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Predominance of *Giardia duodenalis* AII sub-assemblage in young children from Salvador, Bahia, Brazil

Predominio del subconjunto AII de *Giardia duodenalis* en niños pequeños de Salvador, Bahía, Brasil

***Giardia duodenalis* AII sub-assemblage in young children**

Flávia Thamiris Figueiredo Pacheco [1], Renata Kelly Novaes Rodrigues Silva [1], Silvia Souza de Carvalho [1], Felipe Carvalho Rocha [1], Gisele Maria Trindade das Chagas [1], Daisy Chagas Gomes [1], Hugo da Costa-Ribeiro Junior [2], Tereza Cristina Medrado Ribeiro [2], Ângela Peixoto de Mattos [2], Luciano Kalabric Silva [3], Neci Matos Soares [1], Márcia Cristina Aquino Teixeira [1]

[1] Faculdade de Farmácia, Universidade Federal da Bahia, Salvador, Brazil

[2] Centro Pediátrico Professor Hosannah de Oliveira, Universidade Federal da Bahia, Salvador, Brazil

[3] Fundação Oswaldo Cruz, Centro de Pesquisa Gonçalo Moniz, Salvador, Brasil

Corresponding author:

Márcia Cristina Aquino Teixeira, Av. Barão de Jeremoabo, Nº 147, Campus Universitário de Ondina 40170-115, Salvador, Bahia, Brasil.

Telephone: (5571) 3283 6950/6954

marciat@ufba.br

Authors' contributions:

Márcia Cristina Aquino Teixeira: designed the study.

Flávia Thamiris Figueiredo Pacheco and Renata Kelly Novaes Rodrigues Silva: performed the molecular analysis of samples.

Silvia Souza de Carvalho, Felipe Carvalho Rocha, Gisele Maria Trindade das Chagas and Daisy Chagas Gomes: collected the specimens, performed the parasitological and coproantigen test for *Giardia* diagnosis.

Luciano Kalabric Silva: analyzed the nucleotides sequences and deposited at GenBank.

Hugo da Costa-Ribeiro Junior, Tereza Cristina Medrado Ribeiro and Ângela Peixoto de Mattos: responsible for recruitment and analysis of clinical status of children.

Flávia Thamiris Figueiredo Pacheco, Márcia Cristina Aquino Teixeira and Neci Matos Soares: wrote the manuscript.

All authors revised and approved the final version of the manuscript.

Introducción. Incluir esta sección.

Objetivo. Caracterizar los aislamientos moleculares de *Giardia duodenalis* en niños de Salvador, Bahia, Brasil.

Materiales y métodos. Las muestras fecales positivas para *G. duodenalis* se obtuvieron de 71 niños de dos guarderías y de 39 usuarios de un laboratorio público de análisis clínicos. Las muestras fueram analizadas por PCR-RFLP de los genes de glutamato deshidrogenasa (*gdh*) y *β-giardin*, y por secuenciación de *β-giardin*.

Resultados. De las 110 muestras de *G. duodenalis*, 80 (72,7%) amplificaron uno o ambos genes analizados. De estos, 62 (77,5%) se identificaron como el ensamblaje A y 18 (22,5%) como el ensamblaje B. El subconjunto AII se identificó en el 58,8% (n=47) de los aislamientos, seguido del subconjunto AI (18,8%, n=15), BIV (11,2%, n=9) y BIII (5,0%, n=4). El subconjunto de AII fue el más frecuente en los niños de ambas guarderías, mientras que la AI solo se encontró en el grupo atendido en el laboratorio clínico. Hubo un predominio de sub-ensamblaje AII en niños menores de dos años.

Conclusiones. La mayor frecuencia de subconjuntos AII sugiere que la transmisión antroponótica es más común en Salvador, pero que también existen vías de transmisión zoonóticas, y que un cambio en la susceptibilidad a diferentes patrones moleculares de *Giardia* puede ocurrir durante el crecimiento infantil.

Palabras clave: giardiasis/epidemiologia; niño; guarderías; Brasil.

Introduction. Incluir esta sección.

Objective. To characterize the *Giardia duodenalis* molecular isolates in children from Salvador, Bahia, Brazil.

Methods. *G. duodenalis* positive fecal samples were obtained from 71 children from two daycare centers and from 39 users of a public clinical analysis laboratory. Samples were analyzed by PCR-RFLP of the glutamate dehydrogenase (*gdh*) and *β-giardin* genes, and by sequencing of *β-giardin*.

Results. Of the 110 *G. duodenalis* samples, 80 (72.7%) amplified one or both target genes analyzed. Of these, 62 (77.5 %) were identified as assemblage A, and 18 (22.5%) as assemblage B. The sub-assemblage AII was identified in 58.8% (n = 47) of isolates, followed by sub-assemblage AI (18.8%, n = 15), BIV (11.2%, n = 9) and BIII (5.0%, n = 4). The AII sub-assemblage was the most frequent in children of both day care centers, whereas AI was found only in the group attended at the clinical laboratory. There was a predominance of sub-assemblage AII in children under two years.

Conclusions. The higher frequency of AII sub-assemblage suggests that anthroponotic transmission is more common in Salvador, but that zoonotic transmission pathways are also present, and that a change in susceptibility to different molecular patterns of *Giardia* may occur during child growth.

Key words: Giardiasis/epidemiology; child; escuelas de párvulos; Brazil.

Due to the high prevalence of giardiasis in young children in developing countries and its effects on early childhood diarrhea and malnutrition, giardiasis is of considerable public-health importance (1-3). The high susceptibility of children to *G. duodenalis* infection is usually attributable to the immaturity of their immune system at the first contact with the parasite and to poor hygiene habits, when compared with those of adults (1). The transmission of giardiasis occurs via the fecal-oral route, with infection resulting from the ingestion of cysts present in food or water contaminated with feces (4,5). Direct transmission from person to person also contributes to the dissemination of the parasite among children attending day-care centers and schools (1,6).

Although *G. duodenalis* is considered a unique species, advances in molecular biology techniques have revealed that the protozoan is a complex of species with genetic diversity, but morphologically identical, exhibiting adaptation to different hosts (4,7,8). Therefore, the related *Giardia* genotypes were grouped into the 8 main assemblages A, B, C, D, E, F, G and H and their respective sub-assemblages (4,9,10). Differences in the genic sequences coding assemblages A and B, have made it possible to distinguish genotypes and subgroups, which differ in host specificity (11). Assemblage A was classified into subgroups from AI to AIV, where AI is usually reported in humans and animals; AII is exclusive to man and AIII and AIV are unique to animals (12). The assemblage B includes sub-genotypes III and IV, identified in fecal samples obtained from humans, dogs, cats, horses, calves and wild animals (11,13).

The geographical distribution of *G. duodenalis* human genotypes varies greatly around the world. In countries such as Bangladesh (14); Portugal (15); Germany (16); Uganda (17), and Syria (18), the studies reported the

predominance of genotype A of G*. duodenalis.* However, a higher prevalence of human B-genotype infections was observed in Austria (19), Kenya (20), Libya (21), Canada (22), Egypt (23) e Argentina (24). In Brazil, there are few studies describing the distribution of genotypes of *G. duodenalis* in humans. In Rio de Janeiro, Volotão et al*.* (25) identified only assemblage A, most of which were classified as AII. In São Paulo, the analysis of five isolates of axenic trophozoites presented the same results obtained in Rio, only assemblage A, and most of these were AII (26). However, in another study conducted in São Paulo with isolates from day-care children, the predominance of assemblage B was shown (27). In Fortaleza, Kohli et al. (28) amplified 58 isolates, among which assemblage B was found in 74.1%, genotype A in 15.5% and mixed infections (A + B) in 10.3%, whereas in the state of Minas Gerais, only type B was found (29). Recently, assemblage B was also reported in patients from the metropolitan area of Rio de Janeiro, showing a change in frequency patterns of genotypes A and B over the five-year study (30).

Notwithstanding the high frequency of *G. duodenalis* infection in Brazil, mainly in young children, the molecular epidemiology of the parasite has been poorly studied, especially in the Northeast region. In this work, we have characterized *G. duodenalis* isolates from preschool and schoolchildren, from the city of Salvador, Bahia, Brazil.

Materials and Methods

Origin of samples

Giardia-positive stool samples were obtained from children, up to 6 years old, from two daycare centers (46 from daycare 1 e 25 from daycare 2) supported by philanthropic institutions and from 39 children under 14 years of age, seen at

the Clinical Analysis Laboratory of Pharmacia College, Federal University of Bahia, Brazil. All children were users of health public services and from low-income families. Positive samples were identified by centrifugal-sedimentation in water (31), centrifugal-fluctuation in zinc sulfate (32) and/or by coproantigen detection using a specific commercial enzyme immunoassay (ELISA; RIDASCREEN® *Giardia*, R-Biopharm AG, Alemanha). For comparisons of frequencies of specific protozoa assemblages and sub-assemblages, children infected with *G. duodenalis* were divided according to their age and gender.

***Molecular characterization of* G. duodenalis**

a. DNA extraction from feces and PCR conditions

DNA from *G. duodenalis* cysts was purified using QIAamp DNA Stool Mini Kit (Qiagen, Hilden, Germany) according to the manufacturer's instructions, with some modifications. For example, the time and temperature of the cell lysis step were increased to 10 min at 95°C, and the DNA elution volume was reduced to 100 µL of buffer.

A 753-bp fragment of the *β-giardin* gene was amplified using forward primer G7 and reverse primer G759 (7). In the sequential nested PCR reaction, a 511-bp fragment was amplified using forward primer G99 and reverse primer G609 (33). In all cases, the PCR mixture consisted of 1x buffer containing 1.5 mM $MgCl_2$, 200 µM of each dNTP, 10 pmol of each primer, 2.5 units of Taq DNA polymerase (Invitrogen), and 1 µl of purified DNA in a final volume of 25 µl. The PCR reactions were performed as follows: an initial denaturation step of 5 min at 94°C for the first PCR and 15 min at 95°C for the nested-PCR, followed by 35 cycles of 30 sec at 94°C, 30 sec of annealing (65°C for the primary *β-giardin* PCR and 55°C for the nested PCR), and 60 sec at 70°C, with a final extension of 7 min at 72°C.

Additionally, the isolates of *G. duodenalis* identified as genotype A, through the analysis of the *β-giardin* gene, were submitted to a semi-nested PCR (sn-PCR) for amplification of the 384-bp fragment, using the direct primers G376 and reverse G759, under the same PCR conditions used for the amplification of 753-bp *β-giardin* fragment (7).

A 432-bp fragment of the *gdh* gene was amplified using semi-nested PCR, as described (34). In the primary PCR reaction, the DNA fragment was amplified using forward primer *GDH*eF and reverse primer *GDH*iR. In the sequential semi-nested PCR reaction, a 432-bp fragment was amplified using forward primer *GDH*iF and reverse primer *GDH*iR. In all cases, the PCR mixture consisted of 1x buffer containing 2 mM $MgCl_2$, 200 µM of each dNTP (GC:TA = 3:1), 12.5 pmol of each primer, 1 unit of Taq DNA polymerase (Invitrogen), and 1 µl of purified DNA in a final volume of 25 µl for the primary PCR and 50 µl for the sn-PCR. The PCR reactions were performed as follows: an initial denaturation step of 5 min at 94°C, followed by 40 cycles consisting of 30 sec at 94°C, 20 sec of annealing at 65°C, and 45 sec at 72°C, with a final extension of 7 min at 72°C. All PCR products were analyzed by electrophoresis on ethidium bromide-stained 1% agarose gels.

b. Amplicon analyses by RFLP and sequencing

For characterization of *Giardia* assemblages, 10 µL of the 511 bp *β-giardin* amplicon was digested overnight with 10 U of *Hae*III in a final reaction volume of 32 µL at 37°C (7). For identification of A sub-assemblages (AI, AII/AIII), the 384 bp fragment produced by snPCR was digested with the endonuclease *Hha*I, as described above (33). The *gdh* gene was digested overnight, at 37°C, using 10 µL of the 432 bp amplicon of the snPCR and 10 U of the enzyme *Nla*IV

(*BspL*I) in a final volume of 32 µL. Samples indicating the presence of assemblage B had the amplicons also digested with a second endonuclease, the *Rsa*I, under the same conditions, to specify sub-assemblages BIII and BIV (34). Restriction fragments were analyzed by 3% agarose gel electrophoresis, using a 50 bp molecular weight standard. The electrophoresis run was performed at 100 volts for two hours.

The isolates with mixed genotype patterns or inconclusive results in RFLP were submitted to amplicon sequencing of the *β-giardin* gene. PCR products were purified and sequenced by the Macrogen Inc. (Macrogen Inc., Seoul, Korea) sequencing service. Nucleotide sequences and electropherograms were analyzed and edited using the program CLC Main Workbench, version 8.0 (CLC Bio, Qiagen). To determine the genotype of each sample, the tree phylogenetic analysis was performed using the Neighbor-Joining method using the MEGA 6 software (35). *β-giardin* gene references corresponding to the different genotypes of *G. duodenalis* were obtained from GenBank (AY072723, subgenotype AII; KR051224, subgenotype AI; GQ337974, genotype B; AY072726, subgenotype BIII; AY072725, subgenotype BIV; and GQ337973, genotype E). Sequences were deposited on GenBank under accession numbers MG845536 to MG845549.

Statistical analysis

The data were analyzed using the IBM SPSS software for Windows, with statistical analyses performed with the GraphPad Instat program (GraphPad Software, Inc., San Diego, California, USA). The chi-squared ($\chi 2$) test was used to compare the frequency of *G. duodenalis* genotypes and subgenotypes according to the age and gender of children and the Kruskal-Wallis followed by

Dunn post test was performed to compare numerical variables. A probability of less than 0.05 was considered significant.

Ethical considerations

The Ethics Committee of Nursing School, Federal University of Bahia, Brazil, approved the study (project approval number 907.867). Children whose parents agreed to participate in the study and signed an informed consent form were enrolled during the research period. Children over 8 years old were also informed about the research and signed a consent form. All parasitological tests results were sent to the children's parents and individuals with parasitic infections were adequately treated by pediatricians when necessary.

Results

Genotyping and subgenotyping of the G. duodenalis *isolates*

From the 110 samples that were positive for *G. duodenalis*, 80 (72.7%) had the DNA successfully amplified in one or both genes (Table 1). Fifty-three (48.2%) isolates were amplified in both loci analyzed, 6 (5.4%) amplified only *β-giardin* and 21 (19.1%) only *gdh* (Table 1).

The PCR-RFLP analysis of both target genes and sequencing of *β-giardin* of the 80 amplified samples revealed genotype A as the most frequent in the general population, being found in 77.5% (62/80) of the isolates ($P<0.05$). Assemblage B was identified in 18 *G. duodenalis* samples (22.5%) (Table 2). When each group of children was analyzed separately, assemblage A was significantly more frequent than B ($P<0.05$) in samples from daycare 1 and laboratory users, whereas in daycare 2 there was no statistical difference in the occurrence of these two genetic types (Table 2). Considering the cases of assemblage B in children, it was significantly more frequent in daycare 2 (11/18,

61.1%, P<0.05) when compared to the other groups analyzed. Overall, the characterization of sub-assemblages in each group identified the AII as the most frequent (47/80, 58.8%), followed by AI (15/80, 18.8%). Of the 18 *G. duodenalis* samples identified as assemblage B, 13 were successfully sub-classified, with 5.0% BIII and 11.2% BIV.

G. duodenalis sub-assemblages distribution also differed among groups. The AI subgenotype was found only in children seen at the routine laboratory, and 10 of the 15 isolates (66.7%) were from children under six years old, the same age range as the daycare children. A significant predominance of the AII sub-assemblage (P<0.05; 83.9%) was observed in daycare 1. On the other hand, in daycare 2, although AII was the most frequent (54.2%); there was no significant difference when compared to the occurrence of assemblage B (45.8%). In both daycare centers, among the isolates of *G. duodenalis* identified as genotype A, only AII was detected.

Distribution of subassemblages according to gender and age of children

There was no significant difference in the distribution of *G. duodenalis* sub-assemblages in relation to the gender of the children. However, a different molecular isolates occurrence according to age of children was observed. The AI sub-genotype was more frequently detected in children aged between 3-10 years, while the AII was predominant in children under 2 years (Table 3). Although few BIV isolates have been characterized, they were most identified, as with AII, in young children, up to 2 years.

Discussion

Advances in molecular biology studies of *G. duodenalis* have shown that the parasite is a multispecies complex, with little variation in their morphology but with

a great genetic variability. This species is classified into eight distinct assemblages (A-H), but only A and B are regularly found in humans, although they can be detected in other domestic and wild animals (8,10,36,37).

Despite the high prevalence of giardiasis in Brazil, there are few studies on the genetic diversity of *G. duodenalis*. It is rare to find reports in the Northeast region of the country and there are no data of assemblage distribution in the state of Bahia. In our study, we performed the molecular characterization of 110 isolates of *G. duodenalis* from children living in Salvador, the capital of Bahia, divided into two groups: children who attended daycare centers and those who were seen in a public clinical laboratory. All the isolates were submitted to PCR for amplification of *β-giardin* and *gdh* gene fragments. Eighty (72.7%) samples were successfully amplified in at least one of the genes analyzed, with a slightly greater success in the amplification rate of the *gdh* gene (67.2%) than in the *β-giardin* gene (53.6%). These genes are often used to detect and/or genotype *Giardia* isolates from fecal samples, but differences in their amplifications have been reported (27,30), suggesting that the presence of divergences between the genomic sequences and primers used for PCR may result in the reduction or even lack of amplification (37,38).

Thirty (27.3%) isolates of *G. duodenalis* included in this study did not amplify any of the genes tested. The negative PCR results can be due to the presence of fecal DNA polymerase inhibitors, such as bilirubin, bile salts, hemoglobin, phenolic compounds and complex polysaccharides, which are co-purified during the extraction of genomic DNA (39,40). These PCR inhibitors may vary in amount and specific characteristics, according to the diet of each individual.

In this study, assemblage A (77.5%) and B (22.5%) were detected, with a significant predominance of the former, as in other countries such as Spain (41); Germany (16), Portugal (15), Uganda (17), Egypt (42), Syria (18) and Jamaica (43), and contrasting with Austria (19), Kenya (20), Libya (21), Canada (22) and Afghanistan (44) where a higher prevalence of assemblage B was observed. In Latin America, there was a predominance of assemblage B in Colombia and Argentina, assemblage A in Mexico (45,46) and no difference in the occurrence of these *G. duodenalis* molecular groups was observed in Cuba (47).

In Brazil, due to the huge territorial dimension, the prevalence of *G. duodenalis* assemblages varies between regions. Recent studies with daycare children in São Paulo showed a predominance of assemblage B (27) while in pre-school children from a Rio de Janeiro slum, assemblage A was predominant (26). In Fortaleza (28), Minas Gerais (29) and Paraná (48), there was a higher frequency of B molecular isolate. However, in studies conducted in Amazonas (49), Rio de Janeiro (30), São Paulo (50) and Santa Catarina (51), assemblages A and B were found in similar proportions. It is important to note that among the Brazilian studies, the majority performed a molecular characterization of less than 50 *G. duodenalis* isolates (26,29,48,50-53). In contrast, in our study, 80 isolates of *G. duodenalis* from different groups of children were analyzed.

Considering our sampling, differences in distribution of *G. duodenalis* assemblages were observed according to the group studied. In daycare 1, assemblage A was most prevalent (83.9%; $P<0,05$), while no significant differences between A (54.2%) and B (45.8%) occurrences were observed in

daycare 2. The dissemination of *G. duodenalis* cysts through person-to-person contact, common in childcare centers, can promote the concentration of certain molecular isolates (27); a fact that may justify the predominance of assemblage A in daycare 1. In addition, the presence of more than one assemblage in daycare 2 reflects multiple sources of exposure, which may be associated with the socioeconomic vulnerability of children seen at this center (54). Considering that the children from the public laboratory group were from different locations in the city of Salvador, with no interrelationship between them, the predominance of genotype A (92%) suggests a higher frequency of environmental dissemination in Salvador of this molecular type, either through contaminated drinking water and/or food, greens, and other vegetables.

Related to the sub-assemblages characterization, AII was the most frequently detected in 58.8% of cases (47/80), followed by AI (18.8%), BIV (11.2%) and BIII (5.0%). Similarly, other studies have reported a predominance of the AII sub-genotype in children from other countries (18,41,55,56), as well as in some studies conducted in Brazil (30,51,53). However, our result contrasts with a study carried out in Rio de Janeiro, where most of the isolates were identified as AI (25), as well as in Paraná (48) and in a daycare center in São Paulo (27), with a predominance of BIV. The higher frequency of the AII sub-genotype in our study suggests that transmission of giardiasis occurs mainly through an anthroponotic route (direct or indirect), since this subtype is predominantly isolated from humans (8,10).

When we specifically analyzed the distribution of the sub-assemblages in the samples of the two daycare centers, we observed that there was a higher occurrence of AII, followed by BIII and BIV. The detection of these subtypes

corroborates reports of the role of person-to-person transmission of giardiasis due to agglomeration of individuals in childcare centers, since they are predominantly found in humans (36,57). This hypothesis is also supported by the absence of the AI sub-genotype, which is frequently found in domestic and livestock animals (36). On the other hand, in children seen at the public clinical laboratory, the AI sub-assemblage was detected in the majority of cases (60.0%, 15/25). This molecular type is more associated with infection in animals than in humans (8,46), suggesting that poor treatment of drinking water, contamination of water reservoirs with animal excreta and/or contact with pets such as dogs and cats, may be factors involved in exposure to the parasite in this group.

The occurrence of mixed human infections involving different molecular isolates of *G. duodenalis* has been reported in previous studies, with rates varying from 2 to 21%, being higher in developing countries (7,18,20,33,45,58). In this work, isolates presenting RFLP pattern suggestive of mixed infections were not confirmed by *β-giardin* gene sequencing. The occurrence of mixed infections by various assemblages/sub-assemblages of *G. duodenalis* reflects the complex circulation of the parasite in the environment, the exposure of this population to multiple sources of infection, and the lack of cross-immunity between different molecular isolates (20). On the other hand, the occurrence of RFLP profiles, suggestive of the concomitant presence of two or more genotypes in the same sample, can also be attributed to the heterozygous allelic sequence of the target gene (13), as demonstrated by Morrison et al. (59) in the genome project of *G. duodenalis*.

There were no significant differences in the frequency of *G. duodenalis* sub-assemblages according the gender of children analyzed in this study, which is in agreement with previous reports (18,60), although a study have found *G. duodenalis* molecular type B as the most frequent in females (61). Our results also showed that the frequency of the AII sub-assemblage was significantly higher in the age group of 0-2 years, while the AI was higher in children 3-6 years-old. These results corroborate with studies that reported a higher prevalence of AII genotype in younger children (18,62). The high infection rate of AII in younger children can be explained by sub-standard hygiene habits; facilitating the transmission of this sub-assemblage genotype, which is predominantly anthroponotic. However, the higher frequency of AI infection in the age range of 3-6 years may be due to progressive contact with pets, which would facilitate the dissemination of this zoonotic isolate. Nevertheless, we cannot exclude the possibility of an intestinal colonization by a new molecular type of *G. duodenalis* due to active immunological memory against a previous eliminated isolate. In fact, studies conducted in the city of Rio de Janeiro, at different periods, suggest the substitution of one genetic isolate of *G. duodenalis* for another in the population (25,30).

In our study, 91.8% of the children infected with *G. duodenalis* did not present diarrhea or relevant complaints of gastrointestinal symptoms at the time of fecal analysis. Among the cases of giardiasis analyzed, 9 children (8.2%) were symptomatic, all of whom were seen at a clinical analysis laboratory. Of these, 7 had the genotypes characterized, with only two children presenting diarrhea (both infected with AI sub-assemblage). The other 5 had other gastrointestinal symptoms (2 were infected with sub-assemblage AII, 2 with AI and 1 with

assemblage B). Thus, due to the limited number of symptomatic individuals in our study, it was not possible to evaluate associations between molecular isolates and symptoms. However, it is important to emphasize that asymptomatic children play a role as disseminators of cysts both in daycare centers and in the environment.

Despite that most *G. duodenalis* carriers studied were asymptomatic, with predominance of assemblage A, it is important to highlight the similar distribution of A and B molecular groups in one of the daycares, suggesting that factors intrinsic to the host (age, nutritional status, immunological response, intestinal microbiota) are more relevant in triggering disease, rather than the molecular type of parasite involved.

This is one of the few studies of genetic characterization of *G. duodenalis* undertaken in Northeastern Brazil and the first study carried out in the state of Bahia. The results show that although there is a predominance of AII sub-assemblage in the analyzed population, suggesting that anthroponotic transmission is more common in our environment, there is a high molecular variability of *G. duodenalis* isolates, showing that zoonotic transmission routes can also be present. Apparently, in early childhood there is a preferential susceptibility to AII *G. duodenalis* sub-assemblage, changing to AI, and possibly BIV, from three years old onward, which could be related to the development of a sub-assemblage-specific immune response. More studies analyzing different groups parasitized by *G. duodenalis*, with a variety of clinical conditions are necessary for a better understanding of the molecular epidemiology of giardiasis.

Conflict of interest

The authors declare that they have no conflict of interest.

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Table 1. Frequency of *β-giardin* and *gdh* genes amplification.

Target gene	N (%)
β-giardin	6 (5.4)
gdh	21 (19.1)
β-giardin + *gdh*	53 (48.2)
Non-amplified	30 (27.3)
Total	110 (100.0)

Table 2. Distribution of assemblages of *G. duodenalis* in children groups.

	Frequency of assemblages and sub-assemblages in children groups N (%)			
	Daycare 1 (n=31)	Daycare 2 (n=24)	Laboratory users (n=25)	TOTAL (n= 80)
Assemblages				
A	26 (83.9)[a,b]	13 (54.2)	23 (92)[c]	62 (77.5)[d]
B	5 (16.1)[a,e]	11 (45.8)[e]	2 (8)[e]	18 (22.5)[d]
Sub-assemblages				
AI	-	-	15 (60.0)	15 (18.8)[c]
AII	26 (83.9)[a,b]	13 (54.2)[b]	8 (32.0)[b]	47 (58.8)[c,d,e]
B (non-subtyped)	1 (3.2)	3 (12.5)	1 (4.0)	5 (6.2)
BIII	4 (12.9)[a]	-	-	4 (5.0)[d]
BIV	-	8 (33.3)	1 (4.0)	9 (11.2)[e]

[abcde] Equal letters indicate statistically significant differences ($P<0.05$, $\chi 2$ test) in the frequency of assemblages and sub-assemblages among the groups of children.

Table 3. Distribution of sub-assemblages of *G. duodenalis* according to gender and age of children groups.

Frequency of sub-assemblages in children groups N (%)						
Gender	N	**AI** (n=15)	**AII** (n=47)	**BIII** (n=4)	**BIV** (n=9)	***B** (n=5)
Female	43	10 (66.7)	24 (51.1)	1 (25)	5 (55.6)	3 (60)
Male	37	5 (33.3)	23 (48.9)	3 (75)	4 (44.4)	2 (40)
Age (years)						
0 - 2	40	2 (13.3)[a,b]	27 (57.4)[c]	1 (25.0)	7 (77.8)	3 (60)
3 - 6	31	8 (53.3)[a]	16 (34.0)[c]	3 (75.0)	2 (22,2)	2 (40)
7- 10	5	5 (33.3)[b]	0 (0.0)	0 (0)	0 (0.0)	0 (0.0)
11 - 14	4	0 (0)	4 (8.5)[c]	0 (0)	0 (0.0)	0 (0.0)

[abc] Equal letters indicate statistically significant differences ($P<0.05$, $\chi 2$ test) in the frequency of sub-assemblages among the groups of children. *Assemblage B samples non-subtyped.