with *Blastocystis sp.* only and *Blastocystis* with other parasites According to Age Groups.

Age Groups	*Blastocystis* only n(%)	*Blastocystis* and other parasites n(%)	Total
<10	6(21.4%)	22(78.6%)	28(100%)
11-20	13(39.4%)	20(60.6%)	33(100%)
30-21	28(56%)	22(44%)	50(100%)
31-40	23(62.1%)	14(37.9%)	37(100%)
41-50	20(55.6%)	16(44.4%)	36(100%)
>50	44(57.9%)	32(42.1%)	76(100%)
Total	134(51.5%)	126(48.5%)	260(100%)

P= 0.008

Table 14: Frequency of individuals infected with *Blastocystis* subtypes According to Nationality.

Subtypes(n) / Nationality	ST1 n(%)	ST2 n(%)	ST3 n(%)	ST4 n(%)	ST1-ST2 n(%)	ST1-ST3 n(%)	ST1-ST4 n(%)	ST2-ST3 n(%)	ST2-ST4 n(%)	ST3-ST4 n(%)	ST1-ST2-ST3 n(%)	ST1-ST3-ST4 n(%)	ST2-ST3-ST4 n(%)	ST1-ST2-ST3-ST4 n(%)	Total
Italian	24 (40.7%)	12 (57.1%)	25 (39.1%0	14 (73.7%)	2 (33.3%)	19 (43.2%)	0 (0%)	3 (60%)	1 (50%)	6 (60%)	3 (27.3%)	5 (41.7%)	1 (25%)	0 (0%)	115
African	24 (40.7%)	4 (19%)	22 (34.4%)	4 (21.1%)	4 (66.7%)	15 (34.1%)	1 (100%)	1 (20%)	1 (50%)	1 (10%)	2 (18.2%)	4 (33.3%)	1 (25%)	2 (100%)	86
Asian	6 (10.2%)	0 (0%)	6 (9.4%)	0 (0%)	0 (0%)	5 (11.4%)	0 (0%)	0 (0%)	0 (0%)	1 (10%)	2 (18.2%)	1 (8.3%)	0 (0%)	0 (0%)	12
South Americans	4 (6.8%)	1 (4.8%)	9 (14.1%)	1 (5.3%)	0 (0%)	5 (11.4%)	0 (0%)	0 (0%)	0 (0%)	2 (20%)	3 (27.3%)	0 (0%)	1 (25%)	0 (0%)	26
non-Italian Europeans	1 (1.7%)	4 (19%)	2 (3.1%)	0 (0%)	0 (0%)	0 (0%)	0 (0%)	1 (20%)	0 (0%)	0 (0%)	1 (9.1%)	2 (16.7%)	1 (25%)	0 (0%)	12
Totale	59 (100%)	21 (100%)	64 (100%)	19 (100%)	6 (100%)	44 (100%)	1 (100%)	5 (100%)	2 (100%)	10 (100%)	11 (100%)	12 (100%)	4 (100%)	2 (100%)	260

P= 0.125

Table 15: Frequency of individuals infected with *Blastocystis sp.* According to Nationality and Gastrointestinal Symptoms.

Nationality	GI n(%)		Total
	No	Yes	
Italian	48(41.7%)	67(58.3%)	115(100%)
African	49(57%)	37(43%)	86(100%)
Asian	14(66.7%)	7(33.3%)	21(100%)
South Americans	18(69.2%)	8(30.8%)	26(100%)
non-Italian Europeans	6(50%)	6(50%)	12(100%)
Total	135(51.9%)	125(48.1%)	260(100%)

P= 0.031

Table 16: Frequency of individuals infected with *Blastocystis sp.* According to Nationality and Itching.

Nationality	Itching n(%)		Total
	No	Yes	
Italian	89(42.2%)	26(53.1%)	115
African	73(34.6%)	13(26.5%)	86
Asian	18(8.5%)	3(6.1%)	21
South Americans	20(9.5%)	6(12.2%)	26
non-Italian Europeans	11(5.2%)	1(2.1%)	12
Total	211(100%)	49(100%)	260

P= 0.508

Table 17: Frequency of individuals infected with *Blastocystis sp.* only and *Blastocystis* with other parasites According to Nationality.

Nationality	*Blastocystis* only n(%)	*Blastocystis* and other parasites n(%)	Total
Italian	71(61.7%)	44(38.3%)	115(100%)
African	42(48.7%)	44(51.3%)	86(100%)
Asian	5(23.8%)	16(76.2%)	21(100%)
South Americans	12(46.2%)	14(53.8%)	26(100%)
non-Italian Europeans	4(33.3%)	8(66.7%)	12(100%)
Total	134(51.5%)	126(48.5%)	260(100%)

P= 0.009

Table 18: Frequency of individuals infected with *Blastocystis* subtypes according to have GI symptoms.

Subtypes(n)		ST1 n(%)	ST2 n(%)	ST3 n(%)	ST4 n(%)	ST1-ST2 n(%)	ST1-ST3 n(%)	ST1-ST4 n(%)	ST2-ST3 n(%)	ST2-ST4 n(%)	ST3-ST4 n(%)	ST1-ST2-ST3 n(%)	ST1-ST3-ST4 n(%)	ST2-ST3-ST4 n(%)	ST1-ST2-ST3-ST4 n(%)	Total
GI	No	24 (40.7%)	14 (66.7%)	31 (48.4%)	10 (52.6%)	4 (66.7%)	28 (63.6%)	0 (0%)	2 (40%)	2 (100%)	7 (70%)	6 (54.5%)	4 (33.3%)	2 (50%)	1 (50%)	159
	Yes	35 (59.3%)	7 (33.3%)	33 (51.6%)	9 (47.4%0	2 (33.3%)	16 (36.4%)	1 (100%)	3 (60%)	0 (0%)	3 (30%)	5 (45.5%)	8 (66.7%)	2 (50%)	1 (50%)	101
Totale		59 (100%)	21 (100%)	64 (100%)	19 (100%)	6 (100%)	44 (100%)	1 (100%)	5 (100%)	2 (100%)	11 (100%)	11 (100%)	12 (100%)	4 (100%)	2 (100%)	260

P= 0.353

Table 19: Frequency of individuals infected with *Blastocystis sp.* only and *Blastocystis* with other parasites According to have GI symptoms.

		Blastocystis only n(%)	*Blastocystis* and other parasites n(%)	Total
GI	No	66(49.3%)	69(54.8%)	135
	Yes	68(50.7%)	57(45.2%)	125
Total		134(100%)	126(100%)	260

P= 0.222

Table 20: Frequency of identified parasites in subjects in our study according to have GI symptoms

	GI n(%)		Total
	No	Yes	
Blastocystis only	66(48.9%)	68(54.4%)	134
Blastocystis - E. dispar	11(8.2%)	11(8.8%)	22
Blastocystis - D. fragilis	26(19.3%)	27(21.6%)	53
Blastocystis - S. stercoralis	7(5.2%)	2(1.6%)	9
Blastocystis - G. intestinalis	4(3%)	2(1.6%)	6
Blastocystis- Schistosoma spp	6(4.5%)	3(2.4%)	9
Blastocystis- E. histolytica	1(0.7%)	0(0%)	1
Blastocystis- H. nana	2(1.5%)	0(0%)	2
Blastocystis- E. dispar- D. fragilis	3(2.2%)	4(3.2%)	7
Blastocystis- E. dispar- S. stercoralis	2(1.5%)	1(0.8%)	3
Blastocystis- E. dispar- G. intestinalis	1(0.7%)	1(0.8%)	2
Blastocystis- D. fragilis- S. stercoralis	2(1.5%)	0(0%)	2
Blastocystis- D. fragilis- G. intestinalis	1(0.7%)	1(0.8%)	2
Blastocystis- D. fragilis- E. histolytica	1(0.7%)	0(0%)	1
Blastocystis- D. fragilis- Schistosoma spp	0(0%)	1(0.8%)	1
Blastocystis- D. fragilis- H. nana	0(0%)	1(0.8%)	1
Blastocystis- G. intestinalis- H. nana	1(0.7%)	1(0.8%)	2
Blastocystis- E. dispar- D. fragilis- S. stercoralis	1(0.7%)	1(0.8%)	2
Blastocystis- E. dispar- D. fragilis- G. intestinalis	0(0%)	1(0.8%)	1
Totaal	135(100%)	125(100%)	260

P= 0.754

Table 21: Distribution of *Blastocystis sp* between individuals infected with *Blastocystis sp*. only and coinfected with other parasites.

Subtypes	*Blastocystis* only n(%)	*Blastocystis* and other parasites n(%)	Total
ST1	26(19.4%)	33(26.2%)	59
ST2	9(6.7%)	12(9.5%)	21
ST3	37(27.6%)	27(21.4%)	64
ST4	10(7.5%)	9(7.1%)	19
ST1-ST2	2(1.5%)	4(3.2%)	6
ST1-ST3	21(15.7%)	23(18.3%)	44
ST1-ST4	0(0%)	1(0.8%)	1
ST2-ST3	3(2.2%)	2(1.6%)	5
ST2-ST4	1(0.7%)	1(0.8%)	2
ST3-ST4	7(5.2%)	3(2.4%)	`10
ST1-ST2-ST3	6(4.5%)	5(4%)	11
ST1-ST3-ST4	10(7.5%)	2(1.6%)	12
ST2-ST3-ST4	2(1.5%)	2(1.67%)	4
ST1-ST2-ST3-ST4	0(0%)	2(1.6%)	2
Total	134(100%)	126(100%)	260

P= 0.401

List of Figures

Figure 1: 2.5% agarose gel electrophoresis of first Nested PCR Products.

Lane 1 displays 100 bp DNA Step Ladder (Sigma), Lanes 2-4 are first step PCR product (607 bp)

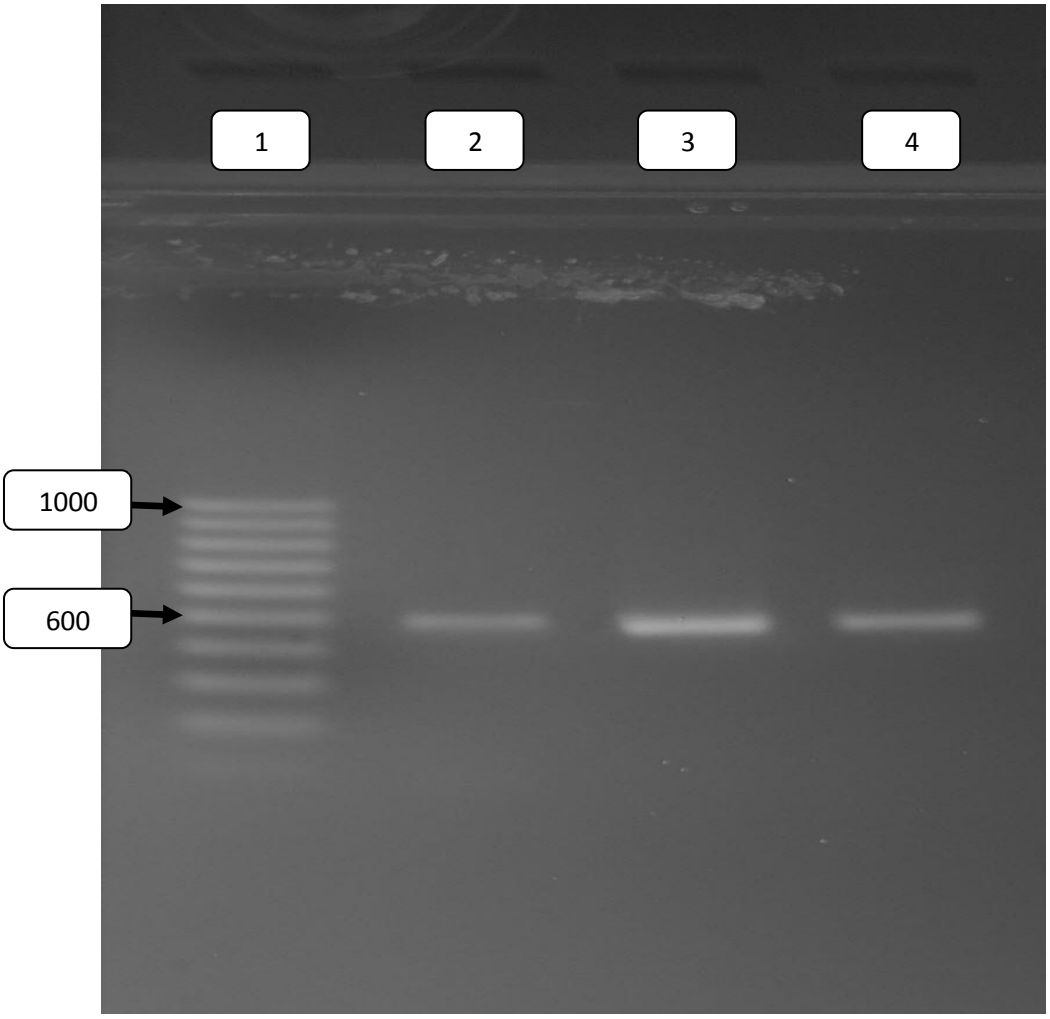

Figure 2: Nested PCR for *Blastocystis* genotype detection on agarose gel.

From left to right, lane 1 displays 50 bp DNA Step Ladder (Sigma), lane 2 is negative control, lane 3 displays first step PCR product (607 bp) and lines 4, 5, 6, and 7 display second step PCR products, respectively: ST1(427 bp), ST2(459 bp), ST3(433 bp) and ST4(399 bp).