Detection of risk factors associated to *Blastocystis hominis* infection

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Master Thesis

Master in Zoonoses and One Health

Facultat de Veterinària

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Laura Hidalgo	Dr. Fernando Salvador	Dr. Mariano Domingo

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Abbreviations

EMR	Electronic Medical Record
HDI	Human Development Index
HIV	Human Immunodeficiency Virus
IBS	Irritable Bowel Syndrome
Ig	Immunoglobulin
IPI	Intestinal Parasitic Infection
NHC	Clinical Story Number
OR	Odds Ratio
qPCR	Quantitative Polymerase Chain Reaction
RNA	Ribonucleic Acid
ST	Subtype
TMP-SMX	Trimethoprim/Sulfamethoxazole

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Abstract

Introduction: *Blastocystis hominis* is the most common intestinal parasite isolated in humans. However, a lot of controversies still surround it. Even it has a worldwide distribution, with a higher prevalence in developing countries, its burden is still under-estimated. Nowadays, interest concerning it is increasing due to its potential role as a human pathogen. The aim of the study is to detect the risk factors associated to *Blastocystis hominis* infection.

Materials and Methods: a case-control retrospective study was carried out at Vall d'Hebron University Hospital, a reference Hospital that receives all microbiological tests performed in Barcelona at primary care level. Eligible patients were those adults in whom with three consecutive stool samples that had been examined for parasitic infection diagnosis during 2017. Blagg's technique (merthiolate-iodine-formaldehyde concentration) was used to microscopically examine *Blastocystis* sp. Medical record review and telephonic interviews were carried out in order to gather clinical and epidemiological information.

Results. Overall, 4174 patients were eligible for the study, from whom 1928 (15.4%) had *Blastocystis* sp. infection. 170 cases (infected) and 170 controls (non-infected) were randomly selected for inclusion. Most of the participants were females (60.6%), with a median age of 46.7 (17-88) years. 126 (37.1%) of them were immigrants, mostly from America (20.3%), and had been living in Spain for 11 years, on average. 171 (50.3%) patients had travelled out of Spain the year before the sampling, mostly to European countries (19.4%). The majority of individuals had jobs with direct contact with other people (health personnel, teachers, caregivers) (85.6%) and 29.4% of individuals were in usual contact with animals (mostly dogs and cats). Regarding clinical information, 68.2% of patients presented digestive symptoms at the time of stool analysis, 3.5% presented an immunosuppressed status and 6.5% were infected by other parasites. When comparing cases with controls in the multivariate analysis, variables associated to *Blastocystis* sp. infection were: being born in Africa (p=0.012, OR 5.216), having travelled abroad (p=0.004, OR 1.932) and working in direct contact with other people (p=0.004, OR 2.654). However, having an intestinal parasitic co-infection seems to have a protective value (p=0.046, OR 0.37).

Conclusions. Significant risk factors detected in the present study include having an African origin, working directly with the public and recent travelling are risk factors for acquiring *Blastocystis* sp. infection. Our study provides new insights into the epidemiology of *Blastocystis* sp. infection in industrialized countries, although larger and prospective studies must be performed.

Keywords: *Blastocystis hominis*, Intestinal protozoa, Risk factors

1 Introduction

Parasitic diseases contribute greatly to the burden of infectious diseases worldwide, being a cause of morbidity and mortality both in developed and developing countries [23]. Intestinal parasitic infections (IPIs) are a major public health problem in rural and urban environments of tropical of subtropical regions, with protozoan or helminths infections affecting about one third of the world's population. Zoonotic diseases represent most of parasitic diseases distributed worldwide. Despite their huge animal, human and economic consequences, it has not been until recent years that there has been an increasing interest and concern by public health and veterinary authorities in human-to-animal transmission of parasites [13, 22].

Nowadays, worldwide interconnections are rapidly increasing, leading to constant changes in ecosystems. This offers unpredictable opportunities to microbes, which are more varied, numerous, and adaptable than has ever been expected [13]. It is known that anthropogenic activity is affecting and accelerating natural climate change events, producing direct threatening effects on global health and security such as increases in temperatures and changes in weather patterns. It is suggested that these changes will likely increase the risk and incidence of infectious diseases [27]. Nonetheless, indirect consequences to these effects such as changes in parasitic diseases endemic ranges are not that easy to realize. Regarding parasitic diseases, tropical and subtropical regions are usually more affected because their warmer climates promote species richness. Moreover, usually, these are also areas with lower hygienic conditions. Changes in the environment may alter occurrence, prevalence and pathogenicity of parasites. Even though parasitic diseases are not a cause of high mortality rates, they can feed poverty in disadvantaged communities by debilitating and hampering their growth [59]. On the other hand, diarrhea and enteritis, which are typical symptoms of parasitic diseases infections, increase morbidity and produce significant economic losses in developed countries, such as those linked to medical and loss of productivity [55]. *Blastocystis hominis* distribution has been recently studied regarding climatic and socioeconomic conditions, and these have been suggested as factors that directly affect its prevalence [25, 31].

Even being a big concern globally, zoonotic infectious diseases have a bigger impact in developing countries, where lifestyle is more dependent on animals. On the other hand, in developed countries bacterial and viral infection capture most of the attention and therefore, parasitic infections are usually downplayed. While enteric protozoa such as *Entamoeba* sp., *Cryptosporidium,* and *Giardia* are mostly found in developing countries, *Blastocystis* sp. and *Dientamoeba fragilis* are more frequent in developed countries [22, 23].

Blastocystis hominis

History

Blastocystis hominis is an enteric obligate anaerobic protozoan that resides in humans' colon and cecum. It is the most common intestinal parasite isolated in humans, with up to 1 billion humans colonized or infected around the world, being developing countries the most affected ones [6,17,38], where prevalence can reach 100% of population [20]. *Blastocystis hominis* was discovered in the early 1900s, however, little is known about its pathogenicity, genetic diversity, and available treatment options [16, 57]. From its discovery until recent days, its classification has ranged from a yeast to a fungus, but it was not until phylogenetic analysis were done that it was finally positioned as the only known zoonotic *Stramenophile* [66]. The fact that it does not present the typical features of this group is useful to understand how difficult it has been to classify it correctly[62].

Morphology

Blastocystis hominis presents different life-cycle stages, so its size varies from 5 to 40 μm (Fig. 1). It reproduces asexually, usually by binary fission [60]. Four main stages have been described, including amoeboid, vacuolar, granular and cystic. However, these have been reported to be mainly indistinguishable under the microscope [66]. Vacuolar form is a multinucleated stage that presents a large and central vacuole that occupies around 90% of the cell's volume. The amoeboid form, which is rarely reported in stool samples, presents pseudopod-like cytoplasmic extensions even though it appears to be nonmotile. The granular form is a multinucleated stage that presents a central vacuole surrounded by granules. Cysts, which are the most frequent stage, have a rounded shape and are protected by a multilayered cyst wall, which can and cannot be covered by a loose surface coat [66]. Avacuolar and multivacuolar cells and cells containing filament-like inclusions have also been described but appear to be less frequent. These are rarely seen in stool samples [50].

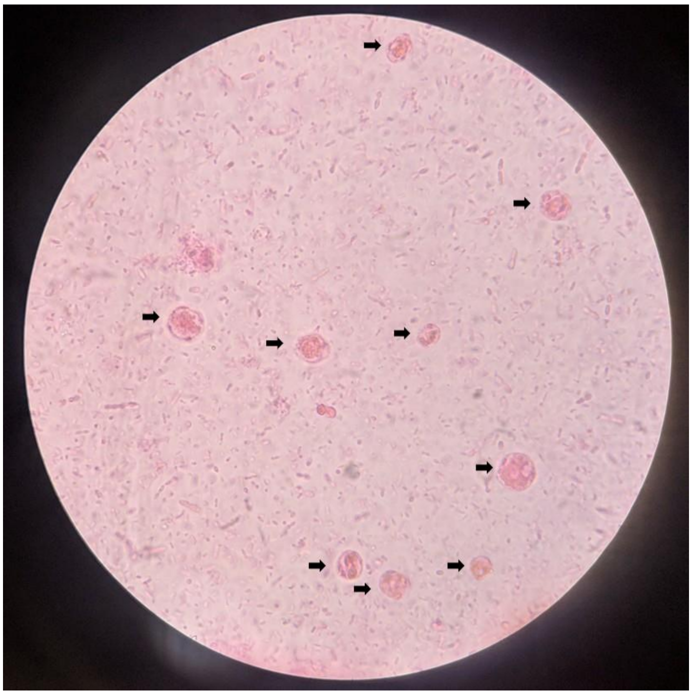

Figure 1: Stool sample with *Blastocystis hominis*. Detection was performed by microscopic examination of stool samples following concentration using the Blagg's concentration technique (merthiolate-iodine-formaldehyde concentration). 40x field microscopy. Image obtained from the Microbiology department from the Vall d'Hebron University Hospital.

Life cycle

Blastocystis hominis is commonly found in stools from humans and a wide range of animals, including mammals, birds and amphibians [54]. Transmission has been stated to occur from human to human, human to animal or animal to human [50, 61]. By now, only chickens and rats have been demonstrated to be suitable experimental models for *Blastocystis hominis* [30], but the lack of a reliable animal model for this infection complicates further investigation of its pathogenesis [15, 30, 67]. Furthermore, the non-standardization of diagnostic techniques, high genetic diversity and studies with small size samples lead to current controversies surrounding *Blastocystis* [66].

Humans and animals are externally infected by fecal cysts, which, after ingestion pass down the intestinal tract and undergo a change by blending cytoplasmic vesicles into a vacuole in the large intestines [22] (Fig. 2). The vacuolar form can reproduce by binary fission and may change to an amoeboid or granular form or may form a cyst in the host's lumen. In the host intestine, vacuolar form will encyst and create a fibrillar layer, that will be lost before getting to the external environment again. The transition from the vacuolar to the amoeboid form reflects the progression from an asymptomatic to a symptomatic stage [58]. However, transition from amoeboid to vacuolar form, and from vacuolar to cyst form is not fully understood. Whereas the amoeboid form is mostly associated with pathogenicity, cysts are the only infective and transmissible form [73], and are likely to be excreted in faeces, restarting the infective cycle. There are two types of cysts: thin walled, which contain schizonts and allow auto-infection, and thick walled, which are responsible for the external transmission [10]. As cysts are highly resistant to the environment conditions and can survive outside the host for more than 1 or 2 months in water at 25°C or 4°C, respectively, they are mainly involved in external transmission. However, they are fragile at extreme temperatures and in common disinfectants [50, 60, 66].

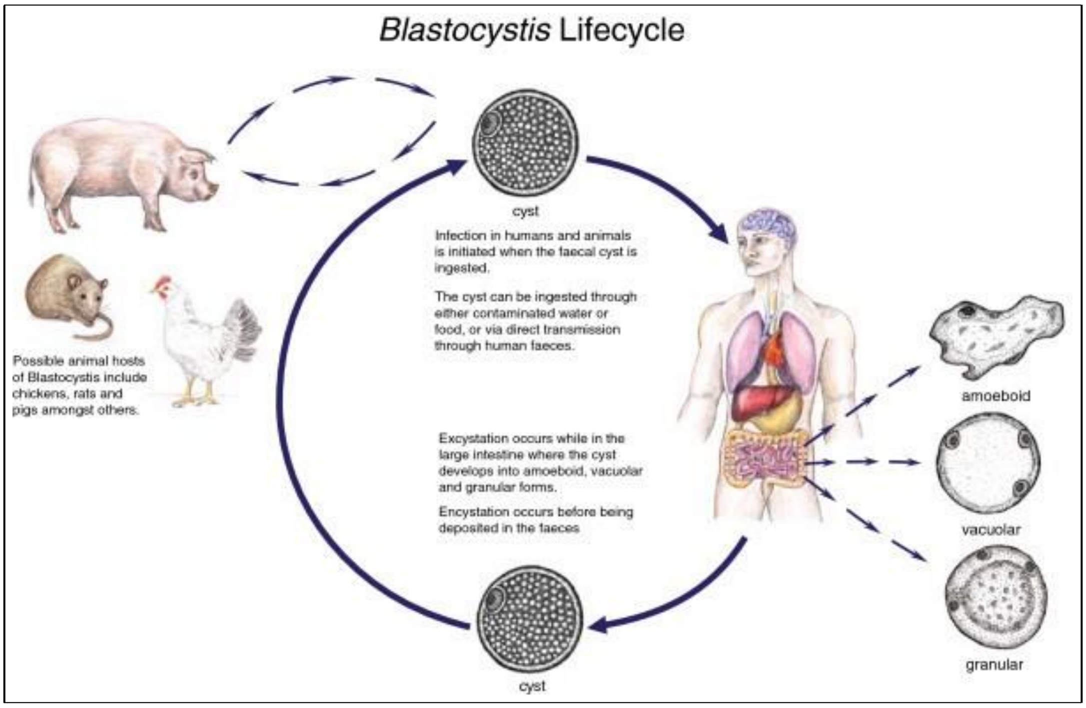

Figure 2: Proposed life cycle for *Blastocystis hominis*. Extracted from [54]

Genetic diversity

Recent studies based on the comparison of the nuclear small subunit rRNA gene show that *Blastocystis* has an extensive molecular diversity. Up to 17 genetic distinct ribosomal lineages have been described, and 5 more have been recently suggested [32], being subtypes (STs) 1-9, specially ST3 [2, 50], the most found in human epidemiological studies [54]. In addition, STs 1-8 and 12 are shared both by humans and animals, suggesting that animals may be reservoirs for *Blastocystis* and may be involved in zoonotic transmission. Amongst these, ST1 is considered the most virulent. [50]. ST8 has been found in zookeepers working with non-human primates [1], as well as pig workers were infected with ST5 in South East Queensland, Australia [69]. In spite of this, STs 1-4 are more common in humans, appearing in 90% of isolations, rather than STs 5-8, which are usually found in hoofed animals (ST5), birds (STs 6 and 7) and in non-human primates (ST8). At this moment, ST 9 is the only one known to be restricted to humans [62], whereas STs 10, 11, 13-17 have only been detected in animals [37].

Prevalence and proportion of STs is known to vary across the world. For example, a recent study found that ST4 is very common in Europe and the United States whereas ST2 mainly predominates in non-industrialized populations [8]. However, any clear explanation has been found to this fact. Different epidemiological and demographic characteristics such as climate, geography, cultural habits, exposure to reservoir hosts and way of transmission are the reflection of the differences in diversity and prevalence of STs isolated from humans [2].

Epidemiology

Blastocystis distribution around the world is not homogeneous, as prevalence can vary from up to 10% in developed countries to 50-60% in developing countries [44]. This large variance may be due to miss-reporting and miss-detection in some clinical laboratories, what suggests that prevalence may be higher than recorded in some studies [28]. The high prevalence in developing countries is usually related to poor hygiene practices and to consumption of contaminated food or water [9]. Therefore, *Blastocystis* rate may be a reflect of the sanitation conditions of a population. In addition, recent studies have also strongly related its distribution to the socioeconomic and geographic situation [31]. However, developed countries also show high prevalence rates of infection. Animals have been suggested as the sources of infection since genetic similarity has been demonstrated [31]. Immigrants, travelers and people who live in close contact with animals are also susceptible to suffer *B. hominis* infection. Prevalence does not only vary from one country to another, but it may also change between regions from the same country [10, 15]. Even so, it has been shown that in developing countries, infection still affects more lower socio-economic status communities [60]. Furthermore, factors such as climate have been shown to have an effect on *Blastocystis* prevalence [31].

Pathogenesis

Nowadays, there is a growing recognition about *B. hominis* pathogenic potential, although its virulence mechanisms are not fully understood [6]. Illness can be either acute or chronic if the parasite accommodates in the human gastrointestinal system, with symptoms persisting from weeks to years [10, 11]. However, no consensus has been reached yet, and many authors still put on doubt *Blastocystis* role in human disease [12, 35, 43, 60]. Even so, it is accepted to be a potential pathogen for immunocompromised people. Pathogenicity has been suggested to be subtype dependent, being STs 1,4 and 7 the most pathogenic ones, and STs 2 and 3 nonpathogenic [22, 67]. Moreover, it has also been related to intensity and host immunity [50].

Transmission is mostly accepted to happen feco-orally both by direct or indirect ways, like contact with infected people or animals or through the ingestion of contaminated water, respectively. Food-borne transmission has also been reported as a source of infection [9, 21]. Due to its controversial pathogenicity -it can be found both in symptomatic and asymptomatic patients-, the role of *Blastocystis* sp. as a truly pathogen or as a commensal organism has been widely debated, and its real human health impact is still uncertain [19]. The different behavior shown could be associated with the high genetic diversity found in this species [5]. However, and even it is usually an asymptomatic parasite, it is thought to cause nonspecific gastrointestinal symptoms including, diarrhea, abdominal pain, flatulence, dyspepsia or vomiting [9]. Moreover, cutaneous manifestations such as chronic urticaria have been reported as well as it has been associated to blastocystosis, especially in patients with irritable bowel syndrome (IBS) [57, 63, 70].

Risk factors

Factors affecting prevalence of intestinal parasites may vary between countries according to geographical and climate conditions of the area, sociocultural characteristics, nutrition culture, poverty, malnutrition, basic sanitary conditions and population density [28,41,42]. There are several risk factors that have already been described. Some of them, such as close contact with domestic animals and livestock, gender, age, work place, seasonality, region of location, immunocompromised status and diabetes may be shared both by developed and developing countries. However, whereas overcrowding, poor personal and environmental hygiene, lack of safe water supply and poor sewerage and waste removal services may be determinant factors in developing countries, this may not be crucial in the developed ones. On the other hand, travelling to tropical and under-developed countries, specially to tropical and subtropical ones, may have a higher importance in developed countries [60].

Data regarding gender is inconclusive, as both females and males have been identified as a potential risk factor by different studies [45, 51]. On the other hand, some authors have not found any correlation between infection and gender [10]. Differences and divisions in men and women type of work performed together with environmental, ecological, economic and cultural factors may affect the risk of infection. In countries where the rift between genders is bigger education opportunity, nutritional status and access to medical facilities may even have a bigger importance [24]. Regarding age groups, children have been sometimes related to a higher risk to get infected by *Blastocystis* sp., even though it may be acquired in subsequent years [62]. Reasons for relate it to children include the lack of natural resistance and differences in behaviors and habits [52]. Prevalences as high as 100% were found in children from a Senegalese community [20]. However, other studies found older age groups to be more prevalent [65] whereas others found no differences between age groups [55]. Therefore, no consensus has been reached regarding age.

A recent study was the first to show the strong effects that Human Development Index (HDI), an indicator name by the United Nations (UN), has on the prevalence of *Blastocystis* [31]. HDI is used to rank countries depending on income, quality and expectancy of life and education. Socioeconomic factors such as water quality, contact with animals and sanitary conditions have been related with *Blastocystis* prevalence [4]. Given the viability and resistance of cysts to water, it has been demonstrated to be a possible source for *Blastocystis* transmission [18]. Consumption of untreated or unboiled water has also been related to a higher risk of getting *Blastocystis* infection [36]. If water treatment has not been performed correctly it can still contain infectious parasite forms. Even so, developed countries do not usually consider water-borne transmission as a reason for disease, however, parasites may both be transmitted by water in both developed and developing countries [14, 22]. Moreover, improper hygienic conditions facilitate *Blastocystis* transmission [53]. In addition, *Blastocystis* infection has sometimes been associated to other parasites, including *Trichomonas hominis*, *Giardia lamblia*, *Entamoeba histolytica* and *Dientamoeba fragilis* [15, 36]

It has been demonstrated that people who are in close contact with animals are at a higher risk to be infected with *Blastocystis* [56], what reinforces the hypothesis that it is a zoonotic infection [15]. Therefore, those occupations that involve exposure to animals are also at a higher risk to suffer infections by this parasite [66] Amongst pets, dog ownership has been described as a potential risk factor to suffer the infection [9]. Regarding domestic farm animals, pigs' potential as zoonotic reservoirs has also been demonstrated. In rural areas, education in personal hygiene and increase of sanitation facilities could help to decrease the prevalence of infections [72].

Seasonality has also been thought to be related to *Blatocystis* infection. A study performed in Spain found a higher peak of infection in adults during spring [26] whilst another study conducted in France found a higher peak in summer [19]. On the other hand, a study performed in the United States during year 2000, obtained a higher prevalence between August and October [3]. However, this data should be interpreted with caution, as seasonal differences between geographical location depend on a wide variety of factors rather than the season itself [26].

Patients with a history of recent travel (during the year before stool examination) to low-income countries have been reported to have a higher prevalence of infection [7]. In addition, a study detected that individuals who had travelled more than once to countries at risk in the previous year had even a higher prevalence [19].

An immunocompromised state has also been related to a higher risk to get infected by *Blastocystis*, what may be a threaten for these patients [45]. HIV-positive population has been stated to have a higher prevalence of infection [48]. Moreover, digestive disorders are more frequent amongst this population group [19]. Some authors have stated *Blastocystis* implication in irritable bowel syndrome (IBS) [50, 62, 71], whereas others have refused it [61, 68]. However, the number of patients in the latter may have been too low to show any difference, as only 84 patients participated, of which just 25 where controls. Higher levels of IgG2 sub-class of antibodies against *Blastocystis hominis* have been detected in patients with IBS [60]. A recent study demonstrated that patients with diabetes mellitus are at a higher risk to suffer parasitic infections [45]. In addition, amongst all infections, *Blastocystis*' was the highest detected.

Diagnosis

Diagnosis is usually made through observation of characteristic form of the parasites in fecal samples. Microscopic methods usually stain *Blastocystis* with trichrome. However, these techniques have low sensitivity and may present false-negative results. Cultivation are less sensitive than microscopic methods when detecting *Blastocystis*. However, these are usually time-consuming methods (2-3 days). PCR detection of the small-subunit ribosomal RNA (SSU rRNA) gene is a powerful method, which is little by little getting widely used for detecting enteric parasites. It provides not only a higher sensitivity than the methods commented before but a higher specificity for detecting the organism's DNA too [50]. Nevertheless, the lack of recognition of different morphological forms of the parasite causes misinterpretation and may lead to different prevalences depending on the detection method used. It has been demonstrated that direct-light microscopy or *in vitro* cultures are less sensitive than qPCR, which has a higher sensitivity specificity, being able to detect all forms of *Blastocystis* parasites [20, 49]. Even so, molecular methods are more expensive and are not affordable in some countries [39].

Treatment

Both controversy on the pathogenesis and the non-standardization of a diagnostic criteria difficult finding a consensus within the treatment [43]. Metronidazole is normally the first-line drug used for treatment and eradication, presumably causing *Blastocystis* apoptosis. However, its effectiveness is not been clearly stated, obtaining eradication rates as low as 0% [46, 54] and as high as 100% [64], so there is yet no agreement for what treatment to use nor when to offer it [15, 34, 60]. However, in asymptomatic patients, treatment is not usually indicated [15]. Resistance to metronidazole has been suggested to explain metronidazole failure in completing eradication of *Blastocystis* in some patients, particularly those with severe infections [58]. The most common alternative to it is trimethoprim/sulfamethoxazole (TMP-SMX), but limited efficacy has also been reported. Other treatments include emetine dihydrochloride, furazolidone, iodoquinol, which have been shown to have and inhibitory effect on *Blastocystis hominis* growth *in vitro* [60]. Even so, the use and combination of these has not been demonstrated to be effective neither [46]. So, to treat *Blastocystis* infection, metronidazole and TMP/SMX are clearly effective for some individuals, but not for all. Some of them may not even need any treatment to get rid of the parasite, as happened in a study conducted in Northern Taiwan [33]. However, reasons for these differences are not known yet [43].

2 Justification and Objectives

Throughout these lines, it has been stated that the public health burden of *Blastocystis* sp. is still under-estimated, and so the interest in performing large-scale epidemiological surveys in industrialized countries. Parasitological studies in developed countries are not common. In Spain, studies regarding *B. hominis* infection are limited [26, 47, 57] focus on specific populations groups and are out of date [40]. Furthermore, we have highlighted the importance that emerging zoonoses are acquiring nowadays not only because animals and humans are affected, but because it creates an economical burden both in developed and developing countries.

Despite all the efforts done by numerous researchers, especially in closer years, *Blastocystis* is still surrounded by controversy. But, what does make *Blastocystis* such a moot organism? Some reasons for *Blastocystis* being the most common parasite isolated in human may refer to a low pathogenicity and unprecise symptomatology that make physicians not to treat it habitually. The main controversy is its role as a pathogen [61]. A wide genetic diversity, small sample sizes as well as the non-standardization of detection techniques may lead to data misinterpretation [66, 67]. The fact that no animal model has been found, makes it difficult to demonstrate experimentally the pathogenic potential of *Blastocystis* [29]. Regarding treatments, large randomized controlled clinical treatment trials are also missing, and those existing present discrepant results [15]. The large number of drugs available with a wide efficacy variety contributes to the difficulty to assess infected individuals. [64]. Moreover, ST and sensitivity to drugs are thought to be related, with specific subtypes unsusceptible to metronidazole [54, 58, 64].

Therefore, the main objective of the present study was to determine the risk factors associated to *Blastocysitis hominis* infection. The independent variables studied were those that have been associated with risk of *Blastocystis hominis* infection i.e. age, gender, country of origin, and time living in Spain (in case the patient was born in another country), seasonality, residence location, contact with animals, travelling out of Spain the year before to the stool examination, digestive symptoms when the coproparasitological examination was done, diabetes mellitus and immunosuppression status.

3 Material and Methods

Study design

A retrospective case and controls study was performed at the Vall d'Hebron University Hospital, a tertiary hospital included in the International Health Program of the Catalan Health Institute (PROSICS, Barcelona, Spain).

Ethical issues

Prior to data collection, the study protocol was reviewed and approved by the Ethics Committee of the Vall d'Hebron University Hospital.

Study size

Epidat 4.2 non-lucrative project was used to select the study cohort. A proportion of exposed cases of 50% was selected i. e. those infected by *B. hominis*. On the other hand, a proportion of 35% was agreed for exposed controls i. e. those who were not infected. Moreover, one control was assigned to every case with an Odds Ratio (OR) established at 1,857. The power of the study was established at 80% with a confidence level of 95%. With the previous data, a sample size of 170 cases and 170 controls was obtained, which were selected among hospital patients by means of a randomized selection.

Subjects

The Vall d'Hebron University Hospital (Barcelona, Spain) receives all microbiological tests performed in Barcelona at primary care level. After consulting the Microbiology Department registry, 34.158 stool samples from all patients that were analyzed at the hospital from January to December 2017 were selected. Those from patients younger than 18 years old when study was performed, who had not been practiced only 3 coproparasitological examinations or who had been given a previous treatment, were discarded. Only patients with 3 stool samples were selected because of an agreement with earlier studies that state the need for multiple stool examinations with microscopy before reporting a negative result for *Blastocystis* [7]. 12.522 samples remained, which corresponds to 4.174 patients. From this, 1.928 (15,4%) were infected with *Blastocystis hominis* (Fig 3).

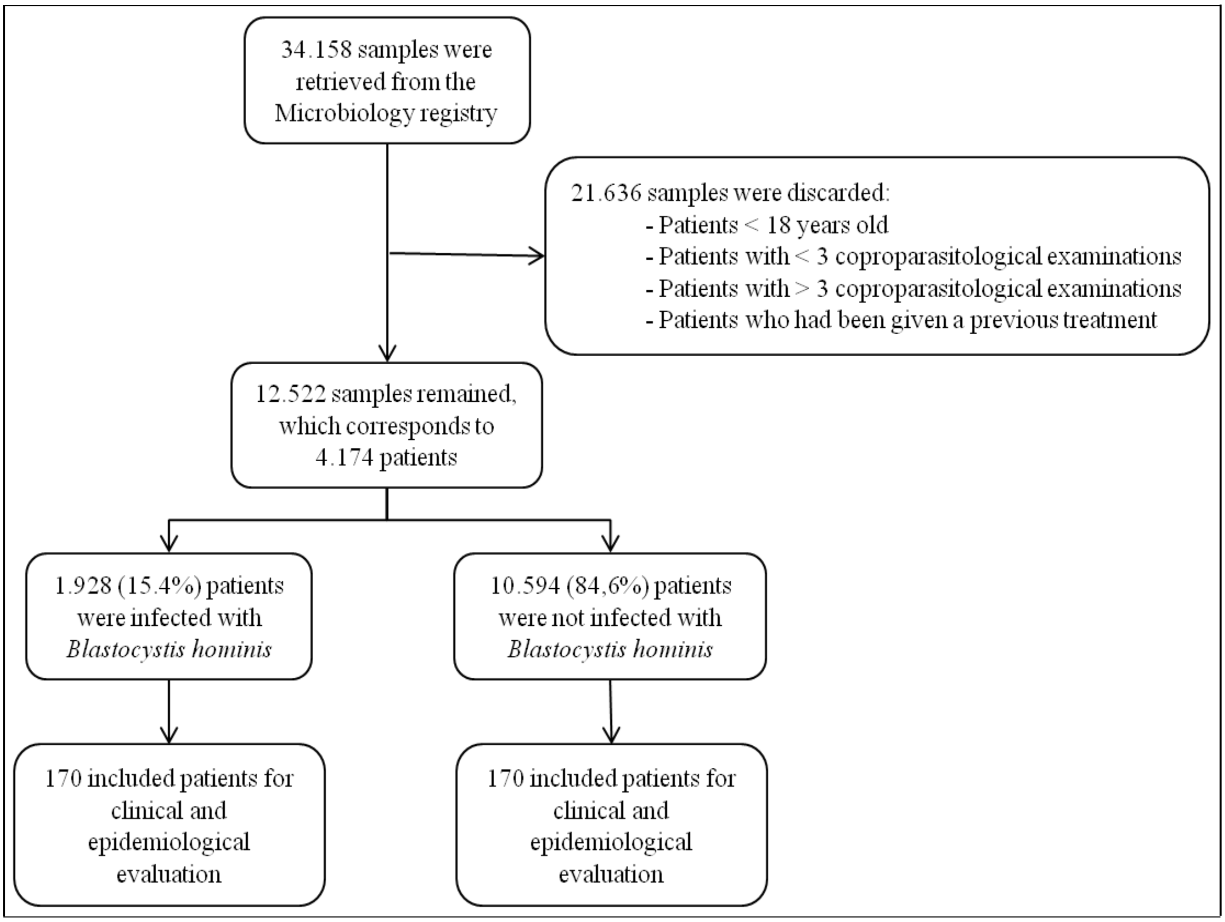

Figure 3: Flow diagram depicting study cohort size and selection

Questionnaire

Based on reviews of the literature on intestinal parasitic infections, we developed a standardized questionnaire to determine the demographic and clinical characteristics of patients, in order to be able to later assess the correlation between the questionnaire's answers and potential infection risk factors (Fig. 4) [57, 60, 62, 63, 70]. It included questions regarding date of inclusion, age, sex, origin, type of work, type of environment, travels, regular contact with animals and gastrointestinal symptoms. The questionnaire also included questions about clinical issues such as number of positive stools, diabetes mellitus, immunosuppression and co-infection with other parasites, which were obtained by looking at the medical record of patients. Factors related to hygiene and quality of water were not included in the questionnaire given their lower impact in developed countries. All questions were asked related to the moment of the stool sampling. As it has been demonstrated that *Blastocystis* can persist for months and colonize the gut [8], questions referring to previous travels took into account those done between year 2016 until the time when stool sampling was done.

Data collection

Eligible patients were contacted by telephone and, after a brief description of our research, they gave their consent to answer the questionnaire and use their data for a scientific purpose and to elicit their medical record. Contact telephones were obtained from the electronic medical record (EMR) at our institution. Patients were called at the most three times before passing to another randomized patient. Amongst cases, 259 patients were contacted until gathering 170 responses. On the other hand, among controls, 263 individuals had to be contacted.

Statistical analysis

IBM SPSS software platform version 15 was used to perform the statistical analysis. Categorical data were presented as absolute numbers and proportions, and continuous variables were expressed as means and standard deviations (SD) or medians and interquartile ranges (IQR) depending on the distribution. The Kolmogorov-Smirnov test was used to evaluate the normal distribution of variables. The χ^2 test or Fisher exact test, when appropriate, was used to compare the distribution of categorical variables, and the t-Student test for continuous variables. Multivariate logistic regression analysis was made to identify factors associated with *Blastocystis hominis* infection. Variables were entered in the model if $p<0.20$ on univariate analysis. Results were considered statistically significant if the 2-tailed P value was <0.05.

4 Results

Overall demographic profiles

The epidemiological characteristics of patients are summarized in Table 1. A total of 340 individuals aged 17 to 88 were interviewed in this study with an overall median age of 46.7 ± 18.24 years, most of them females (n=206, 60.6%). Most of the participants lived in Barcelona city (n=326, 95.9%), whereas the rest lived mainly in the surrounding areas. 126 (37.1%) of participants were immigrants and had been living in Spain, on average, 11 years. Among them, the majority of immigrants came from America, encompassing 69 (20.3%) patients. Specifically, 54 (15.9%) came from South America, 14 (4.1%) from Central America and 1 (0.3%) from North America. 24 (7.1%) immigrants came from Asiatic countries, and 16 (6.96%) from European countries. Finally, 17 (5%) patients came from Africa.

Jobs were subdivided into two categories, depending on the presence of direct contact with other people or not. Hospital personnel, teachers, restaurant workers and caregivers were included in this group. Nevertheless, most of the people did not work with the public (n=291, 85.6%). Only 4 (1.2%) people were in contact with the rural. Regarding daily life, 100 (29.4%) patients were in usual contact with animals, 70 (20.6%) of them with dogs and 28 (8.2%) with cats. Other pets included birds (n=6, 1.8%), rabbits (n=7, 2.1%), turtles (n=2, 0.6%), fishes (n=1, 0.3%), hamsters (n=3, 0.3%) and chickens (n=3, 0.3%). Summer was clearly the season with the highest prevalence, with 122 (35.9%) samples collected during those months. It was followed by 86 (25.3%) samples on autumn, 67 (19.7%) in winter and 65 (19.1%) in spring. In addition, 171 (50.3%) patients travelled out of Spain during the year before the stool sampling. The most visited continent was Europe, with 66 (19.4%) individuals, followed by 62 (18.9%) visitors to America and 34 (10%) to Asia.

Cases demographic profiles

Among the 170 *Blastocystis hominis* positive individuals, age ranged from 18-85, with a median age of 43.95 ± 17.9 years. 98 (57.6%) participants were females, and *Blastocystis* was mostly found in the three of the stools (141/170). Urban environment predominated amongst participants (n=169, 99.4%), who were mainly born in Spain (n=93, 54.7%). Nevertheless, the 77 (45.3%) immigrant *Blastocystis* positive patients, had been living in the country 11 years on average. Regarding their birth place, America was the country with the highest percentage of individuals, with 42 (20.3%) of them. Specifically, 30 (17.6%) had been born in South America, 11 (6.5%) in Central America and only 1 (0.58%) in North America. 14 (8.2%) participants were born in Africa, 13 (7.6%) in Asia and 8 (4.7%) in other European countries apart from Spain.

Jobs with no regular contact with people predominated, with 137 (80.6%) people working on them. Only 47 (27.6%) people had regular contact with animals, 31 (18.2%) of them with a dog, 13 (7.6%) with a cat and the 11 remaining with birds (n=6, 1.8%), rabbits (n=4, 2.4%), chickens (n=1, 0.6%), fishes (n=1, 0.6%), turtles (n=1, 0.6%) or hamsters (n=1, 0.6%). 66 (38.8%) patients were diagnosed in summer, whereas only 31 (18.24%) were during winter. During spring and autumn, a similar number of samples were collected (21.76% and 24.17%, respectively). European and American countries were both visited by 36 (21.2%) people. From these, Southern Americans were the most visited, with 24 (14.1%) people, followed by North and Central American countries, each of them with 6 (3.5%) travelers.

Controls demographic profiles

On the other hand, the 170 *Blastocystis hominis* negative individuals were aged 17-88, with an average of 49.44 ± 18.2 years, with women also being the prevalent population (n=108, 63.5%). Only 3 (1.8%) people lived in a rural environment. The average of time living in Spain for the 49 (28.8%) immigrants was of 11 years, and they mostly came from American countries (n=27, 15.9%). From these, 24 (14.1%) were from South America, 3 (1.8%) from Central America and none from the northern part.

153 (90%) people had no regular contact with others at work, whereas 16 (9.4%) worked directly

with public and only 1 (0.6%) had contact with the rural. Regarding pets, 53 (31.2%) people was in possession of them. 39 (22.9%) people had a dog whereas 15 (8.8%) had a cat. The remaining 9 (5.8%) had birds (n=3, 1,8%) or rabbits (n=3, 1,8%), hamsters (n=2, 1.2%) or turtles (n=1, 0.6%). Summer was again the most prevalent season for stool examination, with 56 (32.93%) samples, followed by autumn with 50 (29.4%), winter with 36 (21.2%) and spring with 28 (16.5%). 99 (58.2%) people travelled out of Spain amongst negative population for *Blastocystis*. 30 (17.6%) people visited Europe.

Table 1: Univariate and multivariate analysis for epidemiological characteristics of patients with and without *Blastocystis* sp. infection attended in Barcelona (2017). Data are reported as number of patients (%) or median (range). Abbreviations: BH+, *Blastocystis hominis* positive patients; BH-, *Blastocystis hominis* negative patients.

Characteristics	Overall (n = 340)	BH+ (n = 170)	BH- (n = 170)	Univariate analysis P value	Multivariate analysis (OR, 95%CI, and P value)
Age, years	46.69 (17-88)	43.95 (18-85)	49.44 (17-88)	0.005	OR 0.994 (0.98-1.008) p=0.396
Gender, female	206 (60.6%)	98 (57.6%)	108 (63.5%)	0.267	
Origin					
Immigrant	126 (37.1%)	77 (45.3%)	49 (28.8%)	0.002	OR 1.301 (0.753 - 2.2251), p=0.346
Spanish	214 (62.9%)	93 (54.70%)	121 (71.2%)		
Immigrant’s origin continent					
North America	1 (0.3%)	1 (0.58%)	0 (0%)	0.317	
Central America	14 (4.1%)	11 (6.5%)	3 (1.8%)	0.029	OR 3.418 (0.881-13.256), p=0.075
South America	54 (15.9%)	30 (17.6%)	24 (14.1%)	0.373	
Africa	17 (5%)	14 (8.2%)	3 (1.8%)	0.006	**OR 5.216 (1.441-18.879), p=0.012**
Asia	24 (7.1%)	13 (7.6%)	11 (6.5%)	0.672	
Other European countries	16 (6.96%)	8 (4.7%)	8 (4.7%)	1.000	
Travelling	171 (50.3%)	99 (58.2%)	72 (42.4%)	0.003	**OR 1.932 (1.229-3.037) p=0.004**
North America	10 (2.9%)	6 (3.5%)	4 (2.4%)	0.521	
Central America	13 (3.8%)	6 (3.5%)	7 (4.1%)	0.777	
South America	39 (11.5%)	24 (14.1%)	15 (8.8%)	0.126	OR 1.692 (0.790-3.624) p=0.176
Africa	24 (7.1%)	17 (10%)	7 (4.1%)	0.034	OR 1.346 (0.438-4.131) p=0.604
Asia	34 (10%)	20 (11.8%)	14 (8.2%)	0.278	
Europe	66 (19.4%)	36 (21.2%)	30 (17.6%)	0.411	

[Continues in the following page]

Characteristics	Overall (n = 340)	BH+ (n = 170)	BH- (n = 170)	Univariate analysis P value	Multivariate analysis (OR, 95%CI, and P value)
Type of work					
Contact with people	49 (14.4%)	33 (19.4%)	16 (9.4%)	0.009	**OR 2.654 (1.356-5.197), p=0.004**
No contact with people	291 (85.6%)	137 (80.6%)	154 (90%)		
Contact with animals	100 (29.4%)	47 (27.6%)	53 (31.2%)	0.475	
Dog	70 (20.6%)	31 (18.2%)	39 (22.9%)	0.283	
Cat	28 (8.2%)	13 (7.6%)	15 (8.8%)	0.693	
Bird	6 (1.8%)	3 (1.8%)	3 (1.8%)		
Rabbit	7 (2.1%)	4 (2.4%)	3 (1.8%)		
Chicken	1 (0.3%)	1 (0.6%)	0 (0%)		
Fish	1 (0.3%)	1 (0.6%)	0 (0%)		
Turtle	2 (0.6%)	1 (0.6%)	1 (0.6%)		
Hamster	3 (0.9%)	1 (0.6%)	2 (1.2%)		

Overall clinical characteristics

Clinical characteristics of patients are summarized in Table 2 Most of the patients presented diverse digestive symptoms (n=232, 68.2%), and only 22 (6.5%) of participants were co-infected with other organisms, mainly *Endolimax nana* (n=9, 2.6%) and *Giardia lamblia* (n=6, 1.8%). Others include *Dientamoeba fragilis* (n=4, 1.2%), *Entamoeba coli* (n=3, 0.9%) and *Entamoeba histolytica/E. dispar* (n=3, 0.9%), *Strongyloides stercoralis* (n=2, 0.6%) and *Enterobius vermicularis* (n=1, 0.3%). 25 (7.4%) patients were diabetic and 12 (3.5%) presented an immunocompromised status. Amongst immunosuppressions, most patients were HIV positive (n=6, 1.8%). There were 14 patients (4.1%) with a diagnosis of irritable bowel syndrome.

Cases clinical profiles

Regarding *Blastocystis* positive patients, 116 (68.2%) presented digestive symptoms, and only 7 (4.1%) were co-infected with other parasites. Among parasitosis, *Endolimax nana* predominated, with 5 (2.9%) people infected. *Giardia lamblia* (n=1, 0.6%), *Dientamoeba fragilis* (n=2, 1.2%), *Entamoeba coli* (n=2, 1.2%) and *Entamoeba histolytica/E. dispar* (n=1, 0.6%) were also found among stool samples. 9 (5.3%) patients were diabetic, and 6 (6.5%) individuals presented and immunocompromised status and only 4 (2.4%) had irritable bowel syndrome.

Controls clinical profiles

When it comes to *Bastocystis* negative patients, also 116 (68.2%) patients had digestive symptoms, and 15 (8.8%) presented other parasites. *Giardia lamblia* was the most found among these, infecting 5 (2.9%) people. *Endolimax nana* was also found in the samples, and infected 4 (2.4%) people. Other parasites found were *Dientamoeba fragilis* (n=2, 1.2%), Entamoeba coli (n=1, 0.6%), *Entamoeba histolytica/E. dispar* (n=2, 1.2%), *Strongyloides stercoralis* (n=2, 1.2%) and *Enterobius vermicularis (n=1, (0.6%)*. Lastly, 10 (5.9%) people presented irritable bowel syndrome.

Univariate statistical analysis

Age, immigration, coming from Africa or Central America, working with the public and travelling, specifically to Africa or South America were statistically significant for the univariate analysis ($P<0.05$). *Blastocystis hominis* positive patients were younger (median age 43.95 years *vs* 49.44 years, P = 0.005) and included a larger proportion of immigrants (45.3% *vs* 28.8%, P = 0.002), people who worked directly with public (33 *vs* 16, P = 0.009) and individuals who had travelled out of Spain (58.2% *vs* 42.4%, P = 0.03). When it comes to immigrants, those coming from Central America or Africa were also found to be significant (P = 0.029 and P = 0.006, respectively). Regarding travelling, only Africa was found to be a statistically significant (P = 0.034) continent. Seasonality was also considered in the analysis in order to know if differences in the number of samples during the year were statistically significant, but no correlation was obtained (P = 0.194).

Multivariate statistical analysis

After doing the multivariate analysis, the variables associated to suffer *Blastocystis hominis* infection were coming from Africa or Central America, having travelled out of Spain and working with the public. Moreover, being infected by other parasites resulted to be a protective factor. Having an African origin was the most strongly correlated factor, with an OR of 5.216 (95%CI, 1.441-18.879), p=0.012). Moreover, working directly with public was also strong associated factor, with and OR of 2.654 (95%CI, 1.356-5.197, p=0.004). Travelling out of Spain was also related *Blastocystis hominis* infection, with an OR of 1.932 (95%CI, 1.229-3.037, p=0.004). Finally, being infected with other parasites was found to be a protective factor, with an OR 0.370 (95%CI, (0.14-0.98), p=0.046).

Table 2: Univariate and multivariate analysis for clinical characteristics of patients with and without *Blastocystis* sp. infection attended in Barcelona (2017). Data are reported as number of patients (%) or median (range). Abbreviations: BH+, *Blastocystis hominis* positive patients; BH-, *Blastocystis hominis* negative patients.

Characteristics	Overall (n = 340)	BH+ (n = 170)	BH- (n = 170)	Univariate Analysis P value	Multivariate analysis (OR, 95%CI, and P value)
Presence of digestive symptoms	232 (68.2%)	116 (68.2%)	116 (68.2%)	1.000	
Other intestinal parasitosis	22 (6.5%)	7 (4.1%)	15 (8.8%)	0.078	**OR 0.370 (0.14-0.98), p=0.046**
Emdolimax nana	9 (2.6%)	5 (2.9%)	4 (2.4%)		
Giardia lamblia	6 (1.8%)	1 (0.6%)	5 (2.9%)		
Dientamoeba fragilis	4 (1.2%)	2 (1.2%)	2 (1.2%)		
Entamoeba coli	3 (0.9%)	2 (1.2%)	1 (0.6%)		
Entamoeba histolytica/E. dispar	3 (0.9%)	1 (0.6%)	2 (1.2%)		
Strongyloides stercoralis	2 (0.6%)	0 (0%)	2 (1.2%)		
Enterobius vermicularis	1 (0.3%)	0 (0%)	1 (0.6%)		
Diabetes mellitus	25 (7.4%)	9 (5.3%)	16 (9.4%)	0.146	OR 0.586 (0.236-1.452) p=0.248
Presumptive immunocompromised status	12 (3.5%)	6 (3.5%)	6 (3.5%)	1.000	
HIV	6 (1.8%)				
Crohn's disease	2 (0.6%)				
Common variable immunodeficiency	1 (0.3%)				
Breast cancer	1 (0.3%)				
Hypogammaglobulinemia	1 (0.3%)				
Psoriasis	1 (0.3%)				
Irritable bowel syndrome	14 (4.1%)	4 (2.4%)	10 (5.9%)	0.101	OR 0.334 (0.98-1.137), p=0.079

5 Discussion

Key results

We retrospectively studied 340 patients, divided into cases and controls, whose stool samples were analyzed at the Vall d'Hebron University Hospital during year 2017. We used standardized questionnaires and medical records with the aim to gather information to detect risk factors related to *Blastocystis hominis* infection. Most of the samples were analyzed in summer (35.9%), and belonged to Spanish women (62.9% of individuals were Spanish, 60.6% were female and were 47 years old, on average) who lived in Barcelona and did not work directly with the public (85.6% of participants did not have contact with people at work). The majority of participants (70.4%) did not have regular contact with animals. Among all the variables studied, we found out being African, working with the public and travelling to other countries to significantly increase the risk of getting infected by the parasite. Moreover, being infected by other parasites was found out to be a protective factor.

Even having a worldwide distribution, developing countries are the most affected by intestinal parasitic infections [6]. Regarding our study, only people from Africa were reported to have a stronger risk of infection. A higher incidence, however, not significant, was also seen among people coming from Central America. Most immigrants had been living in Spain for 11 years, so no differences were established.

A story of recent travel has been stated to be a risk factor to acquire this infection, being tropical and under-developed destinations those at increased risk of acquiring infection [15, 19]. A recent study from El Safadi et al. concluded that patients who had travelled during the last year before stool sampling were at higher risk than those who had not [19]. A similar conclusion can be extracted from our study since statistical significant data was obtained for the same variable. Among all possible destinations, Southern American and African countries had higher risk of infection, but it was not significant enough. It is interesting to point out that both immigrants coming from Africa and people travelling to Africa are both at a higher risk to get infected. However, the latter is not significantly important.

Seasonal distribution of *Blastocystis hominis* has been previously reported by some authors [19]. In our study, even though the number of samples gathered was higher in summer, we did not find significant differences in relation to the rest of the year. This agrees with other studies performed in the same geographical area [26, 57].

Age has sometimes been stated as a risk factor, usually describing children in developing countries as the most prevalent group, reaching 100% of infected population [20]. Children were not included in our study, but, even it was not statistically significant, younger adult age groups were at a higher risk than older, in accordance with a study conducted in France. However, it is important to highlight the fact that young people usually travels more, so this may increase the prevalence [19].

Some studies have associated *Blastocystis* infection to female gender [45], relating it to differences on the jobs, but also to environmental, ecological, economic and cultural factors [24]. In our case, women presented a higher prevalence of infection than men, but it was not statistically significant.

Contact with animals has been stated to be a risk factor for *Blastocystis* infection [9, 51, 56], but not in our case. However, most of the studies which have proven this fact have been conducted in developing countries such as Thailand, The Philippines or Malaysia, where animal hygienic conditions and treatment may be different. In these studies, population is mostly rural and is in contact with livestock animals, whereas in our case dogs and cats were the main pets reported.

The fact that the present study was conducted by using samples which had been analyzed at the Vall d'Hebron University Hospital, in Barcelona, conditioned the origin of our samples, so most people came from an urban environment. Therefore, only 4 people out of 340 declared to live in a rural environment. This finding made difficult to evaluate this variable and limits our study. Therefore, our results are based on an urbanized population, from an industrialized country.

As *Blastocystis* infection has been stated to happen from human to human, participants job was divided into two groups, depending on their contact with other people or not [50]. Surprisingly, we found out that people working with other people were at a higher risk than those who did not. The majority of people who were at this group included caregivers, hospital personnel, restaurant workers and teachers. These occupations can be related to working with groups of people who are at a higher risk to get infected, meaning that the risk of those surrounding them may also be increased. Hospital personnel are usually in contact with immunocompromised people. Restaurant workers are in constant contact with food, which has also been proved to be a possible source of transmission. Caregivers may be in contact with faeces from their guests, so feco-oral transmission, the main route, may happen [9]. Teachers spend most of their time in contact with children, whose age group has sometimes been noted to be at a higher risk [20]. Therefore, not taking the adequate prevention may increase significantly the risk of infection. Unfortunately, from all he participants almost no one worked in contact with animals or the rural (only 2 people did), so we cannot make any elucidation regarding this possible risk factor.

Blastocystis hominis pathogenicity has been one of the main controversial issues regarding this parasite. It is nowadays thought to cause gastrointestinal symptoms. Even so, this was not a significant factor to take into account in our study. Curiously, the same number of participants among cases and controls reported presence of digestive symptoms. However, some studies suggest that pathogenicity may be related to different *Blastocystis* subtypes, and it has also been demonstrated that these subtypes vary along with geographical variation [2,8].

Extensive research shows that immune suppression and the use of immunosuppressive drugs increases the risk of getting an infection [39,44,45]. However, in this study no correlation was found among these factors. Some studies have stated that [62] *Blastocystis* may be linked to irritable bowel syndrome (IBS). In our case, no significant differences were found. A study by Mohtashamipour et al. stated that diabetic patients are at a higher risk of getting a parasitic infection [45]. In this case-control study they also found out that *Blastocystis hominis* was significantly the most prevalent parasite. In our case, however, no differences were observed for this factor. A significantly lower prevalence of *Blastocystis* sp. was reported among patients who were infected by other parasites than those who were not. Enteric pathogens infection by different parasites has commonly been detected in recent studies [36,47]. In our study, it has been found out to be protective factor.

Limitations

Given its retrospective nature, our study presents some limitations. Firstly, conventional microscopic methods, which have been stated to be less sensitive, were used for diagnosis rather than molecular methods [52]. Besides, the small sample size could also be a limiting factor, but the power of the study was high enough to show differences among cases and controls. Finally, some people stated not to remember some of the issues asked during the questionnaire (mostly related to travelling or to the presence of digestive symptoms), what could suppose a bias for the results.

6 Conclusions

To summarize, a case and control study was conducted in the Vall d'Hebron University Hospital including 340 patients whose samples were collected during year 2017. The results of the present study suggest that African people, recent travelling, working with the public and co-infections with other intestinal parasites are risk factors for acquiring *Blastocystis hominis* infection. Our study provides new insights into the epidemiology of *Blastocystis* sp. in industrialized countries. Further research including molecular and epidemiological studies should be done. This would help to map prevalence and sub type distribution and to clarify the importance of risk factors depending on the area and would improve intervention strategies that could help to mitigate *Blastocystis* transmission.

Annex

Standardized questionnaire

Detection of risk factors associated to *Blastocystis* hominis infection

Questionnaire

1. NHC
2. Data of inclusion
3. Gender
4. Age
5. In which country were you born?
    a. If you were born out of Spain, please specify where
    b. If you were born out of Spain, please specify for how long have you been living in Spain
6. Which is your residence municipality?
7. Do you live in a urban or in a rural environment?
8. What is your profession?
9. Do you have regular contact with animals?
    a. If yes, please, specify which
10. Did you travel out of Spain during year 2016 and until the moment of the stool sampling?
    a. If yes, please specify where
11. Did you present digestive symptoms when you stool sampling was requested?

Medical Record

1. Number of positive samples
2. Other intestinal parasitosis
    a) If yes, specify which
3. Diabetes mellitus
4. Immunosuppressed state
    a) If yes, specification

Figure 4: Standardized questionnaire and observations from the medical record requested from all participants

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