RESEARCH ARTICLE

Spatial and Molecular Epidemiology of *Giardia intestinalis* Deep in the Amazon, Brazil

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Abstract

**Background**

Current control policies for intestinal parasitosis focuses on soil-transmitted helminths, being ineffective against *Giardia intestinalis*, a highly prevalent protozoon that impacts children’s nutritional status in developing countries. The objective of this study was to explore spatial and molecular epidemiology of *Giardia intestinalis* in children of Amerindian descent in the Brazilian Amazon.

**Methodology/Principal Findings**

A cross sectional survey was performed in the Brazilian Amazon with 433 children aged 1 to 14 years. Fecal samples were processed through parasitological techniques and molecular characterization. Prevalence of *G. intestinalis* infection was 16.9% (73/433), reaching 22.2% (35/158) among children aged 2–5 years, and a wide distribution throughout the city with some hot spots. Positivity-rate was similar among children living in distinct socioeconomic strata (48/280 [17.1%] and 19/116 [16.4%] below and above the poverty line, respectively). Sequencing of the β*-giardin* gene revealed 52.2% (n = 12) of assemblage A and 47.8% (n = 11) of assemblage B with high haplotype diversity for the latter. The isolates clustered into two well-supported *G. intestinalis* clades. A total of 38 haplotypes were obtained, with the following subassemblages distribution: 5.3% (n = 2) AII, 26.3% (n = 10) AIII, 7.9% (n = 3) BIII, and 60.5% (n = 23) new B genotypes not previously described.

**Conclusions/Significance**

*Giardia intestinalis* infection presents a high prevalence rate among Amerindian descended children living in Santa Isabel do Rio Negro/Amazon. The wide distribution observed in a small city suggests the presence of multiple sources of infection, which could be related to environmental contamination with feces, possibly of human and animal origin, highlighting...
the need of improving sanitation, safe water supply and access to diagnosis and adequate treatment of infections.

Introduction

Among the intestinal parasites, *Giardia intestinalis* stands out for its high frequency in different socioenvironmental scenarios and its prevalence in both developed and developing countries [1–5]. *G. intestinalis* presents high levels of genetic diversity, which have been classified into eight assemblages (A–H). Parasites isolated from humans belong to the globally distributed assemblages A and B, which also have other animals as hosts, being potentially zoonotic [6]. Genotypes C and D have been described in domestic and wild canines, genotype E in domestic ruminants and pigs, F in cats, G in mice and rats and H in seals [7].

Infections with *G. intestinalis* occur after the ingestion of cysts in contaminated water, directly from person to person by fecal-oral contamination or, occasionally, from food [7]. Low-income populations residing in environments with poor household sanitation level and without safe water supply are more vulnerable to water and excreta-related diseases. Contaminated central water supplies can be the source of community-wide outbreaks or spreading of *G. intestinalis* [4]. Giardiasis prevalence ranges to 20–30% in developing countries and 2–7% in developed countries, being, in the latter, frequently related with day care center disease and public pools outbreaks, and also to travel-associated diarrhea [4,8].

Although *G. intestinalis* is an important cause of diarrhea, most infections have chronic and asymptomatic character [9]. The pathogenicity of *G. intestinalis* includes apoptosis of enterocytes, epithelial cell damage, and consequent malabsorption [10]. Importantly, *G. intestinalis* infection has been shown to impact the nutritional status of children, with the potential of seriously compromising their physical development [11–15].

While control policies for intestinal parasitoses have been successful against soil-transmitted helminths, these same policies are ineffective against protozoan parasites as the treatment for the diseases they cause requires different drugs and more complex ministration schedules [16–18]. Here we assessed the prevalence, spatial distribution and molecular epidemiology of *G. intestinalis* infection in children of Amerindian descent that live in a remote municipality in the Brazilian Amazon.

Materials and Methods

This study was a cross-sectional survey performed in 433 children from Santa Isabel do Rio Negro in 2011 (Fig 1A). This small city in Brazilian Amazon was occupied mainly by Amerindians, descendent from the Tukano and Aruak speaking societies. Although the overall population of this area was approximately 18,000 people, this study was conducted in the urban area, comprised of approximately 5,000 inhabitants, distributed among six districts: Aparecida (APA), Centro (CEN), Santa Inês (SI), São José Operário (SJO), São Judas Tadeu (SJT), and Santana (SAN). All children included in our study were at maximum 14 years old. None of them presented with diarrhea during the study. Containers without preservatives were distributed for stool samples collection, and parasitological tests were performed using ether sedimentation technique [19].

Georeferencing was performed with a Global Position System in the SAD-69 geodetic datum. Spatial data were analyzed in a GIS platform using ArcGis 9.3® software (Environmental Systems Research Institute, Redlands, CA-USA). Maps were generated using the kernel...
density estimation method, and only first order effects were evaluated. The maps were made using data provided by OpenStreetMap available under the Open Database License (https://www.openstreetmap.org/copyright).

DNA was extracted, in a field laboratory, only from parasitologically confirmed *G. intestinalis*-positive stool samples using the ZR Fungal/Bacterial DNA kit (ZymoResearch, Irvine-USA). For the amplification of the 753-bp β-giardin (βG) gene fragment we utilized the G7-G759 primers, as described by Cacciò et al. [20]. Products were purified using the Illustra-GFX kit (GE Healthcare, Pittsburgh, PA-USA) and sequenced with the ABI-BigDye Terminator kit (Applied Biosystems, Foster City, CA-USA) using ABI 3730 (Applied Biosystems) automated sequencer. In addition, sequences that presented double peaks were cloned using pGEM-1 T-Easy (Promega, Madison, WI-USA). Briefly, the inserts were amplified by PCR using the M13 primer and sequenced [21]. We used the Bioedit-7.1 and Mega-6.0 in order to edit and align the sequences.

Bayesian and maximum-likelihood phylogenetic trees based on 657-bp βG sequences were inferred with BEAST-1.8 and PhyML-3.0, respectively. The Akaike and Bayesian Information Criteria of jMODELTEST-2 were used to elect Tamura-Nei with four gamma categories as the best-fit evolutionary model for the dataset. Eighteen orthologous sequences representing the diversity of *G. intestinalis* (six of the eight known assemblages) were retrieved from GenBank and added to the analyses. Genealogies were reconstructed with Network-4.6 (Fluxus-Engineering, Inc.) using the median-joining method with maximum-parsimony post-processing.

**Ethics Statement**

This study was approved by the Evandro Chagas Research Institute Committee for Ethics on Research of FIOCRUZ (0011.0.009.000–3). The parent or legal guardian of all children included in this study provided written informed consent on their behalf.
Results and Discussion

The prevalence of *G. intestinalis* infection was 16.9% (73/433). Infection was more frequent among children aged 2–5 years old and among males (Table 1). In addition, giardiasis was observed in distinct income strata with similar frequencies.

SI presented a significantly higher *G. intestinalis* positivity rate than the other districts (Table 1). A similar trend was observed with the kernel analysis that identified infection hot-spots in the APA and SI districts (Fig 1B).

Since the extraction was performed in a field laboratory, it was possible to obtain DNA from 50/73 (68.5%) positive stool samples. From these, 23 (46.0%) were good-quality sequences (fragment of 657-bp). The isolates clustered into two well-supported *G. intestinalis* clades (Fig 2A). The assemblage frequencies were 52.2% (n = 12) for A and 47.8% (n = 11) for B. While assemblage A sequences obtained did not contain double-peaks, most assemblage B sequences did (n = 7), and were thus cloned. Up to five different haplotypes could be retrieved from a single sample in five clones analyzed.

*G. intestinalis* assemblages A and B are widely distributed in the studied region (Fig 2B). Assemblage A exhibited low haplotype diversity, with two haplotypes separated from each other by two mutation steps. One haplotype of assemblage A was observed in five of the six districts, and the other one only in APA. On the other hand, assemblage B exhibited high haplotype diversity (Hd = 0.85), with 22 different haplotypes separated by 1–10 mutation steps.

### Table 1. Distribution of *Giardia intestinalis* infection according to sociodemographic characteristics in Santa Isabel do Rio Negro-AM, Brazil, 2011.

| Characteristic                        | Number of *Giardia intestinalis* positive / examined subjects (% positive) | p-value<sup>a</sup> |
|---------------------------------------|--------------------------------------------------------------------------|----------------------|
| Località                              |                                                                          |                      |
| Aparecida                             | 7/47 (14.9%)                                                             | 0.026                |
| Centro                                | 3/20 (15.0%)                                                             |                      |
| Santana                               | 18/104 (17.3%)                                                           |                      |
| Santa Ines                            | 25/87 (28.7%)                                                            |                      |
| São José Operário                     | 11/104 (10.6%)                                                           |                      |
| São Judas Tadeu                       | 9/71 (12.7%)                                                             |                      |
| **Sex**                               |                                                                          |                      |
| Female                                | 29/208 (14.0%)                                                           | 0.157                |
| Male                                  | 44/225 (19.6%)                                                           |                      |
| **Age (years)**                       |                                                                          |                      |
| 0–1                                   | 10/60 (16.7%)                                                            | 0.164                |
| 2–5                                   | 35/158 (22.2%)                                                           |                      |
| 6–11                                  | 25/192 (13.0%)                                                           |                      |
| 12–14                                 | 3/17 (17.6%)                                                             |                      |
| Unknown                               | 0/6 (0%)                                                                 |                      |
| **Income per capita per month (USD)** |                                                                          |                      |
| USD 1 = BRL 4                         |                                                                          |                      |
| Below the poverty line (≤ 38.5)       | 48/280 (17.1%)                                                           | 0.854                |
| Above the poverty line (> 38.5 and ≤ 330) | 19/116 (16.4%)                                              |                      |
| Unknown                               | 6/37 (16.2%)                                                             |                      |

<sup>a</sup>Fisher exact test.

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These were present in four districts and only one haplotype was shared between two localities, APA and SI.

A total of 38 haplotypes were obtained, with the following subassemblages distribution: 5.3% (n = 2) AII, 26.3% (n = 10) AIII, 7.9% (n = 3) BIII, and 60.5% (n = 23) new B genotypes not previously described (Table 2). Two distinct epidemiological scenarios were observed. While children living in the same house were infected by the same assemblage, surprisingly children in one house were infected by distinct assemblages (AIII and new B haplotypes).

*G. intestinalis* infection was distributed throughout the city, with some hotspots of higher frequency. Interestingly, *G. intestinalis* positivity is not associated with income stratum. The wide distribution observed in a small city suggests the presence of multiple sources of infection, which could be related to environmental contamination with feces, possibly of human and animal origin [22]. Many houses do not have access to potable water, being served by two sources: “black water”, which is drawn from the Rio Negro and chlorinated in a plant, and “white water”, which is taken up in wells. The vast majority of homes do not have septic tanks or latrines and the disposal of feces is done directly in the river. This practice may facilitate the spread of different haplotypes of *G. intestinalis*. The observed hotspots on the margins of the Rio Negro suggest that people who live closer to the river are at greater risk of becoming infected.

The high genetic divergence between A and B (5.5–6.3%) supports previous proposal for their separation in two taxa, *G. intestinalis* and *Giardia enterica* [23, 24]. Assemblage A haplotypes detected in the present study are identical to European strains [20], evidencing their low
genetic divergence. In contrast, assemblage B haplotypes were highly diverse, being possible to observe up to five different clones in a single sample. Previous genome sequencing analysis evidenced a 10-fold difference in heterozygosity levels between assemblages A and B [24], but the reasons for such difference are still unknown and deserves further investigation.

The Amazon region is the largest drainage basin in the world and harbors one-fifth of the fresh water reserves on the planet. Paradoxically, the living conditions of many people who inhabit this basin are substandard, favoring the transmission of fecal-oral diseases such as giardiasis. It has been proposed that the routine water treatment practices usually employ

| Community          | Sample ID | Assemblage | Subassemblage | GenBank accession number |
|--------------------|-----------|------------|---------------|--------------------------|
| Aparecida          | S17       | A          | All           | KU504725                 |
| Aparecida          | S23       | A          | AllI          | KU504729                 |
| Aparecida          | S35       | A          | AllI          | KU504735                 |
| Aparecida          | S36       | A          | AllI          | KU504736                 |
| Aparecida          | S38       | A          | All           | KU504737                 |
| Centro             | S8        | B          | New           | KU504707                 |
| Santa Inês         | S3C1      | B          | New           | KU504702                 |
| Santa Inês         | S3C2      | B          | New           | KU504703                 |
| Santa Inês         | S3C3      | B          | New           | KU504704                 |
| Santa Inês         | S9        | B          | New           | KU504708                 |
| Santa Inês         | S11C1     | B          | New           | KU504712                 |
| Santa Inês         | S11C2     | B          | New           | KU504713                 |
| Santa Inês         | S11C3     | B          | New           | KU504714                 |
| Santa Inês         | S11C4     | B          | New           | KU504715                 |
| Santa Inês         | S11C5     | B          | AllI          | KU504716                 |
| Santa Inês         | S12       | A          | AllI          | KU504717                 |
| Santa Inês         | S15       | B          | New           | KU504720                 |
| Santa Inês         | S18       | B          | New           | KU504726                 |
| Santa Inês         | S24C1     | B          | New           | KU504730                 |
| Santa Inês         | S24C2     | B          | New           | KU504731                 |
| Santa Inês         | S24C3     | B          | AllI          | KU504732                 |
| Santa Inês         | S42       | A          | AllI          | KU504738                 |
| São José Operário  | S2        | A          | AllI          | KU504701                 |
| São José Operário  | S7        | A          | AllI          | KU504706                 |
| São José Operário  | S21C2     | B          | New           | KU504727                 |
| São José Operário  | S21C3     | B          | New           | KU504728                 |
| São Judas Tadeu    | S29       | A          | AllI          | KU504733                 |
| São Judas Tadeu    | S30       | A          | AllI          | KU504734                 |
| Santana            | S4        | A          | AllI          | KU504705                 |
| Santana            | S10C3     | B          | New           | KU504709                 |
| Santana            | S10C4     | B          | New           | KU504710                 |
| Santana            | S10C5     | B          | New           | KU504711                 |
| Santana            | S13C1     | B          | New           | KU504718                 |
| Santana            | S13C2     | B          | New           | KU504719                 |
| Santana            | S16C1     | B          | New           | KU504721                 |
| Santana            | S16C2     | B          | New           | KU504722                 |
| Santana            | S16C4     | B          | New           | KU504723                 |
| Santana            | S16C5     | B          | AllI          | KU504724                 |

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concentrations of chlorine able to inactivate only bacterial and viral pathogens, but not *Giardia* cysts [25–26]. Thus, adequate control of giardiasis, particularly in Amazon region requires the improvement of drinking water quality and reduction of environmental contamination with feces [27]. Despite the high prevalence of giardiasis and its health impact worldwide, large-scale interventions—as those implemented for STH control—are lacking in developing countries. In this context, enteric protozoa infections emerge as neglected conditions in the STH control era [18].

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**Author Contributions**

Conceived and designed the experiments: BCN MNB FACC. Performed the experiments: BCN MGP LHJ KJLM FACC. Analyzed the data: BCN MGP LHJ SCCX FAM MNB FACC. Contributed reagents/materials/analysis tools: SCCX FAM MNB FACC. Wrote the paper: BCN MGP LHJ KJLM SCCX FAM MNB FACC.

**References**

1. Ehsan AM, Geurden T, Casaert S, Parvin SM, Islam TM, Ahmed UM, et al. Assessment of zoonotic transmission of *Giardia* and *Cryptosporidium* between cattle and humans in rural villages in Bangladesh. PLoS One. 2015; 10(2):e0118239. doi:10.1371/journal.pone.0118239 PMID: 25695662
2. Barry MA, Weatherhead JE, Hotez PJ, Woc-Colburn L. Childhood parasitic infections endemic to the United States. Pediatr Clin North Am. 2013; 60(2):471–85. doi:10.1016/j.pcl.2012.12.011 PMID: 23481112
3. Jenkins EJ, Castrodale LJ, de Rosemond SJ, Dixon BR, Elmore SA, Gesy KM, et al. Tradition and transition: parasitic zoonoses of people and animals in Alaska, northern Canada, and Greenland. Adv Parasitol. 2013; 82:33–204. doi: 10.1016/B978-0-12-407706-5.00002-2 PMID: 23548085
4. Fletcher SM, Stark D, Harkness J, Ellis J. Enteric protozoa in the developed world: a public health perspective. Clin Microbiol Rev. 2012; 25(3):420–49. doi: 10.1128/CMR.00033-10 PMID: 22763633
5. David ÉB, Guimarães S, de Oliveira AP, Goulart de Oliveira-Sequeira TC, Nogueira Bittencourt G, Moraes Nardi AR, et al. Molecular characterization of intestinal protozoa in two poor communities in the State of São Paulo, Brazil. Parasit Vectors. 2015; 15(8):103. doi: 10.1186/s13071-015-0714-8 PMID: 34238665
6. Feng Y, Xiao L. Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. Clin Microbiol Rev. 2011; 24(1):110–40. doi: 10.1128/CMR.00033-10 PMID: 12123509
7. Garbossa G, Pia Buyayisqui M, Geffner L, López Arias L, de la Fournière S, Haedo AS, et al. Social and environmental health determinants and their relationship with parasitic diseases in asymptomatic children from a shantytown in Buenos Aires, Argentina. Pathog Glob Health. 2013; 107(3):141–52. doi: 10.1179/2047773213Y.0000000087 PMID: 23683369
8. Kotloff KL, Nataro JP, Blackwelder WC, Nasrin D, Farag TH, Panchalingam S, et al. Burden and aetiology of diarrhoeal disease in infants and young children in developing countries (the Global Enteric
10. Luther A, Bartelt LA, Sartor RB. Advances in understanding Giardia: determinants and mechanisms of chronic sequelae. F1000Prime Rep. 2015; 7:62. doi: 10.12703/P7-62 PMID: 26097735

11. Carvalho-Costa FA, Gonçalves AQ, Lassance SL, Silva Neto LM, Salmazo CA, Bóia MN. Giardia lamblia and other intestinal parasitic infections and their relationships with nutritional status in children in Brazilian Amazon. Rev Inst Med Trop São Paulo. 2007; 49(3):147–153. PMID: 17625691

12. Ignatius R, Gahutu JB, Klotz C, Steininger C, Shyirambere C, Lyng M, et al. High prevalence of Giardia duodenalis Assemblage B infection and association with underweight in Rwandan children. PLoS Negl Trop Dis. 2012; 6(6):e1677. doi: 10.1371/journal.pntd.0001677 PMID: 22720102

13. Nematiian J, Gholamrezanezhad A, Nematiian E. Giardiasis and other intestinal parasitic infections in relation to anthropometric indicators of malnutrition: a large, population-based survey of schoolchildren in Tehran. Ann Trop Med Parasitol. 2008; 102(3):209–214. doi: 10.1179/136485908X267876 PMID: 18348775

14. Quihui L, Morales GG, Méndez RO, Levy JA, Esparza J, Valencia ME. Could giardiasis be a risk factor for low zinc status in schoolchildren from northwestern Mexico? A cross-sectional study with longitudinal follow-up. BMC Public Health. 2010; 10:85. doi: 10.1186/1471-2458-10-85 PMID: 20170531

15. Verhagen LM, Incani RN, Franco CR, Ugarte A, Sierra Ruiz CI, et al. High malnutrition rate and soil-transmitted helminths infections in children in the Chaco region, Bolivia. Am J Trop Med Hyg. 2015; 92(4):794–796. doi: 10.4269/ajtmh.14-0039 PMID: 25711609

16. Turkeltaub JA, McCarty TR 3rd, Hotez PJ. The intestinal protozoa: emerging impact on global health and development. Curr Opin Gastroenterol. 2015; 31(1):38–44. doi: 10.1097/MOG.0000000000000135 PMID: 25394233

17. Macchioni F, Segundo H, Gabrielli S, Totino V, Gonzales PR, Salazar E, et al. Dramatic decrease in diarrheal diseases in the Brazilian Amazon. Rev Inst Med Trop São Paulo. 2007; 49(3):147–153. PMID: 17625691

18. Robertson LJ, Lim YAL. Waterborne and Environmentally-Borne Giardiasis. In: Luján HD, Svärd S. Giardia: A model organism. Springer Wien, New York; 2011.pp. 29–61.