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Microscopic examination after concentration techniques for *Blastocystis* sp. detection in serial faecal samples: How many samples are needed?

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ABSTRACT

Objective: *Blastocystis* sp. is one of the most frequently observed intestinal parasites in humans. It is suggested that sensitivity of classical parasitological tests for the *Blastocystis* sp. diagnosis increases when increasing the number of investigated samples, although there is a lack of information. The aim of the study is to evaluate the sensitivity of classical parasitological tests for the *Blastocystis* sp. diagnosis depending on the number of investigated samples and to determine risk factors associated to high parasite burden.

Methods: Retrospective study where patients in whom three consecutive stool samples were examined for parasitic diagnosis through microscopic examination at Vall d'Hebron University Hospital (Barcelona, Spain) from January to April 2019 were included. To determine risk factors associated to high parasite burden, a case-control study was performed including patients with at least one positive stool sample for *Blastocystis* sp.: cases were those patients with only one or two positive stool samples, and controls were those with all three stool positive samples). Clinical records were reviewed from included patients to collect clinical and demographic information.

Results: In 2771 patients three consecutive stool samples were examined for parasitic diagnosis, with an overall prevalence of *Blastocystis* sp. detection of 23.3%. The proportions of positive cases depending on the number of investigated samples were: 22.3% when taking into account the first sample, 22.9% when taking into account the first and second samples, and 23.3% when taking into account the three samples, with no statistically significant differences among them. For the case-control study we finally included 63 cases and 133 controls. No differences were found regarding clinical and demographic characteristics among groups.

Conclusion: Prevalence of *Blastocystis* sp. infection was high in our study (23.3%). The sensitivity of classical parasitological methods for *Blastocystis* sp. diagnosis did not increase when increasing the number of investigated samples, and no risk factors associated to high parasite burden were identified.

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1. Introduction

Blastocystis sp. is an intestinal parasite with a worldwide distribution, with higher prevalence reported in developing countries (50–60%). It is an anaerobic protozoan that resides in the intestines of humans and a wide range of animals. Transmission can occur via faecal-oral from human to human, human to animal or animal to human (Mohamed et al., 2017). Studies based on the comparison of the nuclear small subunit rRNA gene show that *Blastocystis* has an extensive molecular diversity, with up to 17 genetic distinct ribosomal lineages described (Kazmarekk et al., 2017; Alfellani et al., 2013).

Despite *Blastocystis* sp. was described >100 years ago, scarce information is available regarding its pathogenesis, genetic diversity, host range and available treatment options (Roberts et al., 2014). Clinical manifestations associated with *Blastocystis* sp. carriage include gastrointestinal symptoms (diarrhea, abdominal pain), and cutaneous manifestations (urticaria) (Salvador et al., 2016). Nevertheless, some investigators report no association between the presence of clinical manifestations and *Blastocystis* sp. infection (Leder et al., 2005).

There are also controversies regarding the best diagnostic methods to detect *Blastocystis* sp. in feces. Diagnosis relies in most of the cases in microscopic visualization of the parasite through different classical parasitological methods. These microbiological techniques are easy to perform, cheap and available. However, they may lack of sensitivity, which is suggested to increase when increasing the number of investigated faecal samples (Tan, 2008). In the last decades, different molecular biology techniques to detect *Blastocystis* sp., such as the polymerase chain reaction (PCR), have been developed, showing higher sensitivity than classical parasitological methods and the possibility of subtype (ST) detection (Roberts et al., 2013; Udonsom et al., 2018; Paulos et al., 2018). However, these techniques are more expensive and only available in reference centres.

The aim of the present study is to evaluate the sensitivity of microscopic observation with increasing number of faecal samples for the *Blastocystis* sp. infection diagnosis, and determine risk factors associated to high parasite burden.

2. Material and methods

This is a retrospective observational study 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). The hospital receives all stool samples collected in Barcelona at primary care level for microbiological investigation. Eligible patients were those in whom three consecutive stool samples were examined for parasitic diagnosis for any reason from January to April 2017.

During the study period, the diagnosis of intestinal parasites, including *Blastocystis* sp. were performed by microscopic examination of concentrated stool sample using a commercial dispositive (Midi Parasep SF. APACOR. England). Our laboratory routinely uses a low centrifugation method (1500 rpm for 3 min) to decrease the risk of lysis of trophozoites (especially in the case of *Dientamoeba fragilis*) and other non-cystic forms of intestinal protozoa (such as the vacuolar form of *Blastocystis* sp). The merthiolate-iodine-formalin (MIF) method for staining the samples. Moreover, the fluorescent auramine staining is routinely performed in children <5 years old and in patients with any immunosuppressant condition to detect *Cryptosporidium* sp. and other coccidian parasites.

For the evaluation of possible risk factors associated to high parasite burden, a nested case-control study was performed including all patients with at least one positive stool sample for *Blastocystis* sp. Cases were defined as patients with only one or two positive stool samples for *Blastocystis* sp. Controls (patients with all three stool samples positive for *Blastocystis* sp. detection) were randomly selected with a 1:2 ratio. Clinical records from included patients were reviewed to collect the following information: gender, age, co-infection with other intestinal parasites, symptoms, immunosuppression, other comorbidities such as diabetes, concomitant treatment.

SPSS software for Windows (Version 19.0; SPSS Inc., Chicago, IL, USA) was used for statistical analyses. Categorical data were presented as absolute numbers and proportions, and continuous variables were expressed as means and standard deviation (SD). 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. To evaluate the sensitivity of the microscopic examination for *Blastocystis* sp. diagnosis depending on the number of stool samples investigated, the two-sample test of proportions was performed. Results were considered statistically significant if the 2-tailed *P* value was <0.05.

Procedures were performed in accordance with the ethical standards laid down in the Declaration of Helsinki as revised in 2013, and the study protocol was approved by the Ethical Review Board of the Vall d'Hebron University Hospital (reference number PR (AG) 99/2018).

3. Results

After consulting the Microbiological Department registry of the Vall d'Hebron University Hospital, we detected 2771 patients in whom three consecutive stool samples were examined for parasitic diagnosis from January to April 2017 (see the flow diagram of patients in Fig. 1). Overall, 647 (23.3%) patients had at least one positive sample for *Blastocystis* sp., which represents the overall prevalence. When analyzing the prevalence depending on the number of investigated stool samples, we obtained the following results: 22.3% when taking into account the first sample, 22.9% when taking into account the first and second samples, and 23.3% when taking into account the three samples. When comparing the proportions of positive patients for *Blastocystis* sp. detection depending on the number of investigated stool samples, no differences were found (see Fig. 2).

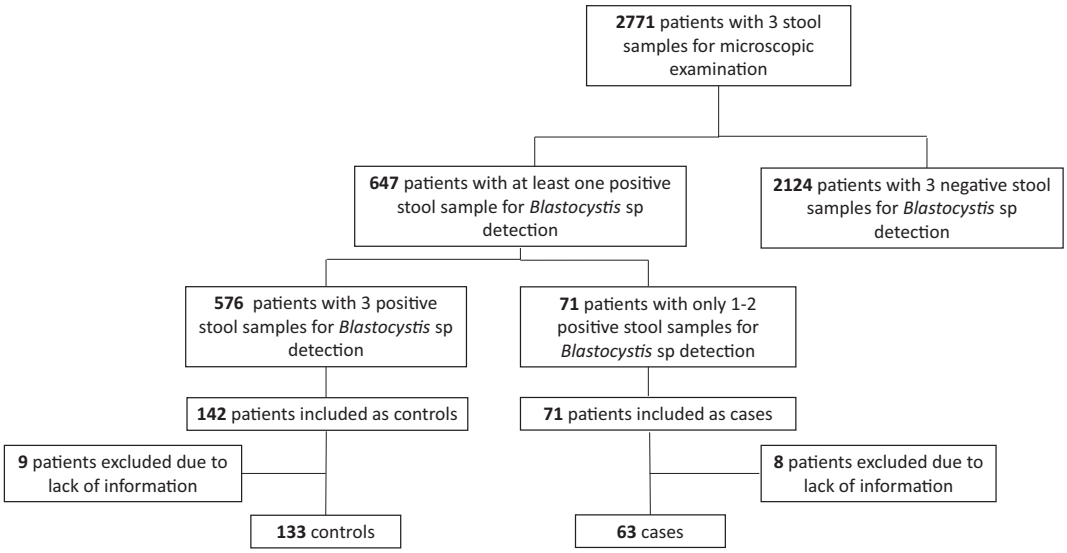

Fig. 1. Flow diagram of included patients.

For the nested case-control study, 647 patients were eligible (at least one positive sample for *Blastocystis* sp). From these, 71 (10.9%) patients were considered cases (only one or two positive stool samples), and 576 (89.1%) patients were considered control (all three samples positive for *Blastocystis* sp), from whom 142 patients were randomly selected. After revising the clinical records, 17 patients (8 cases and 9 controls) were excluded due to lack of information, hence 63 cases and 133 controls were finally included (see Fig. 1).

Of the 196 included patients, 90 (45.9%) were male, with a mean age of 34.9 (SD 20.9) years. At the moment of intestinal parasites investigation, 110 (59.1%) patients had gastrointestinal symptoms, only 2 (1.1%) patients had some kind of immunosuppression (both of them with HIV infection), and 8 (4.3%) patients had diabetes mellitus. Regarding concomitant treatment, 9 (4.8%) patients were receiving antacid drugs, and 4 (2.2%) patients were receiving antibiotics. In 75 (35.2%) patients, another intestinal parasitic infection was diagnosed; details from co-infections are summarized in Table 1. When comparing the clinical and demographic characteristics between cases and controls in order to evaluate possible risk factors associated to high parasite burden, we did not find any difference (see Table 2).

4. Discussion

In the this study from 2771 patients in whom three consecutive stool samples were examined for parasitic diagnosis, the overall prevalence of *Blastocystis* sp. detection was 23.3%. When comparing the proportion of positive cases depending on the number of investigated stool samples (up to three consecutive samples), no differences were found. Moreover, in positive cases, no

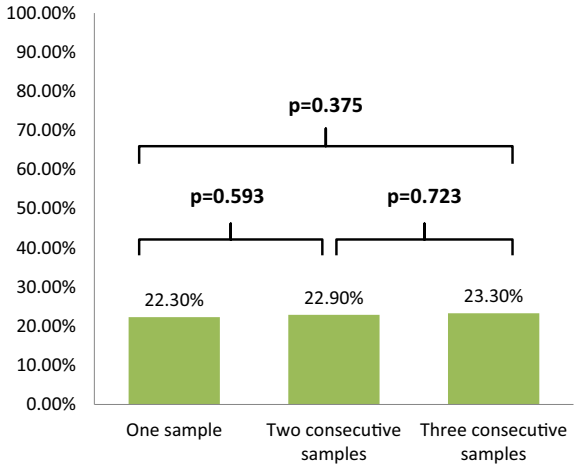

Fig. 2. Comparison of the prevalence of *Blastocystis* sp. infection depending on the number of investigated stool samples.

Table 1
Other intestinal parasites found in the study population.

Intestinal parasites	Number of patients ($n = 196$)
Dientamoeba fragilis	35 (16.4%)
Endolimax nana	26 (12.2%)
Entamoeba coli	16 (7.5%)
Enterobius vermicularis	6 (2.8%)
Giardia lamblia	4 (1.9%)
Entamoeba histolytica/dispar/moshkovskii	3 (1.4%)
Iodamoeba bustchlii	3 (1.4%)
Balantidium coli	1 (0.5%)
Strongyloides stercoralis	1 (0.5%)
Trichuris trichiura	1 (0.5%)

NOTE. Data are reported as number (%) of patients.

differences between patients with only one or two positive stool samples (surrogate marker of low parasite burden) compared with those with all three positive stool samples (surrogate marker of high parasite burden) were found.

Although prevalence of *Bastocystis* sp. is higher in developing countries due to social, economic, and hygienic conditions, this infection is increasingly being diagnosed in developed countries (Fletcher et al., 2012). The diagnosis is performed in most of the cases through microscopic examination of stool samples, which is easy to perform, cheap, and available. The examination can be performed directly in fresh stools, or after concentration techniques, that increase the sensitivity of the test. However, the sensitivity of these microbiological techniques is observer-dependant (Stark et al., 2009).

It has been assumed that the sensitivity of classical parasitological techniques for the diagnosis of intestinal parasites increases when increasing the number of investigated stool samples, but this information comes mostly from studies focused in helminths, where the excretion of larvae and eggs is very irregular. As an example, the study by Nielsen et al. showed how the sensitivity for *Strongyloides stercoralis* diagnosis increased from 53% with one sample to 100% with 7 consecutive samples (Nielsen and Mojon, 1987). These observations have been transferred to intestinal protozoan infections with low evidence. The results of our study showed how the sensitivity of microscopic investigation through a concentration technique did not increase when two or three consecutive stool samples were investigated compared with one stool sample investigation. This may reflect the regular output of *Blastocystis* sp. through the feces. However, we have to take into account that in symptomatic patients may have a broad range of intestinal parasites, and for some of them serial stool samples investigation is needed. Hence, the applicability of these results are almost restricted to studies focused only in *Blastocystis* sp. detection.

A study performed by our group suggested that some epidemiological characteristics (migrants coming from Africa, recent travelling, and working with the public) were risk factors for acquiring *Blastocystis* sp. Conversely, no clinical conditions (presence of symptoms, immunosuppression, diabetes mellitus, and irritable bowel syndrome) were associated (Hidalgo et al., 2019). In the same way, in the current study, no clinical characteristics of patients with *Blastocystis* sp. detection were associated with high parasite burden.

This study has some limitations given the retrospective nature of its design. Moreover, more sensitive diagnostic techniques such as the PCR could increase the detection of *Blastocystis* sp., but this technique is not routinely performed for clinical management. Some studies suggest that the combination of different microscopic techniques could be used for the *Blastocystis* sp. infection diagnosis to ensure better and accurate diagnosis, but this is scarcely affordable in routinely clinical conditions (Mohammad et al., 2018). Moreover, the study has been performed in a single centre with highly trained laboratorial personnel, and it could be difficult to extrapolate the conclusions to other centres with less trained staff.

Summarizing, in our study we observed a 23.3% prevalence of *Blastocystis* sp. carriage measured by microscopic examination through concentration methods. Contrary to what it has been assumed, the increase of the number of investigated stool samples was not translated into an increase in the sensitivity of the diagnostic test. Moreover, among positive patients for *Blastocystis* sp. detection, no risk factors were associated to a high parasite burden. These results should be taken into account when designing prevalence studies for *Blastocystis* sp., since one single stool sample investigation could be enough.

Table 2
Comparison of main clinical and demographic characteristic between cases (one or two positive stool samples for *Blastocystis* sp) and controls (three positive samples for *Blastocystis* sp).

Characteristics	Total (n = 196)	Cases ($n = 63$)	Controls ($n = 133$)	*p*-value
Gender, male	90 (45.9%)	32 (50.8%)	58 (43.6%)	0.346
Age, years	34.9 (20.9)	32.7 (20.8)	35.9 (20.9)	0.321
Presence of gastrointestinal symptoms	110 (59.1%)	38 (62.3%)	72 (57.6%)	0.541
Immunosuppressive conditions	2 (1.1%)	1 (1.6%)	1 (0.8%)	0.550
Antacids drugs	9 (4.8%)	1 (1.6%)	8 (6.4%)	0.275
Antibiotics	4 (2.2%)	0 (0%)	4 (3.2%)	0.305
Diabetes mellitus	8 (4.3%)	1 (1.6%)	7 (5.6%)	0.276
Other intestinal parasites detected	75 (35.2%)	23 (32.4%)	52 (36.6%)	0.543

NOTE. Data are reported as number (%) of patients or mean (SD).

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