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Article

Genotypic and epidemiologic profiles of *Giardia duodenalis* in four Brazilian biogeographic regions

Deiviane A. Calegar[1]*, Beatriz C. Nunes[2], Kerla J. L. Monteiro[3], Polyanna A. A. Bacelar[1], Brenda B. C. Evangelista[1], Mayron M. Almeida[1], Jurecir Silva[4], Jéssica P. Santos[1], Márcio N. Boia[5], Lauren H. Jaeger[6] and Filipe A. Carvalho-Costa[1]*/****

[1] Laboratório de Epidemiologia e Sistemática Molecular, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil; deivianecalegar@gmail.com (D.A.C.); polyannabio_gen@hotmail.com (P.A.A.B.); brendaevangelista5@gmail.com (B.B.C.E.); mayronmorais@outlook.com (M.M.A.); jessica.ssantos87@gmail.com (J.P.S.); guaratiba@ioc.fiocruz.br (F.A.C.C.)
[2] Faculdade de Medicina de Petrópolis (FMP)/ Centro Universitário Arthur Sá Earp Neto (UNIFASE). Rua Machado Fagundes, 326, Cascatinha, Petrópolis, Rio de Janeiro, Brasil; biacoronato@gmail.com (B.C.N.)
[3] Escritório Técnico Regional Fiocruz Piauí, Teresina, Piauí, Brasil; kerla.monteiro@gmail.com (K.J.L.M.)
[4] Instituto Federal de Educação, Ciência e Tecnologia do Piauí, Teresina, PI, Brasil; jurecir.silva@ifpi.edu.br (J.S.)
[5] Laboratório de Biologia e Parasitologia de Mamíferos Silvestres Reservatórios, Instituto Oswaldo Cruz, Fundação Oswaldo Cruz, Rio de Janeiro, RJ, Brasil; mboia@ioc.fiocruz.br (M.N.B.)
[6] Faculdade de Farmácia, Universidade Federal de Juiz de Fora. Rua José Lourenço Kelmer, s/n – Campus Universitário, Juiz de Fora, Minas Gerais, Brasil; laurenhj@gmail.com (L.H.J.)

* Correspondence: guaratiba@ioc.fiocruz.br; Tel.: 55 21 38658182
** Lauren Hubert Jaeger and Filipe Anibal Carvalho-Costa contributed equally to this work.

Abstract: Gut protozoan parasites are neglected and not targeted by specific control initiatives and this have led to a knowledge gap concerning their regional diversity and epidemiology. The present study aims to explore *Giardia duodenalis* genetic diversity and assess the epidemiologic scenario of subclinical infections in different Brazilian biogeographic regions. Cross-sectional surveys (n=1,334 subjects) were conducted in the Amazon, Cerrado, Semiarid and Atlantic Forest. Microscopy of non-diarrheal feces and nucleotide sequencing of a β-giardin gene fragment were performed. Twenty-seven (52.9%) β-giardin sequences were characterized as assemblage A and 24 (47.1%) as assemblage B. In Amazon, assemblage B was the most frequently detected with 2 novel sub-assemblages. Assemblage A predominated in the extra-Amazon region, with 5 novel sub-assemblages. Prevalence rates reached 17.8% in Amazon, 8.8% in Atlantic Forest, 7.4% in Cerrado and 2.3% in the Semiarid. People living in poverty and extreme poverty presented significantly higher positivity rates, reaching 11.9% and 14.5%, respectively. Giardiasis tended to be more frequent in stunted (21.6%) than in eutrophic children (12.9%). In conclusion, subclinical giardiasis in endemic in Brazilian communities in different biogeographic regions, presenting high genetic diversity and a heterogeneous genotypic distribution.

Keywords: *Giardia duodenalis*; Assemblages; Epidemiology; Genetic diversity

1. Introduction

Giardia duodenalis is a cosmopolitan flagellated gut protozoan parasite with higher prevalence rates in developing countries, mainly in regions with poor sanitation and inappropriate drinking water supply [1]. *G. duodenalis* trophozoites inhabits the small intestine and, during the course of infection, a variable proportion of cells continuously encyst and are shed in feces. Cysts can resist in the environment and contaminate water and food, constituting the parasite infective stage. In industrialized regions, *G. duodenalis* causes outbreaks of diarrheal disease which have, as main sources, water contaminated with fecal material [2,3].

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The Global Enteric Multicenter Study (GEMS) assessed the role of *G. duodenalis* as an etiologic agent of diarrheal diseases in children living in African and Asian developing countries in a 3-year prospective, age-stratified, multicentric, and matched case-control study, demonstrating that *G. duodenalis* infected more frequently controls than children aged 12–59 months with diarrhea [4]. Similar results were obtained in Cambodja, where higher *G. duodenalis* positivity rates were found among controls when compared with children with diarrhea [5]. Thus, in endemic areas in developing countries there is growing evidence that giardiasis is a subclinical and chronic infection, with the parasite being detected in non-diarrheal feces and frequently associated with protein-caloric malnutrition. These findings were reported in the Etiology, Risk Factors, and Interactions of Enteric Infections and Malnutrition and the Consequences for Child Health and Development Project (MAL-ED), a multisite birth-cohort study [6-8]. Therefore, in vulnerable communities of developing regions, *G. duodenalis* – through complex pathophysiological mechanisms – impacts the absorption of nutrients and affects both nutritional status and physical development of children in a process that does not depend on the presence of diarrhea [9,10].

G. duodenalis infects a wide range of mammalian species and presents considerable intraspecific genetic variation, with eight distinct assemblages (A to H). Assemblages A and B were identified mainly in humans, C and D in wild and domestic canines, E in ruminants and domestic pigs, F in cats, G in mice and rats and H in seals [11,12]. Giardiasis is a potentially zoonotic infection and cross-host transmission has been well documented [13]. The degree of genetic divergence between assemblages A and B has led to the proposition of two distinct species [14]. Studies conducted in Brazil to characterize *G. duodenalis* genetic variation revealed the predominance of assemblage A in several states, with assemblage B also being detected, to a lesser extent, in specific areas [15]. In Rio de Janeiro, assemblage E was also described in human infections [16]. Studies performed in the Amazon region demonstrate predominance of assemblage B and greater haplotype diversity [17,18].

Brazil occupies most of the South American continent and has great biogeographic and climatic diversity, with specific rainfall regimes with great variation from rain forests to semiarid, associated with different water management strategies, creating regional scenarios for the epidemiology of water-borne infections.

Infections with gut protozoan parasites are not targeted by specific intestinal parasite control strategies, which are based on the mass administration of anthelmintic drugs [19]. This have led to a knowledge gap concerning the prevalence, distribution and factors associated with subclinical giardiasis in many regions, as well as concerning parasite genetic diversity. The present study aims to describe the parasite genetic heterogeneity and the epidemiological scenario of subclinical *G. duodenalis* infection in an urban Amazonian area and three extra-Amazonian Brazilian biogeographic regions.

2. Materials and Methods

2.1 Study setting

Brazil has great social and environmental diversity. Many regions have in common a poor access to drinking water and inadequate sewage systems. The study was conducted in four localities whose sociodemographic, environmental and climatic differences, are presented in Table 1: São João do Piauí (SJPI, semiarid) and Teresina (TER, Cerrado), in the state of Piauí; Bagre (BAG, Amazon), in the state of Pará, and Cachoeiras de Macacu (CAM, Atlantic Forest), in the state of Rio de Janeiro (Figure 1).

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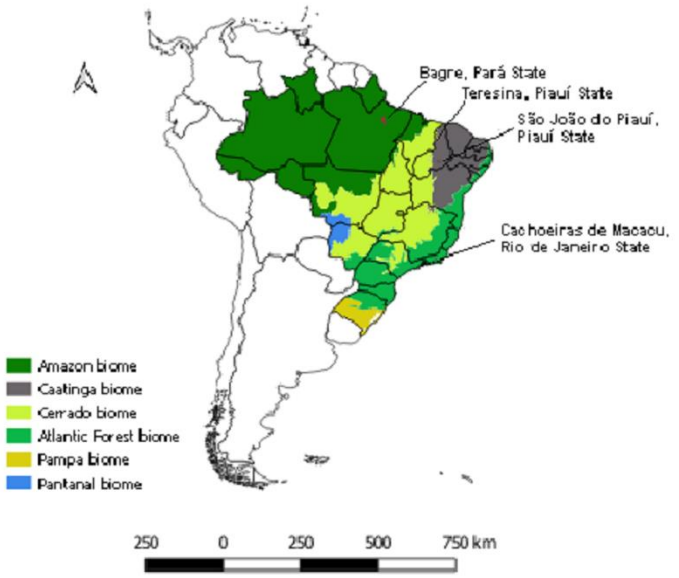

Figure 1. Map showing the location of the studied areas belonging to different Brazilian biogeographic regions. The map was created using QGis 3.12.3.

2.2 *Study design*

Cross-sectional surveys (n = 1,334, Table 1) were performed in order to obtain sociodemographic, anthropometric and sanitation data as well as fecal samples for parasitological and molecular analyses. A fecal sample was obtained from each person and the interviews were conducted face-to-face by the research team in the domiciles. Fecal samples were from non-diarrheal stools, from asymptomatic subjects. Weight, height, and arm circumference measurements were obtained from individuals between 0-14 years of age. The nutritional parameters height-for-age Z-scores (HAZ) and weight-for-age Z-scores (WAZ) were calculated with the Nutrition module of the Epi Info™ v.3.5.1 software, to verify the presence of protein-energy malnutrition characterized by stunting (HAZ < -2) and low weight (WAZ < -2). Extreme poverty was defined when monthly familiar per capita income was below R$ 125, which corresponds to US$ 25 (considering the exchange rate of US$ 1 = R$ 5). Poverty was defined by monthly familiar per capita income between R$ 125 and R$ 250 (US$ 25 – US$ 50). The researchers gathered information about the site of defecation, *i.e.*, if the family had a latrine inside the house or if members of the family practice open defecation in the peridomestic environment. The final destination of the feces was also characterized, being adequate when the feces went to closed septic tanks and inadequate when they were deposited in the ground, in rudimentary holes or directly in a water-body.

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Table 1 Socio-environmental characteristics of the different studied localities.

Characteristic	**Biome/ Localities**			
	Amazon/ Bagre (PA)	**Cerrado/ Teresina (PI)**	**Caatinga/ São João do Piauí (PI)**	**Atlantic Forest/ Cachoeiras de Macacu (RJ)**
Population	30,673	864,845	20,601	58,937
Climate*	Equatorial (Af)	Semi-humid tropical (Aw)	Semiarid (Bsh)	Semi-humid tropical (Aw)
Income (MPCHI)**				
extreme poverty[a]	144/360 (40%)	107/299 (35.8%)	44/131 (33.6%)	159/544 (29.2%)
poverty[b]	114/360 (31.7%)	91/299 (30.4%)	52/131 (39.7%)	113/544 (20.8%)
Human development index	0.471	0.751	0.645	0.700
Gini index	0.37	0.50	0.45	0.45
Water supply	Furo de Santa Maria River (Baía de Marajó)	Artesian wells	Rain water stored in cisterns and artesian wells	Macacu River and artesian wells
Year of study	2020	2017	2018	2018
Localization of districts included	Urban	Rural	Rural	Urban and rural
Fecal samples	360	299	131	544

AM: Amazonas State, **PA**: Pará State, **PI**: Piauí State, **RJ**: Rio de Janeiro State. * World map of the Koppen-Geiger climate classification system. ** **MPCHI** - monthly per capita house income (1/4 of minimum wage, considering R$ 1000). **a**-MPCHI < R$ 125 per capita. **b**- MPCHI= R$ 125 - 250 per capita.

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2.3 Parasitological examinations

Fecal samples were collected in plastic bottles without preservatives and sent to the field laboratory to be examined through light microscopy using the Ritchie and saturated glucose solution flotation techniques.

2.4 DNA extraction, PCR amplification and nucleotide sequencing of β-giardin encoding gene fragment

Polymerase chain reaction (PCR) was performed on 180 fecal samples from humans (137 positive and 43 negative for *G. duodenalis* on microscopy). Genomic DNA was extracted from 200 µL of the sedimented fecal material using the ZR Fungal/Bacterial DNA MiniPrep ™ kit (ZymoResearch, Irvine-USA). PCR was performed using the Platinum Taq DNA Polymerase kit (Invitrogen, Waltham, MA, USA) with a final volume of 50 µL, and targeted a 753 bp region of the β-giardin locus of G. duodenalis, as described [20]. The PCR conditions were: 1X PCR Buffer, 1.5 mM MgCl2, 0.05 mM dNTP, 10 pmol of each primer, 2.5 U of Taq polymerase and ~ 40 ng of template DNA. Amplification parameters included an initial denaturation at 94 °C for 5 min followed by 35 cycles of amplification comprising of denaturation (94 °C for 30 s), annealing (65 °C for 30 s) and extension (72 °C for 30 s), and a final extension at 72 °C for 5 min. The PCR products were purified with polyethylene glycol (PEG). Capillary electrophoresis was performed in an ABI3730 automated DNA sequencer (Applied Biosystems) in PDTIS/Fiocruz Genomic Platform RPT01A.

2.5 Sequence data analysis

The sequences were edited and analyzed using the BioEdit v.7.2.5 software [21]. The Basic Local Alignment Search Tool (BLAST – NCBI https://www.ncbi.nlm.nih.gov/) was used to verify similarity with G. duodenalis sequences. All sequences generated were deposited in the GenBank database under the accession numbers MW679411- MW679461. To determine *G. duodenalis* assemblages (genotypes), an alignment was performed with 55 G. duodenalis orthologous reference sequences retrieved from GenBank in BioEdit v.7.2.5 software. Sequences with degenerate bases were not included. Further details of reference strains can be found in Supplementary Table S1. The most appropriate substitution model was estimated using Bayesian Information Criterion (BIC) in MEGA v.7 software [22]. Maximum likelihood (ML) and neighbor joining (NJ) genetic tree was constructed in MEGA v.7 software using a Tamura-Nei model (bootstrap 1,000-replicates). The median-joining (MJ) haplotype network based on distance criteria was constructed using the Network v.10.1.0.0 software (Fluxus Technology Ltd., www.fluxusengineering.com) [23]. The DNA Sequence Polymorphism (DnaSP) v.5.10.01 software was used for editing the files [24]. To evaluate the intraspecific genetic diversity of G. duodenalis, diversity indexes were determined for each population pair using Arlequin v.3.5.2.2 software (http://cmpg.unibe.ch/software/arlequin35/) [25]. The populations were grouped considering: assemblage, geographic origin, and Brazilian regions.

2.6 Statistical analysis

G. duodenalis positivity rates were described in different groups defined by sociodemographic characteristics and nutritional status. Prevalence ratios (PRs) and their respective 95% confidence intervals (CIs) were calculated. The statistical significance of the differences between the positivity rates was assessed by Fisher's exact test. Associations were considered statistically significant when $p < 0.05$. Statistical analyses were performed with Epi Info 2000® (CDC, Atlanta, Georgia, USA).

2.7 Ethics

The study was approved by the Research Ethics Committee (license CAAE 12125713.5.0000.5248) of the Oswaldo Cruz Institute, Fiocruz.

3. Results

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3.1. Genetic diversity of Giardia duodenalis.

Of 180 fecal samples submitted to PCR/sequencing, 51 were successfully genotyped using the β-giardin locus. Overlapping peaks were not observed in the nucleotide sequences. Twenty-seven (52.9%) sequences were characterized as assemblage A and 24 (47.1%) as assemblage B (Table 2).

Table 2. Distribution of *Giardia duodenalis* assemblages and sub-assemblages obtained in this study based on β-giardin locus (592 bp, n = 51).

Assemblage/ sub-assemblage	**Localities**				**Total**
	Bagre (AM)	Cachoeiras de Macacu (RJ)	São João do Piauí (PI)	Teresina (PI)	
A	-	-	2	1	3
AI	1	3	3	1	8
AII	3	4	1	1	9
AIII	-	1	-	1	2
A novel	-	2	-	3	5
B	4	-	-	-	4
BIII	16	-	-	-	16
B novel	2	1	-	1	4
Total	**26**	**11**	**6**	**8**	**51**

In Amazon (BAG), assemblage B was the most frequently detected (22/28 [78.6%]) with de following sub-assemblage distribution: B not specified, n=4; BIII, n = 16, and novel sub-assemblages B, n = 2. In BAG, 4 samples (14.3%) were assemblage A (A not specified, n = 1 and AII, n = 3) (Figure 2).

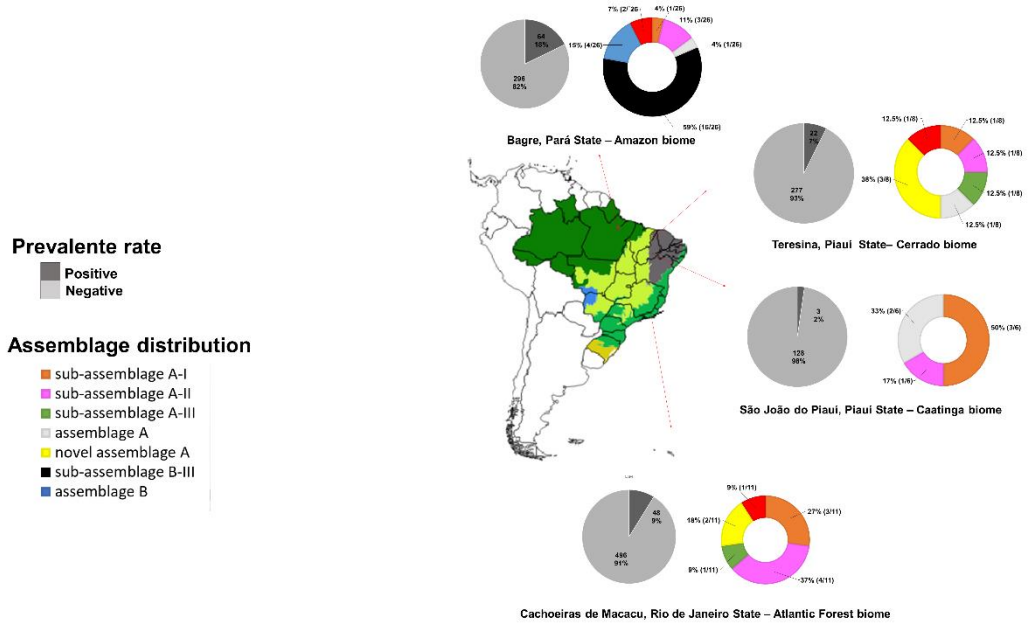

Figure 2. Geographical distribution of the different assemblages and sub-assemblages of *Giardia duodenalis* in the studied localities.

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Conversely, assemblage A predominated in the extra-Amazon region, being characterized in 10/11 (91%) of the sequences from CAM (AI = 3, AII = 4, AIII = 1, and novel sub-assemblages A = 2), 6/6 (100%) from SJPI (A = 2, AI = 3, and AII = 1), and 7/8 (88%) from TER (A = 1, AI = 1, AII = 1, AIII = 1, and novel sub-assemblages A = 3) (Figure 2, Table 3). Among assemblage A sequences, sub-assemblage AII was observed in all studied sites and AI in all sites except in Amazon biome. The MJ haplotype network (Figure 3) showed that the *G. duodenalis* sequences were grouped by assemblages, as expected. Assemblages A and B presented a star-like shape, including the sequences obtained in this study as a central and dominant haplotype (except the novel haplotypes).

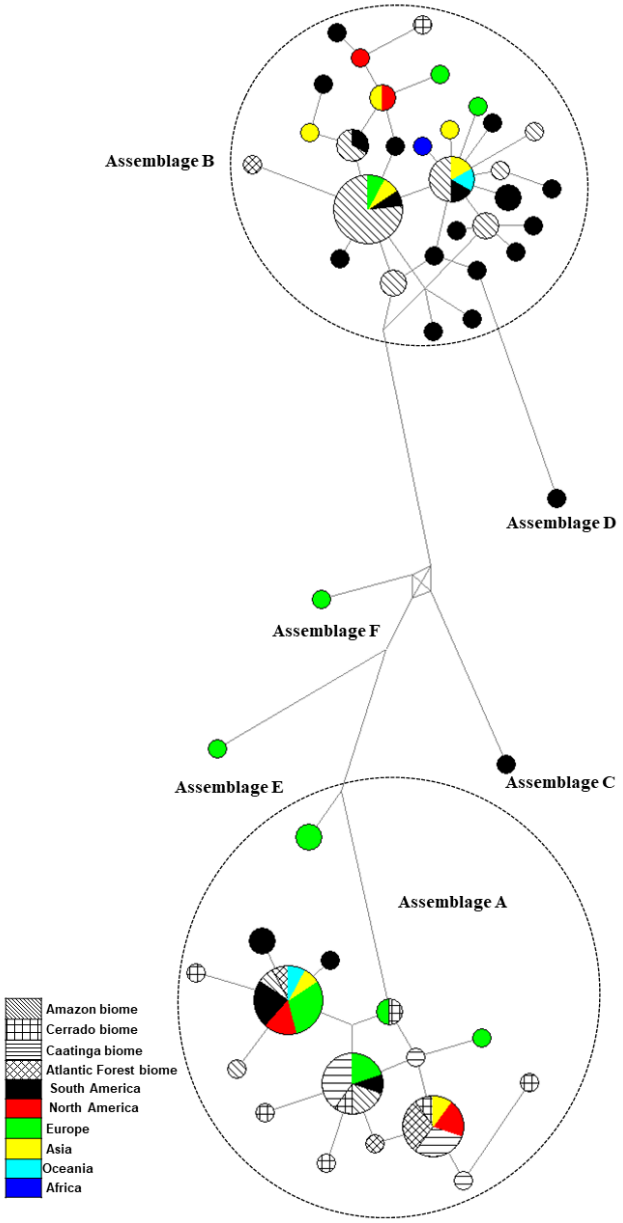

Figure 3. Haplotype network based on *Giardia duodenalis* β-giardin locus (592 bp, n=106). Area of the circle is proportional to number of sequences.

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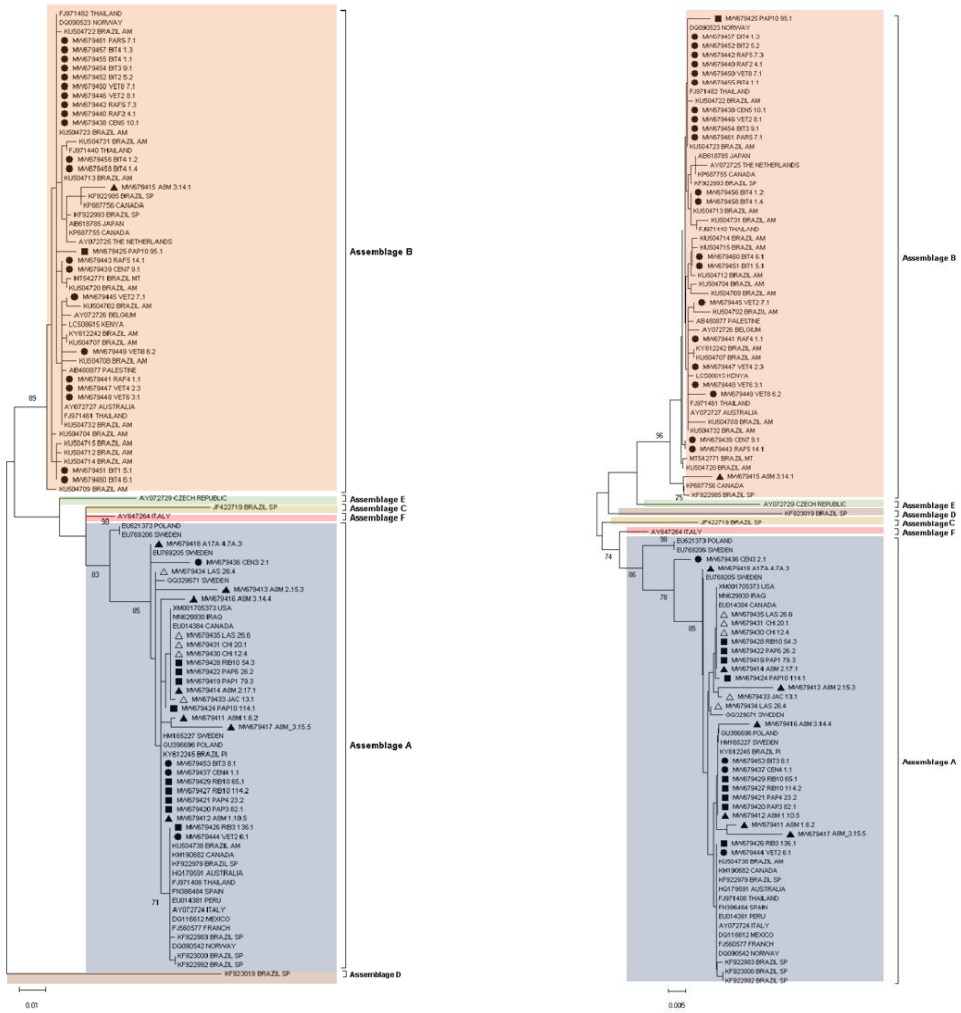
ML and NJ phylogenetic trees (Figure 4) also demonstrated that the *G. duodenalis* sequences were grouped by assemblages. The main difference between the two trees was in NJ tree, the assemblage D shared a common ancestor with Assemblages B and E. Concerning the molecular diversity indexes, in general, assemblage B revealed greater intraspecific diversity when compared with assemblage A (H = 0.921 ± 0.028 vs H=0.854 ± 0.029) (Supplementary Table S2). In Brazil the assemblage A showed greater intraspecific diversity (H = 0.879 ± 0.037) when compared with Europe and North America (H = 0.822 ± 0.096 and 0.666 ±0.204, respectively). In contrast, assemblage B in Brazil showed lower intraspecific diversity when compared with North America, Asia and Europe (H = 0.918 ± 0.033 vs 1.000 ± 0.500, 1.000 ± 0.126 and 1.000 ± 0.272). Assemblage A from Caatinga showed lower diversity when compared with the other biomes in the present study. Assemblage B from Amazon biome showed a lower diversity (H = 0.757 ± 0.086) when compared with reference sequences from the same biome (H = 0.991 ± 0.025) (Supplementary Table S2).



(**a**) (**b**)

Figure 4. (a) Maximum likelihood and **(b)** neighbor joining trees inferred from Giardia duodenalis β-giardin locus (592 bp, n=106). Support for the branching order was determined by 1,000 bootstrap replicates and only values >70% are reported. ●: Bagre – Amazon biome; ▪: Cachoeiras de Macacu – Atlantic Forest biome; Δ: São João do Piauí - Caatinga biome; ▲: Teresina – Cerrado biome. GenBank accession numbers are indicated. Further details of reference strains can be found in Supplementary Table S1.

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3.2. Prevalence rates and factors associated with Giardia duodenalis infection

Table 3 shows positivity rates (by microscopy) in different groups defined by sociodemographic characteristics and nutritional status and the association of giardiasis with other intestinal protozoa (coinfection with *Entamoeba histolytica* / *E. dispar* or with *Entamoeba coli*). The overall positivity rate was 137/1,334 (10.3%). The frequency was significantly higher in BAG, in the Amazon region, reaching 64/360 (17.8%) and lower in SJPI, in the Semiarid (3/131 [2.3%]). The age groups of 3 to 6 years old and 7 to 15 years old had the highest rates of positivity (above 13%), infants and toddlers up to 2 years old were also frequently infected (6.2%). Subclinical giardiasis was more frequent among people living in poor (PR = 2.40; 95% CI = 1.84 - 4.67) and extremely poor (PR = 2.94; 95% CI = 1.46-3.93) families. People living in scenarios of inappropriate disposal of feces also presented significantly higher positivity (PR = 2.41; 95% CI = 1.54-3.76). Infection tended to be more frequent in stunted than in eutrophic children (PR = 1.67; 95% CI = 0.95 - 2.92). Individuals positive for *Entamoeba histolytica* / *E. dispar* or *E. coli* were infected with *G. duodenalis* at frequencies five and two times higher, respectively.

Table 3. Positivity rates in different groups defined by sociodemographic characteristics and the association of giardiasis with other intestinal protozoa (Entamoeba histolytica / Entamoeba dispar or Entamoeba coli).

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	Positivity by microscopy (%)	Prevalence ratio	95% CI	p-value
Locality				
Cachoeiras de Macacu	48/544 (8.8)	1		
Bagre	64/360 (17.8)	2.01	1.42 – 2.85	<0.001
Teresina	22/299 (7.4)	0.83	0.51 – 1.35	0.515
SJ do Piauí	3/131 (2.3)	0.26	0.08 – 0.82	0.009
Total	137/1,334 (10.3)			
Sex				
Male	73/748 (9.1)	1		
Female	64/586 (10.9)	1.12	0.81 – 1.53	0.524
Age group				
0-2	12/194 (6.2)	1		
3-6	42/306 (13.7)	2.21	1.19 – 4.10	0.008
7-15	65/489 (13.3)	2.14	1.18 – 3.88	0.007
16-30	2/79 (2.5)	0.40	0.09 – 1.78	0.363
31-50	6/118 (5.1)	0.82	0.31 – 2.13	0.805
>50	7/103 (6.8)	1.09	0.44 – 2.70	0.808
Unknown	3/45 (6.7)			
Income				
>250	22/445 (4.9)	1		
125-250	44/370 (11.9)	2.40	1.84 – 4.67	<0.001
<125	66/454 (14.5)	2.94	1.46 – 3.93	<0.001
Open defecation				
Yes	36/298 (12.1)	1.21	0.85 – 1.74	0.279
No	96/969 (9.9)	1		
Unknown	5/65 (7.7)			
Inadequate feces disposal				
Yes	105/825 (12.7)	2.41	1.54 – 3.76	<0.001
No	22/415 (5.3)	1		
Unknown	10/94 (10.6)			

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Stunting				
Yes	11/51 (21.6)	1.67	0.95 – 2.92	0.089
No	89/691 (12.9)	1		
Low weight				
Yes	8/54 (14.8)	1.16	0.59 – 2.25	0.674
No	101/790 (12.8)	1		
Entamoeba coli coinfection				
Yes	39/201 (19.4)	2.24	1.59 – 3.14	<0.001
No	98/1133 (8.6)	1		
Entamoeba histolytica/ E. dispar coinfection				
Yes	56/153 (36.6)	5.33	3.96 – 7.17	<0.001
No	81/1181 (6.9)	1		

4. Discussion

In the present study, *G. duodenalis* was identified in fecal samples from children and adults without diarrhea, at varying positivity rates and with some identifiable socioenvironmental associated factors and regional differences, reinforcing the concept that, in endemic regions, giardiasis is a chronic and frequently undiagnosed condition. Data suggests that, in endemic areas, children can remain infected for long periods, without access to diagnosis and treatment [7,26]. This points to the need of inexpensive and widely applicable diagnostic techniques, as well as low-cost and safe single-dose drugs [27,28]. The negative effect of chronic *G. duodenalis* infections on the weight and height of children living in endemic areas has been demonstrated, in different regions, in the multicentric MAL-ED study through a molecular diagnostic approach to enteropathogens, including children from Bangladesh, India, Nepal, Brazil, Peru, South Africa and Tanzania [29]. In our study, chronically malnourished children tended to be more frequently infected with *G. duodenalis*.

Our data demonstrated that *G. duodenalis* positivity was influenced by the living conditions of the studied subjects, as the positivity rate was almost 3 times higher among children within extremely poor families. The detected association of giardiasis with other gut protozoan parasites such as *E. histolytica* / *E. dispar* and *Entamoeba coli* reinforces the vulnerability of the poorest families. Intestinal multiparasitism is related to increased intestinal damage in children [30].

Published data generated by the analysis of *G. duodenalis* complete genomes point to substantial phylogenetic divergence between the two main genotypes infecting humans, *i.e.*, assemblages A and B, and demonstrate that: i) the average amino-acid identity in 4,300 orthologous proteins is not superior to 78% and ii) the full-genome derived similarity between the enzootic assemblage E and the assemblages A and B at the level of amino-acid identity is 90% and 81%, respectively [31]. This reinforces the dissimilarity between the two main anthroponotic genotypes and suggests the existence of putative epidemiological

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and clinical differences between them. Our results based on the β-giardin gene sequencing also demonstrated a greater similarity between assemblage E with assemblages A and B, which, in turn, showed marked phylogenetic divergence. Through the ML analysis, assemblage E sequences obtained in GenBank were more related to our samples characterized as assemblage A, while the NJ analysis demonstrated that assemblage E was closer to assemblage B. In both phylogenetic trees, assemblage A was more closely related to enzootic assemblages C and F than to assemblage B.

Concerning biogeographic differences, our study showed that, in the Amazon region, a scenario of higher prevalence rates, there is a predominance of assemblage B and, probably, this genotype is associated with greater morbidity in this region. In addition, the Amazonian community studied is characterized by being riverside, with the concentrated population in very close contact with water bodies into which faecal waste is thrown, facilitating spread of cysts. Demographic concentration of poor resource populations of Amerindian descent, without sanitation infrastructure, favors the spread of gut protozoan parasites [32]. In previous prevalence surveys in Amazon, subclinical giardiasis prevalence rates ranged from 22% to 33% [17,33]. In addition, we demonstrated that the Amazon region had a much higher prevalence rate than the semiarid northeastern region, where the great water scarcity and the need to water supply by water trucks does not seem to favor the dissemination of *G. duodenalis*, with predominance of assemblage A. We therefore demonstrated a heterogeneous geographic distribution of *G. duodenalis* genotypes in Brazil, with a predominance of assemblage B in the Amazon biome and assemblage A outside Amazon, a trend that has been demonstrated previously [17,20,34]. Five and four novel haplotypes were identified for assemblages A and B, respectively. The detection of new haplotypes with the β-giardin locus in Brazil is not surprising, and 23 new haplotypes for assemblage B were described in a previous study [17].

The association between different clinical presentations and specific *G. duodenalis* assemblages has been explored in some studies. In Saudi Arabia, children infected with assemblage B were predominantly symptomatic, whereas asymptomatic participants harbored assemblages AI and AII [35]. In Egypt, it was demonstrated that iron deficiency anemia and intestinal symptoms were mainly associated with assemblage A [36]. In Brazil, assemblage B was more associated with HIV co-infection than assemblage A [37]. Nevertheless, the association between the different *G. duodenalis* assemblages and the clinical picture presented by the host remains unclear and with conflicting results. In the present study, we demonstrated that both assemblages A and B are associated with chronic infections in non-diarrheal children.

In conclusion, subclinical giardiasis, identified in subjects without diarrhea, is common in poor resource Brazilian communities and with a heterogeneous genotype distribution in different biogeographic regions. The data suggest the need to improve control strategies, including better access to diagnosis and treatment.

Supplementary Materials: The following supporting information can be downloaded at: www.mdpi.com/xxx/s1, Figure S1: title; Table S1: title; Video S1: title.

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Data Availability Statement: All nucleotide sequencing data have been submitted to GenBank as shown in the methods section. The data that support the findings of this study are available on

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request from the corresponding author, Carvalho-Costa FA. The data are not publicly available because contain information that could compromise the privacy of research participants.

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