

Predominance of *Blastocystis* spp, *entamoeba histolytica* /*e.dispar* and other protozoan evaluated through classical and molecular diagnostic techniques in two communities of Niterói-rj/Brazil

Predominância de *Blastocystis* spp, *entamoeba histolytica* /*e.dispar* e outros protozoários avaliados através de técnicas de diagnóstico clássico e molecular em duas comunidades de Niterói-rj/Brazil

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ABSTRACT

Background: Enteroparasites infections remains an important health issue in Brazil as contaminations are still detected among inhabitants of socially vulnerable populations of the country. This study was performed in two low income communities in Niterói, Rio de Janeiro State, Brazil. Methods: Health educational actions in two selected communities were performed and in parallel, 150 stool samples were collected. Coproparasitological diagnosis was carried out employing Hoffmann, Pons & Janer, Willis and Rugai techniques^{5,6,7}. Molecular diagnosis using Paglia & Visca protocol was performed to detect and differentiate *E. histolytica*/*E. dispar* infections⁸. Results: Enteroparasites were found in more than 50% of the samples and protozoan infections prevailed. *Blastocystis* spp was the most prevalent protozoan (55.08%) followed by *Endolimax nana* (16.10%), *Giardia lamblia* (15.56%) and *Entamoeba coli* (11.02%). For helminth species, only *Enterobius vermicularis* and *Strongyloides stercoralis* were found, in 4.24% of positive samples. All samples were negative for *E. histolytica*/*E. dispar* infections through coproscopy, however, PCR analysis of 143 suitable stool samples showed that both species were present - as a complex or not - in 15% of the samples, in both institutions evaluated. Conclusions: The high frequency of protozoan infections may indicate extensive faecal-oral contamination. Additionally, it can ensure the basis for directed intervention actions in some communities to prevent water transmitted parasites providing sanitary infrastructure. Also, educational health programs for inhabitants of low

income communities of Niterói, Rio de Janeiro State, Brazil, are necessary in order to provide a better understanding of enteroparasites and its prophylactic measures.

Keywords: *Blastocystis* spp, *E. histolytica*/*E. dispar*, diagnosis

RESUMO

Antecedentes: As infecções enteroparasitas continuam sendo um importante problema de saúde no Brasil, pois ainda são detectadas contaminações entre os habitantes de populações socialmente vulneráveis do país. Este estudo foi realizado em duas comunidades de baixa renda em Niterói, Estado do Rio de Janeiro, Brasil. **Métodos:** Foram realizadas ações educativas de saúde em duas comunidades selecionadas e, paralelamente, foram coletadas 150 amostras de fezes. O diagnóstico coproparasitológico foi realizado empregando as técnicas Hoffmann, Pons & Janer, Willis e Rugai 5,6,7. O diagnóstico molecular usando o protocolo Paglia & Visca foi realizado para detectar e diferenciar infecções *E. histolytica*/*E. dispar* 8. **Resultados:** Foram encontrados enteroparasitas em mais de 50% das amostras e prevaleceram as infecções por protozoários. *Blastocystis* spp foi o protozoário mais prevalente (55,08%) seguido por *Endolimax nana* (16,10%), *Giardia lamblia* (15,56%) e *Entamoeba coli* (11,02%). Para as espécies helmintas, apenas *Enterobius vermicularis* e *Strongyloides stercoralis* foram encontrados, em 4,24% das amostras positivas. Todas as amostras foram negativas para infecções por *E. histolytica*/*E. dispar* através da coproscopia, entretanto, a análise PCR de 143 amostras adequadas de fezes mostrou que ambas as espécies estavam presentes - como complexas ou não - em 15% das amostras, em ambas as instituições avaliadas. **Conclusões:** A alta frequência de infecções de protozoários pode indicar uma contaminação fecal-oral extensa. Além disso, pode assegurar a base para ações de intervenção direcionadas em algumas comunidades para evitar a transmissão de parasitas da água que fornecem infra-estrutura sanitária. Também são necessários programas educacionais de saúde para habitantes de comunidades de baixa renda de Niterói, Estado do Rio de Janeiro, Brasil, a fim de proporcionar uma melhor compreensão dos enteroparasitas e suas medidas profiláticas.

Palavras-chave: *Blastocystis* spp, *E. histolytica*/*E. dispar*, diagnóstico

1 INTRODUCTION

Intestinal parasitic infections in humans have an important impact on the health of the population of poor regions in countries where they still prevail. Although the transmission of intestinal parasites is well described, the lack of access to information combined with low social and economic conditions contribute to the maintenance of parasites transmitted by water and soil and promote their dissemination in the environment. Health education actions and interventions with the aim of controlling the transmission of intestinal parasitic infections are scarce and punctual, but they are still the most effective procedures for reducing prevalence rates and risk factors in susceptible areas. In Rio de Janeiro, some studies have shown that intestinal parasitic infections still

occur, especially among socially vulnerable communities in the state ^{1,2,3,4}. Despite the efforts of educational and religious institutions to improve the quality of life of those communities, some of them are located in conflict areas that hinder other social interventions, mainly by government and health authorities. In fact, most health and education activities are aimed at children and their families, in order to promote social, environmental and health education bases combined with assistance activities. In this context, although intestinal parasitic infections are well documented in childhood and have an impact on cognitive functions, there are few initiatives aimed at increasing the knowledge and awareness of children and families in the poorest regions of Niterói. However, some actions were implemented in order to provide additional data on infection rates, together with a sanitary education program for children and residents of some communities, carried out in educational institutions in Niterói ^{1,3}. In this study, we report two years of actions in health education and parasitological and molecular diagnosis.

2 MATERIAL AND METHODS

Study area: Niterói is located in the state of Rio de Janeiro and has almost 500.000 inhabitants, most of them in urban areas. The municipality occupies an area of 133,919 km² and the climate is humid subtropical, and temperatures around 20⁰C and above 30⁰C in the winter and summer seasons, respectively. In a report presented by the United Nations Development Program (UNDP) the municipality was described as the one with the highest Human Development Index (HDI) of the country – third among 5700 Brazilian municipalities according to the standards of the United Nations Organization (UNO) However, some regions and neighborhoods still live in poor sanitary and economic conditions and in an unhealthy environment.

Study population: A community-based cross-sectional survey was conducted from May 2016 to September 2017, using two educational institutions of Niterói - at Pendotiba and Piratininga neighborhood - as a basis for contact with participants. Meetings were scheduled by the school staff and the lectures were given by our research group on enteroparasites (transmission, prevention and diagnostic measures) and the objectives of our study.

Stool samples: Participants received two flasks - one with 10% formalin to collect three samples on alternate days - and a dry one, for a single fresh sample. Fresh stool

samples were used to perform Rugai, Mattos & Brisola technique (1954) for live nematodes and aliquots were frozen for molecular analysis⁵. Preserved stool samples were processed through the techniques of Hoffman; Pons & Janer (1934) and Willis (1921)^{6,7}. Duplicate slides of each sample were prepared and examined separately by two operators. All analyzes were performed at the Laboratory of Parasitology of the Hospital Universitário Antonio Pedro, Pathology Department of the Medical School/UFF.

Molecular detection of *Entamoeba histolytica*/*Entamoeba dispar* infections:

220 mg of frozen samples were used for DNA extraction with QIAamp DNA Stool Mini Kit (Qiagen), following the manufacturer's instructions. Five µL of the extracted DNA obtained were amplified through Nested-PCR according to previous protocol for *E.histolytica*/*E. dispar* detection⁸. Initially, the 1,076 bp fragment of the small subunit rRNA gene sequence common to the *Entamoeba* genus was amplified using primers E1 (5-TGCTGTGATTA-AAACGCT-3) and E2 (5-TTAACTATTTCAATCTC-GG-3). Nested-PCR was performed with primers Eh-L (5-ACATTTTGAAGACTTTATGTAAGTA-3) and Eh-R (5-CAGATCTAGAAACAATGCTTCTCT-3) which are specific for *E. histolytica* and amplify a 427bp fragment, and the primers Ed-L (5-GTTAGTTATCTAATTTTCGATTAGAA-3) and Ed-R (5-ACACCACTTACTATCCCTACC-3), specific for *E. dispar* and amplify a 195 bp product. DNA from *E. histolytica* and *E. dispar* culture was used as a positive controls and a sample with no DNA fragment as a negative control. The PCR products were submitted to electrophoresis on 1.5% agarose gel and visualized under ultraviolet light after staining with ethidium bromide.

Epidemiological data and statistical analysis: A self-administered questionnaire was used to collect data in order to assess life style, habits and sanitation conditions of the participants. Categorical variables were expressed as frequency rates and data were tested for significance using the chi-square test with significance levels at 5%, 1% and 0,1% for reject the null hypothesis.

Ethical approval and informed consent

Participants received a written and signed an informed consent. This study was approved by the Ethics Committee of the Medical School of Universidade Federal Fluminense, Niteroi, Rio de Janeiro, Brazil, Protocol number 3.326.585.

3 RESULTS

A total of 150 participants (62,7 % female, 37,3% male), ages ranging from 2 months to 71 years old (SD = 50.09 years) were enrolled in the study.

FREQUENCY OF ENTEROPARASITES IN THE STUDY POPULATION

Infections were detected through the Hoffmann, Pons & Janner technique, however, only two samples presented rhabditiform larval stages of *Strongyloides stercoralis* and all samples were found negative through Willis technique. Enteroparasites were present in stool samples collected at both institutions, and protozoan species (95.76%) were more frequent than helminth ones (4.24%) as only *Enterobius vermicularis* and *S. stercoralis* were found. The protozoan *Blastocystis* spp was detected in high frequency rates in both institutions. Also, it was the most frequently found species among positive samples, prevailing significantly over *Endolimax nana*, *Entamoeba coli* and *Giardia lamblia* in both institutions ($p < 0.05$) (Table 1).

Table 1. Frequency of intestinal parasite in stool samples collected at two educational institutions in Niterói, RJ, Brazil (2016-2017).

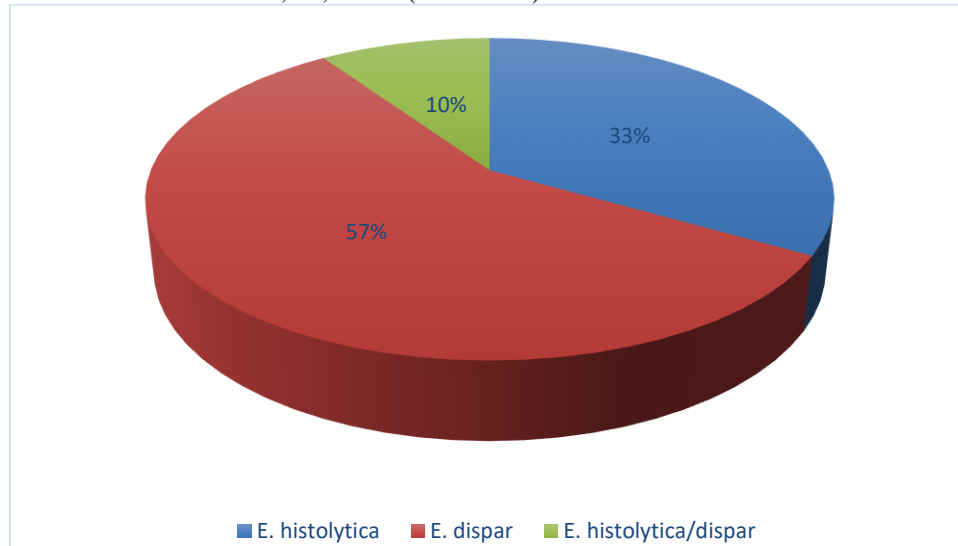
Enteroparasite species	Total (%)
Protozoans	113/118 (95.76%)
<i>Blastocystis</i> spp	65/118 (55.08%)
<i>Endolimax nana</i>	19/118 (16.10%)
<i>Giardia lamblia</i>	16/118 (13.56%)
<i>Entamoeba coli</i>	13/118 (11.02%)
Helminths	5/118 (4.24%)
<i>Enterobius vermicularis</i>	3/118 (2.54%)
<i>Strongyloides stercoralis</i>	2/118 (1.69%)

E. HISTOLYTICA/E. DISPAR INFECTIONS

A total of 150 stool samples were evaluated through coproscopy and all of them were negative for the complex *Entamoeba histolytica/E.dispar*. However, 143 suitable samples for PCR analysis were used to detect and differentiate *Entamoeba histolytica* and *E. dispar* species. In total, species-level identification could be carried out with 21 stool samples PCR positive (15%). A total of 58 samples from Pendotiba were analyzed and 13 (22%) were found positive while among those from Piratininga - total of 85 - 8 (9.4%)

were positive for *Entamoeba* sp. Most of *E. histolytica* infections were detected in Pendotiba based school whereas at the one in Piratininga, *E. dispar* infections prevailed. Mixed infections were found only in Pendotiba (**Figure 1**).

Figure 1. *E. histolytica/E. dispar* infections detected through Nested-PCR among participants at two educational institutions in Niterói, RJ, Brazil (2016-2017).



FREQUENCY OF INTESTINAL PARASITES ASSOCIATED SYMPTOMS

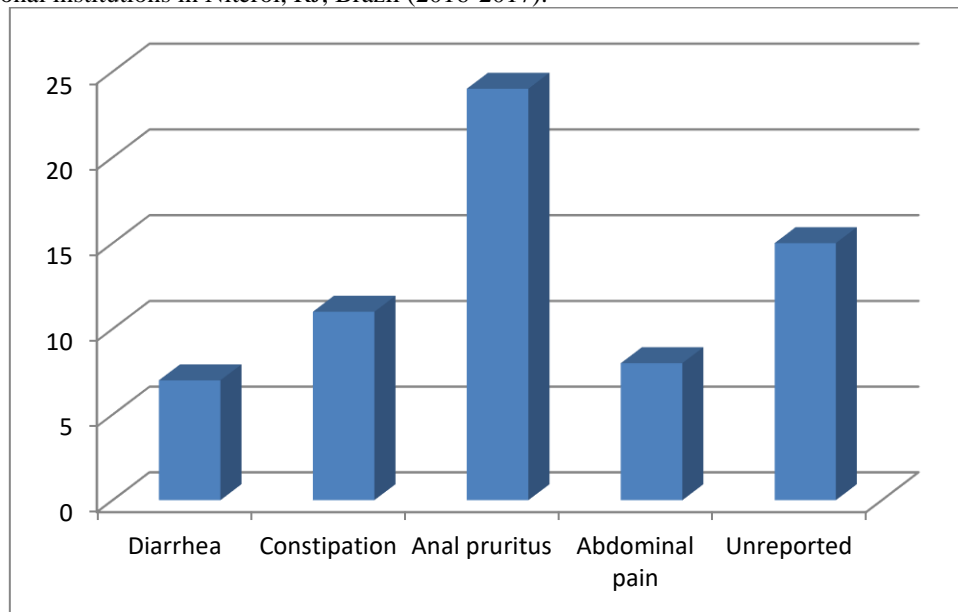
The frequency of intestinal parasites' associated symptoms by infected participants is shown in **Table 2**. In order to apply the chi-square test it was necessary that the expected frequencies were greater than 5. However, only *Blastocystis spp* satisfied this condition. So, considering the null hypothesis for all 5 classes as 20% for each symptom and testing the chi-square test for this hypothesis, the chi-square calculated is 14.62 ($p < 0.001$). It implies that this hypothesis must be reject, i.e., the proportions are no equal and the principal symptoms is anal pruritus (**Figure 2**).

Table 2. Frequency of intestinal parasites associated symptoms reported by infected participants according to parasite species found in both Institutions studied in Niterói, RJ, Brazil (2016-2017).

Enteroparasite	D	C	AP	APAIN	NR
<i>Blastocystis spp</i>	7/65	11/65	24/65	8/65	15/65
<i>E. nana</i>	2/19	2/19	8/19	3/19	4/19
<i>G. lamblia</i>	2/16	1/16	5/16	2/16	6/16
<i>E. coli</i>	1/15	1/15	5/15	2/15	6/15
<i>E. vermicularis</i>	1/2	-	2/2	-	-
<i>S. stercoralis</i>	1/2	-	1/2	-	-

E. coli occurs more than one symptoms reported. Values in table represent: D) diarrhea; C) constipation; AP) anal pruritus; APAIN) abdominal pain; NR) unreported.

Figure 2. Frequencies of associated symptoms reported in *Blastocystis spp* infected participants at two educational institutions in Niterói, RJ, Brazil (2016-2017).



* Frequencies are expressed as percentual (%) found among *Blastocystis spp* infected participants.

Table 2. Frequency of intestinal parasites associated symptoms reported by infected participants according to parasite species found in both Institutions studied in Niterói, RJ, Brazil (2016-2017).

Enteroparasite	D	C	AP	APAIN	NR
<i>Blastocystis spp</i>	7/65	11/65	24/65	8/65	15/65
<i>E. nana</i>	2/19	2/19	8/19	3/19	4/19
<i>G. lamblia</i>	2/16	1/16	5/16	2/16	6/16
<i>E. coli</i>	1/15	1/15	5/15	2/15	6/15
<i>E. vermicularis</i>	1/2	-	2/2	-	-
<i>S. stercoralis</i>	1/2	-	1/2	-	-

E. coli occurs more than one symptoms reported. Values in table represent: D) diarrhea; C) constipation; AP) anal pruritus; APAIN) abdominal pain; NR) unreported.

4 DISCUSSION

The results obtained in the present study corroborate previous information on the prevalence of intestinal parasites in some communities of Niterói, RJ, Brazil ^{1,3}. Indeed, it is known that the distribution of enteroparasites clearly correlates with factors such as climatic conditions, environmental issues, educational pattern of the population as well as sanitation. In short, these conditions are frequently provided in particular areas of developing countries ^{9,10,11}. A total of 150 stool samples were obtained and in general, in both institutions we found similar infection rates. It is important to point out that infections were predominantly caused by protozoan species and only 2% by helminths. Still, in our results the frequency of protozoans (95.76%) were higher than (78.28%) than those obtained in previous work carried out in Rio Grande do Sul, Brazil ¹². Raso ¹³ studied frequency of intestinal parasites in ambulatory patients from Antonio Pedro Hospital,

Niterói, RJ, Brazil and observed inferior frequencies for *Blastocystis spp* (34,6%) and *G. lamblia* (7,7%).

Although all participants reported no previous treatments, it is not uncommon for inhabitants of low income communities to take a single dose of Albendazol 400 mg twice a year as a self-medication practice. If this habit is associated with the low prevalence of helminth infections or may select *S. stercoralis* it is unknown as self medication was not the subject of the present study. In contrast, the protozoan *Blastocystis spp* was the most prevalent enteroparasite in the stool samples present in more than 70% of positive samples. As a matter of fact, *Blastocystis spp* is frequently found in coproparasitological surveys carried out in developing countries where its prevalence can vary from 3 to 100% according to the region¹⁴. In developed countries, *Blastocystis spp* can be found in low rates such as 5% as reported in Japan and Singapore, for example - while in the United States, prevalence rates of 10% were already found¹⁵. Additionally, studies performed in public schools in Brazil found prevalence rates varying from 26-38% for this enteroparasite. In Niterói, rates as high as 46 and 44%, were found among institutions for neurological disorders and from a host house respectively^{1,16,17,18}. However, many doubts concerning biological and pathogenic aspects of this parasite remain. Enteroparasites such as *E. nana*, *G. lamblia* and *E. coli* were also found in the present study, and it is possible that contamination is related to water of consumption or deficient hygiene habits. It is known that infections by *Blastocystis spp* may be related to the appearance of diarrhea, constipation, anal itching, abdominal pain and cutaneous rash^{15,19}. Thus, some traditional symptoms related to infections were evaluated through a questionnaire and most of them were present in the participants infected by *Blastocystis spp*, however, in the present study, anal pruritus prevailed significantly over other symptoms among *Blastocystis*'s infected participants.

Prevalence rates of infections by *E. histolytica* are overestimated, due mainly to epidemiological overlap with other morphologically identical species – *E. dispar* and *E. moshkovskii*, currently composing the *E. histolytica/E. dispar/E. moshkovskii* complex²⁰. The pathogenicity of *E. histolytica* is well established. While this species is able to cause invasive intestinal and extraintestinal disease, *E. dispar* and *E. moshkovskii* are considered non pathogenic even though infection by the first one is more common²¹. Besides, coproparasitological techniques may be affected by parasite load and since both species of the complex *E. histolytica/E. dispar* may have irregular cysts elimination, false negative results can occur in the coproscopy²². Indeed, as they share similar morphology

their species differentiation through microscopy is impaired. Surprisingly, no evolutive forms of the parasite were detected in the stool samples examined through coproscopy. Therefore, a molecular approach was necessary to rule out the possibility of false negative results. As a matter of fact, in the present work only primers for *E. histolytica* and *E. dispar* were employed and both species were found through Nested PCR in stool samples from both institutions. Thus, 5.08% of the studied samples from both institutions were infections by *E. dispar* while 4.24% were *E. histolytica*, and 1.69% for both (*E. histolytica/dispar*). Similar results were found by Calegar et al.²¹ studying stool samples from participants of the semiarid region of Brazil where 57.1% were infections by *E. dispar*, 23.8% were *E. histolytica* and mixed infections *E. histolytica/E. dispar* accounted for 14.3% of analyzed samples. Using the same system in a study carried out among children in Niterói municipality, 37 stool samples were studied by Nested-PCR, and amplification was obtained only when specific primers for *E. dispar* were employed, thus no infections by *E. histolytica* could be observed¹. Santos et al.²³ found a prevalence of 21% for four nucleous amoebae in two slums in Rio de Janeiro, but only two samples were positive for *E. histolytica*. In our results, it was not possible to correlate findings in optical microscopy with PCR, as the stool samples were all negative in coproscopy. Instead, in view of difficulties to purify DNA from stool samples due to hardness of cyst wall and the presence of inhibiting substances, which can inhibit Taq polymerase²⁴ it is possible that some samples had provided false negative results. Nevertheless, most participants with positive results were asymptomatic and the ones with *E. histolytica* positive results were treated by a physician. In short, the high frequency of protozoa infections may indicate extensive water, as well as faecal-oral contaminations. The results in our system emphasize continuous surveillance and programs for health education and medical assistance among inhabitants of low income communities of Niterói, Rio de Janeiro State. Thus, it will be possible a better understanding of transmission, and in hope that all provided data could be employed by municipal government to implement targeted actions towards prophylactic and sanitation measures.

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CONFLICT OF INTEREST

The authors state that there was no conflict of interest to declare.

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