Epidemiol. Infect. (2016), **144**, 1418–1428. © Cambridge University Press 2015
doi:10.1017/S0950268815002514

Genotyping of *Giardia duodenalis* isolates in asymptomatic children attending daycare centre: evidence of high risk for anthroponotic transmission

A. P. OLIVEIRA-ARBEX, E. B. DAVID, T. C. G. OLIVEIRA-SEQUEIRA, G. N. BITTENCOURT AND S. GUIMARÃES*

Parasitology Department, Institute of Bioscience, São Paulo State University (UNESP), Campus de Botucatu, Botucatu, São Paulo, Brazil

Received 5 March 2015; Final revision 10 September 2015; Accepted 24 September 2015; first published online 23 November 2015

SUMMARY

Giardia duodenalis is a common intestinal parasite infecting children attending daycare centres. This study aimed to verify *Giardia* occurrence and the genotypes of isolates infecting children aged 0–6 years and workers at a daycare centre in the state of São Paulo, Brazil. The families of children who tested positive for *Giardia*, were asked to provide stool samples from household members and their dogs. Samples (123 children, 14 centre employees, 44 household members, 19 children after treatment, and 20 dogs) were examined for intestinal parasites using concentration methods. DNA extracted from all samples was submitted for polymerase chain reaction (PCR) testing and the amplicons generated were used for multilocus sequence typing of beta-giardin (*bg*), triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*) genes. *Giardia* was detected in 15·9% and 28·6% of the 220 samples by microscopy and PCR, respectively. Analysis of sequences retrieved from 29 isolates revealed both assemblages A (31%) and B (69%). Sub-assemblages AII, BIII and BIV were identified and the alignment of the *bg, gdh* and *tpi* sequences revealed the presence of some single nucleotide polymorphisms, especially in assemblage B sequences. The higher predominance of assemblage B and the identification of the AII type support the view that anthroponotic transmission appears to be an important route of transmission in environments that concentrate children at an age when poor hygiene practices make them more vulnerable to such infection.

Key words: Anthroponotic, children, daycare, genotyping, *Giardia duodenalis*.

INTRODUCTION

Currently, as many children spend a lot of their time in a childcare facility, the shift to out-of-home care has had a significant impact on the attending child's health [1]. The attendance of younger children at daycare centres increases the risk of acquiring infectious diseases when immune system immaturity and limited hygiene practice training make children more vulnerable to circulating infectious agents in the care environment [2, 3].

Faecal–oral transmitted infections occur frequently in daycare attendees and can be associated with diarrhoeal episodes [2, 3]. In such enclosed environments, the incidence of gastrointestinal disease episodes in children may be twice as high compared to home-cared children [4, 5]. Along with bacterial and viral

* Author for correspondence: Dr S. Guimarães, Parasitology Department, Institute of Bioscience, São Paulo State University (UNESP), Campus de Botucatu, Botucatu, São Paulo, PO Box 510; CEP: 18618-970, Brazil.
(Email: sgviana@ibb.unesp.br)

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enteropathogens, the protozoan *Giardia duodenalis* (syn. *G. intestinalis, G. lamblia*) stands out as one of the most common causes of diarrhoea in children attending daycare centres [3, 4, 6]. Giardiasis may remain asymptomatic or cause acute or chronic diarrhoea but currently, especially in developing areas, there is evidence that it may be associated with failure to thrive and nutrient deficiencies, leading to both growth and cognitive impairment during early childhood [7]. Regardless of the clinical impact, infected children are potential sources of cysts by which infection is transmitted to other children and adults in close contact [8]. According to Thompson, 20–25% of childcare staff and family members may become infected enabling the infection to reach the wider community [8].

Several community studies have revealed high prevalence rates of *Giardia* infection in children, especially in low-income populations. In South America, *Giardia* infection rates ranging from 11% to 30% have been previously documented in children of preschool age in some countries [9, 10], including Brazil [11, 12] where recent surveys have registered prevalence rates of nearly 50% [6, 13] or even 100% [14].

Despite such high prevalence, a better understanding of the transmission dynamics of this parasite in different populations has been pursued over the years [15, 16]. Recent advances in molecular techniques have allowed the recognition that *G. duodenalis* is a complex species comprising morphologically indistinguishable but genetically distinct isolates [17]. Human and animal isolates of *G. duodenalis* are characterized by at least eight distinct groups, referred to as assemblages or genotypes A–H [15, 16]. Assemblages A and B are associated with human infections as well as a broad range of mammals, while the other assemblages have strong animal host specificities [15] although assemblages C–F are occasionally isolated from human infections [15]. Therefore, although animals can harbour both zoonotic and host-specific assemblages, the zoonotic potential of *Giardia* still needs to be further assessed and clarified [16].

In recent years, molecular epidemiological studies have identified assemblages A and B associated with humans infections in different geographical locations and populations. In South America, most studies have focused on childhood infections and both assemblages have been identified [9, 10, 18, 19], with a predominance of assemblage B [9, 18, 19]. In Brazil, assemblages A and B have also been recovered from human infections in different populations including children [14, 20–23], hospital patients [22, 24, 25] and fishing village populations [26]. Assemblages A and B sequences have been further classified to sub-assemblages AI, AII, BIII and BIV. Genetic diversity has also been shown in isolates from companion animals [20, 22–26] as well as from environmental samples [22, 23]. Although companion animals usually harbour the host-adapted assemblages, i.e. C and D for dogs and F for cats, both assemblages A and B [20, 22–26] have been found in these hosts.

Given the need for a better understanding of the clinical epidemiology and public health significance of *G. duodenalis* infection, particularly in endemic areas, we here propose to estimate the prevalence of infection, and evaluate the genetic diversity of isolates, associated with daycare centre attendees and staff, as well as in household members and dogs living in close proximity with the children tested positive for *Giardia* cysts. By using a multilocus sequence typing approach, we aimed to assess the occurrence and frequency of the genotypes circulating in this particular group.

MATERIAL AND METHODS

Study design, area and population

The study was conducted from April 2012 to April 2013 in a daycare centre in Vitoriana, a district of Botucatu municipality, São Paulo State, Southeastern Brazil. Based on the last census in 2010 [27], Botucatu has 127 328 inhabitants, about 3130 of whom live in Vitoriana which is located ~16 km from the city; it is a low socioeconomic area although most of the population has access to safe drinking water and sanitation facilities. The population is attended by a primary healthcare facility and the daycare centre serves most pre-school age children from low-income families.

At the time of the study, 140 children aged 0–6 years were enrolled in the centre with 17 staff. Prior to the work, meetings were held with the daycare staff and the parents/guardians of children to inform them of the purpose and procedures of the study and request their collaboration. All attending children and daycare centre workers were invited to participate in the study and the parents were informed that when their child tested positive for *Giardia*, faecal samples would be requested from household members and their dogs. Further they were advised that all positive cases for *Giardia* and other pathogenic intestinal parasites should be treated with appropriate drugs at the local healthcare centre and

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following therapy completion, a new stool examination should be performed to ensure intestinal eradication. Informed consent was received for all study subjects prior to sample collection and parents/guardians were interviewed with a structured questionnaire covering demographic data (age, sex), socioeconomic status (parents'/guardians' occupation and education level, household income, number of adults and children in families) and, household and environmental conditions (hygiene habits, water supply, source of drinking water, sewage availability, latrine system, type of housing, and contact with household pets). The study was approved by the Research Ethics Committees of the Botucatu Medical School, UNESP and Animal Experimentation/Biosciences Institute/UNESP.

Faecal samples and detection of *G. duodenalis* cysts

All children and staff in the daycare centre and their family members were provided with a package with three pots containing 2·5% potassium dichromate solution, and instructed to collect the stool specimens on alternate days. At the laboratory, the samples from each subject were pooled, filtered through gauze into a centrifuge tube and washed three times with distilled water by centrifugation (800 ***g*** for 3 min) to remove potassium dichromate and to concentrate the material. A small amount of sediment was added to a slide with a drop of Lugol iodine and examined at 40× magnification under a light microscope. In addition, a portion of faecal sediment was processed by the zinc sulfate (specific gravity 1·18) flotation technique [28] and examined microscopically. A positive result from either of these methods was interpreted as indicative of *Giardia* infection. For dogs, a single faecal sample was collected in plastic vials, and processed as above.

Treatment

All study subjects positive for *Giardia* and/or other intestinal parasites positive cases were referred for treatment with a 400 mg albendazol tablet/day, for 5 days. Children aged <2 years received 15–20 mg/kg metronidazole per day, for 5 days. At 21–30 days post-treatment, individuals were asked to provide three further stool samples, which were examined by the same procedures.

DNA extraction

DNA was extracted from all faecal samples regardless of the microscopic test results. To optimize disruption of the cysts, prior to DNA extraction, 200 mg of centrifuged faecal sediment was subjected to three freeze–thaw cycles as follows: two cycles of 5 min in liquid nitrogen and then in a water bath at 70 °C, followed by one cycle in liquid nitrogen for 5 min and 95 °C for 5 min. DNA was extracted from disrupted cysts using the QIAamp Stool mini kit (Qiagen, Germany) according to the manufacturer's instructions. All the DNA samples were stored at −20 °C until further analysis.

Molecular and phylogenetic analysis

All DNA samples were analysed by sequence-based genotyping using three gene loci beta-giardin (*bg*), triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*). Fragments were amplified with a nested *bg* polymerase chain reaction (PCR) [29], a nested *tpi* PCR [30] and a semi-nested *gdh* PCR [31], which yielded the expected amplicons of 511, 530 and 432 bp, respectively.

Positive PCR products were purified using spin columns (QIAquick PCR purification kit, Qiagen) and sequenced on both strands by Sequencing Service (Macrogen Inc., Seoul, Korea). Nucleotide sequences and chromatograms were analysed and edited using CLC Main Workbench software, version 7.0 (CLC Bio, Qiagen). Sequences from this study were aligned with each other and reference sequences downloaded from GenBank (listed below) using Clustal X [32]. The assemblages and sub-assemblages at each locus were identified by BLAST searches against the following reference sequences: *gdh* gene (accession nos. L40509.1 – AI, L40510.1 – AII, AF069059.1 – BIII, L40508.1 – BIV); *bg* gene (accession nos. AY258617.1 – AI, AY072723.1 – AII, AY072726.1 – BIII, AY072725.1 – BIV); *tpi* gene (accession nos. L02120.1 – AI, U57897.1 – AII, AF069561.1 – BIII, L02116.1 – BIV). The nucleotide sequences obtained in the present study were submitted to GenBank and were assigned accession numbers KT334235–KT334293.

Phylogenetic analyses were performed with MEGA software v. 5.0 (www.megasoftware.net) and for each locus assessed, a phylogenetic tree was constructed using neighbour-joining and maximum likelihood algorithms. Bootstrap analysis was applied to evaluate the reliability of clusters by using 1000 replicates.

Data analysis

Statistical analyses were carried out using Epi Info software v. 7.0 (Centers for Disease Control and

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Prevention, USA). Data were summarized by absolute frequency and percentage. Differences between proportions were initially submitted to univariate analysis. χ^2 test or Fisher's exact test were used for dichotomous variables and the Mann–Whitney test for numeric variables. Multivariate analysis (logistic regression) was performed using a change-in-estimate approach for selecting variables [33]. Briefly, a preliminary model included variables with $P<0{\cdot}1$ from the univariate analysis. Other models included also (one by one) all the other study variables. Variables were kept in the final model if they changed the odds ratio of any of the statistically significant values by >10%. $P<0{\cdot}05$ was used to define statistical significance in all analyses.

RESULTS

Characteristics of the study population and intestinal parasite status

Of 140 children formally enrolled in the study, 123 provided the three faecal specimens, but of these children not all parents or guardians were present during the household-based interviews and hence the socioeconomic status could only be determined for 80·5% (99/123). From the 123 daycare children aged 3–81 months participating in the study, 62 (50·4%) were male and 61 (49·6%) were female. The mean age was 48 (S.D. ± 1·49) months with 36- to 60-month-old children accounting for about 40% of the total. Based on 99 questionnaires, the socioeconomic data revealed that 89% of the children lived in brickwork houses and 100% had an indoor toilet. Concerning household sanitary conditions, 76·8% of the houses had access to the public water supply and were connected to a public sewage system and, 91·9% had access to public garbage collection. In the houses outside the public water and sewerage services coverage area (23·2%), the families obtained water from artesian wells and disposed of sewage in septic tanks. Almost half (43·4%) of the children lived in families with an income of about US$ 200·00 per month. Approximately 70% of the parents or guardians had at least 6 years of formal education. Regarding family size, 67·7% belonged to families with up three members. Almost all (91·4%) households kept dogs as the main companion animal.

Microscopic examination revealed that at least one intestinal parasite was detected in 40·6% (50/123) of the children attending daycare, with *Giardia* the most frequent at a prevalence rate of 21·9% (27/123). Other intestinal parasites detected were: *Entamoeba coli* (17/123, 13·8%); *Blastocystis* spp. (11/123, 8·9%); and the helminths *Enterobius vermicularis* (2/123, 1·6%), and one individual with *Ascaris lumbricoides*. According to information from parents/guardians and daycare centre staff, all children were considered asymptomatic, since no episodes of diarrhoea were reported in the 2 months prior to stool collection and during the study period.

Fourteen of 17 daycare workers provided faecal samples but all proved negative for intestinal parasites by microscopy. Only 16 household members of the 27 children who tested positive for *Giardia* agreed to provide faecal samples and thus specimens were obtained from 44 family members (31 adults, 13 children) and from 20 dogs. In these families, *Giardia* cysts were detected in samples from only two boys aged 17 and 90 months who were siblings of children attending the daycare centre. No dog was found positive for *Giardia* and the only intestinal parasite detected was the nematode *Ancylostoma* spp.

Thirty children in a daycare centre and two of their relatives were treated for *Giardia* and/or other pathogenic intestinal parasite infections. However, for follow-up post-treatment, only 19 of these submitted samples for examination. In seven samples, *Giardia* (six subjects) and *Blastocystis* (single subject) were detected after treatment, and none of the household members provided stool specimens for follow-up.

Analysis of prevalence of *Giardia* infection in daycare children according to age, gender and socioeconomic variables, showed no statistically significant differences. However, younger children had a higher risk of acquiring *Giardia* infection [odds ratio (OR) 0·69, 95% confidence interval (CI) 00·49–0·97, $P=0{\cdot}03$) as the risk of infection decreased with older age. In addition, children living in families with higher household densities were more likely to be infected (OR 1·8, 95% CI 1·07–3·07, $P=0{\cdot}03$). A higher risk was observed for 32 daycare children in households with ⩾4 members compared to those in households with fewer persons.

Molecular detection and genotyping of *Giardia* isolates

All *Giardia* microscopically positive and negative samples were submitted for PCR and a positive result was indicated when at least one of the three loci assessed was successfully amplified. As shown in Table 1, of the 35 *Giardia* microscopically positive samples (27

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Table 1. *Detection of* Giardia *infection in daycare children, household members, care workers and dogs by microscopy and polymerase chain reaction (PCR)*

Faecal samples	No. tested	PCR results	
		Positive (%)*	Negative (%)
Children	123		
Positive ($n = 27$)		22 (81·5)	05 (18·5)
Negative ($n = 96$)		20 (20·9)	76 (79·1)
Household members	44		
Positive ($n = 02$)		02 (100·0)	0
Negative ($n = 42$)		12 (28·6)	30 (71·4)
Care workers	14		
Positive ($n = 0$)		0	0
Negative ($n = 14$)		0	14 (100·0)
Follow-up	19		
Positive ($n = 06$)		05 (83·3)	01 (16·7)
Negative ($n = 13$)		02 (15·4)	11 (84·6)
Dogs	20		
Positive ($n = 0$)		0	0
Negative ($n = 20$)		0	20 (100·0)
Total	220		
Positive ($n = 35$)		29 (82·9)	06 (17·1)
Negative ($n = 185$)		34 (18·4)	151 (81·6)

* Amplification at least one of the three loci assessed.

from children attending daycare, two from household members and six from follow-up), 29 were successfully amplified. Of the 185 samples previously identified as negative for microscopy, PCR products were generated in 34 of them (20 from daycare children, 12 from household members and two from follow-up). No amplicons were obtained from the daycare workers' and dogs' samples. Thus, in total, the presence of *Giardia* was detected in 63 (28·6%) and 35 (15·9%) of the 220 samples examined by PCR and microscopy, respectively.

Of the 63 PCR-positive samples, 37 showed products for a single gene, 12 for two genes and 14 for all three genes analysed. For each locus, amplicons were generated in 45 (71%), 35 (56%) and 23 (37%) of the samples for *tpi, gdh* and *bg*, respectively. Sequence analysis on 59 PCR products generated from 29 isolate/subject samples identified 15 sequences for *bg*, 25 for *tpi* and 19 for *gdh* and, of these isolates, 11 were successfully genotyped at all three loci, eight at two loci and 10 at a single locus (Table 2). Furthermore, sequences of these isolates were unambiguously identified as assemblages A or B by at least one of the three loci (Table 2, Supplementary

Table 2. *Genotypic characterization of* Giardia duodenalis *isolates from children attending a daycare centre and household members*

Isolate	Age (months)	Sex	Sub-assemblages		
			bg	*tpi*	*gdh*
DC01	76	F	BIV	BIV	BIV
DC16	14	M	AII	AII	AII
DC18	66	F	AII	AII	—
DC28	17	M	—	BIV	—
DC29	15	F	—	—	BIV
DC33	56	F	AII	—	—
DC34	28	F	BIV	BIV	—
DC35	48	F	AII	AII	AII
DC38	39	F	BIV	—	BIV
DC39	36	M	BIII	BIII	BIV
DC41	52	M	—	BIV	—
DC42	25	F	—	BIV	—
DC43	18	M	—	BIV	BIV
DC43 F-up	—	M	BIV	BIV	BIV
DC46	28	F	—	BIV	
DC50	52	M	BIII	BIII	BIV
DC56	25	F	—	AII	AII
DC59	39	F	—	BIII	BIV
DC90	24	M	—	BIV	—
DC110	64	M	AII	AII	AII
DC110 F-up	—	M	—	BIV	BIV
HM110A	17	M	BIV	BIV	BIV
HM110B	Adult	F	—	BIV	BIV
DC111	13	M	AII	AII	AII
DC112	25	M	—	BIV	—
DC112 F-up	—	M	—	—	BIV
HM112A	90	M	—	BIV	—
DC113	32	F	AII	AII	AII
DC113 F-up	—	F	AII	AII	AII

DC, Daycare isolate; HM, household member isolate; F-up, follow-up (new stool sample provided after treatment).

Figs S1–S3). Twenty (69%) isolates were classified as assemblage B (14 from children, three from household members and three from follow-up test) and the assignment at each locus was seven at the *bg*, 17 at *tpi* and 12 at *gdh* (Table 2). The remaining nine isolates (31%) recovered from eight daycare children (eight samples at baseline and one post-treatment) were typed as assemblage A (8 *bg*, 8 *tpi*, 7 *gdh*). Phylogenetic analysis confirmed the distribution of isolates into distinct clusters (Supplementary Figs S4–S6). The comparison of sequences obtained for *bg, tpi* and *gdh* and assigned as assemblages A and B revealed that 85% of them (50/59) showed 100% identity with reference sequences previously described; the remaining 15% (9/59) showed identity of 98% or 99%.

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All assemblage A isolates were identified as sub-assemblage AII, and of assemblage B isolates, 17 were identified as BIV and three could not be clearly subtyped (Table 2). Thus, at *bg* and *tpi* loci, these three isolates (DC39, DC50, DC59) were identified as BIII and at the *gdh* locus they corresponded to BIV, exhibiting an inconsistency in the separation of these subgroups.

Single-nucleotide polymorphism (SNP) analysis at all loci revealed in some assemblage B sequences at least one nucleotide substitution compared to reference sequences (Table 3, Supplementary Figs S1–S3). Further, some nucleotide substitutions at specific positions were found to discriminate between BIII and BIV sub-assemblages at all loci in the three isolates not clearly subtyped as BIII or BIV (Table 3). No overlapping nucleotide sequences and double chromatogram peaks at specific positions were observed. Of the assemblage AII isolates, only two sequences at the *tpi* locus showed a nucleotide substitution at position 879 (Supplementary Fig. S3).

In the context of the daycare centre, a particular situation was evident in relation to isolates from the same child that were recovered from samples collected at baseline (DC110) and at follow-up ($DC110_{F\text{-}up}$) post-treatment; in the former assemblage A (AII) was identified and in the latter, assemblage B (BIV) (Table 2). Interestingly, the isolates recovered from the samples from two household members (HM110A, HM110B) of this child corresponded to sub-assemblage BIV (Table 2); these sequences ($DC110_{F\text{-}up}$, HM110A, HM110B) were identical on alignment.

The distribution of the typed isolates from children, according to the daycare classrooms is reported in Table 4. With the exception of classroom 1 which comprised the younger children, assemblage B was the most frequent in all other groups. Of the few household members that provided stool samples, isolates were recovered from three samples: two from individuals (one adult, one child) that lived with child DC110 in classroom 6 (mean age 60 months and only sub-assemblage AII identified) and, the other sample from a child relative of DC112 in classroom 4 (mean age 32·8 months and both sub-assemblages AII and BIV identified).

DISCUSSION

Enteroparasitic infections persist as neglected diseases that can lead to adverse consequences, particularly for children of preschool age living in low socioeconomic areas. In this study, intestinal parasitic infections were investigated in children attending a daycare centre and *Giardia* was the most frequent parasite detected (21·9%), corroborating the findings reported in other investigations [14, 34–36]. Furthermore, in this population, it was found that younger children and children living within a family with ⩾4 members were at an increased risk for *Giardia* infection. The association between children's age and family size with *Giardia* infection has been previously reported [35, 37], and this reinforces the possibility of interpersonal transmission in a more susceptible and exposed group.

Recent advances in molecular techniques have provided important tools not only for detecting *Giardia* infection but also for understanding the epidemiology of transmission in target groups. We therefore investigated the genetic diversity of *G. duodenalis* infection in daycare attendees using three widely used markers, the *bg, tpi* and *gdh* genes. *Giardia* positive and negative samples by microscopy were submitted to PCR reactions and amplification was achieved in 28·6% of samples compared to 15·9% by microscopy. It follows that reliance of detection by microscopy alone may significantly underestimate true parasite prevalence. Nevertheless, it is noteworthy that 6/35 microscopy-positive samples found here failed to show amplification products for *bg, gdh* and *tpi* genes despite the proven higher sensitivity and specificity of such methods over conventional techniques; similar results have been reported in other *Giardia* molecular-based studies [38, 39]. These failures to isolate DNA or false-negative amplification from stools may be due to the presence of PCR inhibitors, differences in composition of samples and storage conditions [40]. The exclusion of these factors can be difficult but alternatives such as the use of extraction kits and the choice of an appropriate fixative suspension media (potassium dichromate or ethanol) may help minimize them.

Regarding PCR-positive reactions, different rates of amplification for the three loci were found with amplicons generated in 45 (71%), 35 (56%) and 23 (37%) of the samples for *tpi, gdh* and *bg*, respectively. These genes have been frequently used by others to detect and/or genotype *Giardia* isolates in stool samples, but differences in their performance have been reported [38, 39]. The reasons for this are unclear, but according to Broglia *et al.* [39] as these markers are all single-copy genes the different rates are not explained by a difference in target copy number. However, these authors emphasize that the presence

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Table 3. *Polymorphic sites in the* gdh, bg *and* tpi *sequences in* Giardia duodenalis *assemblage B isolates*

Assemblage	Isolate/Genbank number	Nucleotide position											
Glutamate dehydrogenase (*gdh*)		109	154	157	229	247	308	340	361	370	397	406	412
BIII	**AF069059.1**	**C**	G	T	**T**	**T**	G	**T**	**C**	C	C	C	**G**
BIV	**L40508.1**	**T**	.	.	**C**	**C**	.	**C**	**T**	.	.	.	**A**
	DC39/KT334248	T	***A***	.	C	C	.	T	T	.	.	.	A
	DC50/KT334251	T	***A***	.	C	C	.	T	T	.	.	.	A
	DC59/KT334252	T	.	**C**	C	C	***A***	T	C	.	.	***T***	A
	HM110A/KT334243	T	.	**C**	C	C	.	T	C	***T***	***T***	.	A
	DC110F-up/KT334242	T	.	**C**	C	C	.	T	T	.	.	.	A
	HM110B/KT334244	T	.	**C**	C	C	.	T	C	***T***	***T***	.	A
Beta-giardin (*bg*)		161	167	233	284	287	314	317	329	398	476		
BIII	**AY072726.1**	G	**C**	**G**	C	**C**	**C**	**C**	C	**C**	T		
BIV	**AY072725.1**	.	**T**	**A**	.	**T**	**T**	**T**	.	**T**	.		
	DC01/KT334287	***A***	T	A	.	C	T	T	***T***	T	***C***		
	DC34/KT334288	***A***	T	A	.	C	T	T	***T***	T	***C***		
	DC38/KT334289	***A***	T	A	.	C	T	T	***T***	T	***C***		
	DC39/KT334293	.	C	A	***T***	C	C	T	***T***	C	.		
	DC43 F-up/KT334290	***A***	T	A	.	C	T	T	***T***	T	***C***		
	DC50/KT334291	.	C	G	.	C	T	T	.	C	.		
Triose phosphate isomerase (*tpi*)		83	160	163	187	205	289	424					
BIII	**AF069561.1**	**C**	**C**	**C**	A	**G**	A	G					
BIV	**L02116.1**	**T**	**T**	**T**	.	**A**	.	.					
	DC01/KT334262	T	T	T	.	A	.	***A***					
	DC28/KT334263	T	T	T	.	A	.	***A***					
	DC34/KT334264	T	T	T	.	A	.	***A***					
	DC39/KT334265	T	C	C	***G***	A	.	.					
	DC41/KT334266	T	T	T	.	A	.	***A***					
	DC42/KT334267	T	T	T	.	A	.	***A***					
	DC43/KT334268	T	T	T	.	A	.	***A***					
	DC43F-up/KT334269	T	T	T	.	A	.	***A***					
	DC46/KT334270	T	T	T	.	A	.	***A***					
	DC50/KT334271	C	C	C	.	G	***G***	.					
	DC59/KT334272	C	T	T	.	G	.	.					
	DC90/KT334273	T	T	T	.	A	.	***A***					
	HM110 F-up/KT334274	T	T	T	.	A	.	***A***					
	HM110A/KT334275	T	T	T	.	A	.	***A***					
	HM110B/KT334276	T	T	T	.	A	.	***A***					
	DC112/KT334277	T	T	T	.	A	.	***A***					
	HM112A/KT334278	T	T	T	.	A	.	***A***					

Accession numbers in bold are references sequences from GenBank. Dots indicate identity to the reference sequences. Novel substitution positions are highlighted in bold italics.

of mismatches between the genomic sequences and the primers used for PCR, may result in a marked reduction, or even a lack of amplification.

Our results showed that the successfully sequenced isolates were unambiguously assigned to assemblages A or B, with predominance of the latter (20/29). In recent years, molecular epidemiological studies have reported assemblages A and B associated with human infections in different geographical locations and populations. Similar to our study, the predominance of assemblage B, including in children, has been reported by other Brazilian authors [14, 23] as well as in investigations performed in different countries of Latin America such as Nicaragua [41], Argentina [9], Ecuador [18], Colombia [19]. By contrast, Souza *et al.* [24] and Kohli *et al.* [21] in Brazil found that infections with assemblage A were more prevalent, while Volotão *et al.* [20], also in Brazil,

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Table 4. *Distribution of* Giardia duodenalis *assemblages according to daycare*

Classroom isolate	Age, mean (months)	Sub-assemblage		
		bg	*tpi*	*gdh*
Class 1				
DC16	14	AII	AII	AII
Class 2				
DC28		—	BIV	—
DC29	19·5	—	—	BIV
DC43		—	BIV	BIV
DC46		—	BIV	
Class 3				
DC34		BIV	BIV	—
DC42		—	BIV	—
DC56	25·5	—	AII	AII
DC59		—	BIII	BIV
DC90		—	BIV	—
DC111		AII	AII	AII
Class 4				
DC38		BIV	—	BIV
DC39	32·8	BIII	BIII	BIV
DC112		—	BIV	—
DC113		AII	AII	AII
Class 5				
DC50		BIII	BIII	BIV
DC41	50·5	—	BIV	—
DC35		AII	AII	AII
Class 6				
DC33	60	AII	—	—
DC110		AII	AII	AII
Class 7				
DC01		BIV	BIV	BIV
DC18	71	AII	AII	—

DC, Daycare isolate.

observed that this genotype was the only one infecting humans. On the other hand, similar prevalence of assemblages A and B have been detected in children in Cuba [42] and in inhabitants of fishing villages in Brazil [26]. Despite the weight of evidence, various factors need to be carefully considered to establish the prevalence and geographical distribution of these genetic groups, as methodological aspects (targeted genes, number of loci, primers, downstream procedures, etc.) may influence the findings [15, 16].

In this study, in the 29 isolates assessed, sub-assemblage determination was clearly achieved for 26 of them, including nine AII and 17 BIV. Interestingly, in recent studies in Brazil [22, 23] and Colombia [19], subtype BIV has been reported to be the most frequent sub-assemblage related to human infections, including in children [19], and environmental contamination of sources such as water and vegetables [22, 23]. The higher frequency of assemblage B isolates associated with infections in daycare children is an interesting finding and has been reported in other investigations [14, 22]. Although this genetic group has been identified in isolates from some animals, it is more frequently reported in human infections, and therefore it is closely related to anthroponotic transmission. In the context of a daycare centre, such transmission may play a role in the dissemination of *Giardia* cysts in children attending a particular environment where specific epidemiological characteristics may increase the risks for the parasite's transmission. In addition to the predominance of assemblage B, all the assemblage A isolates were classified as AII, a subgroup that has been commonly associated with human infections. In fact, the two most common sub-assemblages, AI and AII, differ in host preference; however, humans have been frequently found infected with AII isolates [15].

Considering that in the course of the investigation, all children were characterized as asymptomatic, attempts to verify a likely association between assemblages and symptoms were not considered. To the best of our knowledge, findings on possible associations between assemblages and clinical status are still inconsistent [15]. Even so, interestingly some previous investigations had reported a correlation between assemblage A and symptomatic infections and between assemblage B and asymptomatic giardiasis [43–47]. In the context of our results, it is pertinent to emphasize that asymptomatic individuals play a role as carriers, spreading cysts both inside daycare and in their households, increasing the chances to infect other children and adults. This situation, when associated with poor hygiene practices may be crucial for *Giardia* transmission. Recently, a study performed with children living in a low socioeconomic community in northeastern Brazil [21], observed that children infected with isolates classified as assemblage B demonstrated a higher level of cyst shedding and these hosts could easily promote parasite spreading.

An interesting finding here was that three assemblage B isolates were not clearly subtyped at *bg* and *tpi* loci but they were identified as BIII and at *gdh* as BIV. SNP analysis at all loci revealed nucleotide substitutions at specific positions that discriminate between sub-assemblages BIII and BIV. These findings could be suggestive of mixed subtype infections or, to a lesser extent, genetic recombination, but further investigation would require cloning of PCR products. The inconsistency in typing results seems to be more

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related to mixed infections, mainly in infected individuals living in low-income areas [16]. Albeit in high prevalence, the occurrence of mixed infections is not easy to detect by conventional PCR assays, as the most abundant parasite population is preferentially amplified [48]. Recently a new RT–PCR assay based on assemblage-specific primers has been proposed [48] which might offer a more sensitive and precise tool for the detection of mixed infections. In view of our findings, it is probable that the high degree of polymorphism in assemblage B may make it difficult for the consistent determination of sub-assemblages within this group [49]. For this reason, it is possible that the genes currently used for genotyping, such as *tpi, gdh* and *bg* will be unable to consistently define the sub-assemblages because this group is too genetically diverse and the high substitution rate of these genes may obscure the true sub-assemblage patterns [49].

In the present study, the low participation of the families of *Giardia*-infected children was a limitation, hampering the detection of isolates associated with the household members' infections and the possibility of household transmission. Nevertheless, the observation that two genetically different isolates were recovered from the same child before (AII) and after (BIV) treatment is of interest and supports the plausibility of household transmission as the same types were identified in household members. Furthermore, the daycare child attended a classroom where other positive children were infected with AII isolates. Moreover, the scenario of possible reinfection of this child after treatment cannot be discounted.

Here, the predominance of assemblage B suggests that humans are the major source of *Giardia* cysts and that human-to-human transmission is a relevant route. In low-income areas, the lack of sanitation facilities favours the spread of cysts in the environment and potential contamination of water sources. The current study was conducted in such a community but where most of the children have access to safe drinking water and sanitation facilities. However, a lack of hand washing habit undoubtedly plays a major role in the spread and transmission of *Giardia* cysts through food handling by infected persons or directly by person-to-person contact.

In conclusion, despite the low participation of families and relatively small number of isolates examined the results presented herein outline pertinent aspects about the occurrence of *G. duodenalis* assemblages in children attending daycare centres and also provide some insights for future elucidation of giardiasis epidemiology in endemic areas. The study highlights the importance of investigating *G. duodenalis* genetic diversity for a more precise understanding of its epidemiology, while also focusing on improving children's health through prevention and control of giardiasis.

SUPPLEMENTARY MATERIAL

For supplementary material accompanying this paper visit http://dx.doi.org/10.1017/S0950268815002514.

ACKNOWLEDGEMENTS

This work was supported by the Fundação de Amparo à Pesquisa do Estado de São Paulo (FAPESP) through a research grant to S.G. (2011/52100-3). Graduate fellowships were granted to E.B.D. and A.P.O.A. by FAPESP (DD 2011/09963-0) and Coordenação de Aperfeiçoamento de Pessoal de Nível Superior (CAPES, Tropical Diseases Program), respectively. The authors thank all the families that participated in the study and the daycare staff. We also thank Ms. Patrícia Borim for technical support, Dr Carlos Magno Fortaleza and Dr José Eduardo Corrente for statistical assistance and Dr José Ricardo Jensen for language advice.

DECLARATION OF INTEREST

None.

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