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Epidemiological, parasitological and molecular aspects of *Giardia duodenalis* infection in children attending public daycare centers in southeastern Brazil

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ABSTRACT

The purpose of this study was to determine the prevalence, associated risk factors and genotype of *Giardia duodenalis* infection in children attending public daycare centers in the city of Araguari, state of Minas Gerais, Brazil. Fecal samples were collected from 245 children aged 0–5 years, and questionnaires were asked about sociodemographic and hygiene-related characteristics. At the daycare centers where children tested positive, fecal samples were collected from the staff handling food, and from family members and domestic animals. Positive samples were analyzed at the dehydrogenase glutamate (gdh) locus to determine the genotype. The prevalence of *G. duodenalis* was 51.8%, and drinking unfiltered and unboiled water (OR 2.12, CI 1.26–3.69, p<0.001) and washing hands only with water (OR 2.14, CI 1.19–4.04, p < 0.001) were related risk factors. No association was found between test-positive children and their family members, domestic animals and food handlers. An analysis of the sequences of 30 samples revealed that they all belonged to genotype B.

1. Introduction

Despite the scientific and technological advances of recent years, intestinal parasites still pose a public health problem. *Giardia duodenalis* is the most frequent protozoon among the endoparasites, with prevalence rates of 23–30% in developing countries and of 2–5% in industrialized countries.[1]

Giardia duodenalis (synonyms: *G. lamblia, G. intestinalis*) is a common endoparasite in humans and in domestic and wild animals throughout the world,[2] infesting the small intestines of young individuals, especially in their early years. Most infections are asymptomatic and, when present, symptoms vary from diarrhea to abdominal pain to poor intestinal absorption, and the infection is responsible for retarding the growth and development of individuals.[2]

Molecular studies using biomarkers have shown that *G. duodenalis* is a complex species comprising seven genotypes or assemblages (A–G). The analyses of several human isolates from different geographic locations demonstrate that only assemblages A and B are associated with human infection.[3] However, assemblage A and, to a lesser extent, assemblage B are commonly found in wild animals, with the exception of beavers and muskrats, which seemingly

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have a high occurrence of assemblage B. Both assemblage A and B are commonly reported to infect humans. Because they are found in humans and numerous species of mammals, both assemblages A and B are considered to have broad host specificity and can be transmitted zoonotically.[3]

The transmission pathway of *Giardia* is oral–fecal, by direct ingestion of feces or of water and food contaminated by the infecting form, cysts.[2] Studies in industrialized and developing countries on the risk factors for this parasite demonstrate a significant association between infection and the ingestion of contaminated water, exposure to contaminated recreational water, poor basic sanitation, and inadequate personal hygiene and food habits.[4]

Daycare centers are places where children are exposed to the parasite, due to the natural vulnerability of this age group through interpersonal contact and inadequate hygiene habits and conditions.[5] In view of this, the sanitary and environmental situation of daycare centers needs to be investigated to better understand their impact on the health of the children that use these services. Based on this premise, the purpose of this study was to determine the prevalence of *G. duodenalis* in children attending public daycare centers, the daycare center employees, the children's family members and the animals with which they live, to identify the factors considered risks for infection by this parasite, and to molecularly characterise the genotype present in the test-positive individuals.

2. Materials and methods

2.1. Study area and population

This work was carried out from May 2007 to March 2008 in the city of Araguari, state of Minas Gerais, southwestern Brazil (latitude 18° 38′S, longitude 48° 11′W).

The water consumed by the population of Araguari is filtered and treated with sodium hypochlorite. The city has a sewage collection system but no system for treating sewage, which is discharged into a nearby river. The city has 13 public daycare centers: 11 located within the urban perimeter and 2 in the periurban perimeter. An average of 56 children aged 0–5 years from low-income families attend the daycare centers.

The size of the sample was determined based on previous research in the region, which found a prevalence of *G. duodenalis* of 12.6% with a 95% CI and 3% error.[6] The calculated sample size was 230 individuals, and 245 children were included in this study, stratified by daycare center and by sex.

Before starting the study, a meeting was held with the parents and staff of each daycare center to explain the work, how the feces would be collected and how they could help with the research. The parents or guardians received and signed a Free Prior Informed Consent Form.

2.2. Data collection

A pilot study was conducted to prepare the questionnaire, the instruction manual and train the interviewer.

Information was collected by the researcher in charge, and three questionnaires were drawn up according to the focus of the data to be collected. The first was a questionnaire for parents or guardians, containing questions about the child's demographic and social characteristics (age, sex), the parents' socioeconomic status (education level, family income, number of people and children, food purchasing), environmental and home conditions (people per room, number of rooms, presence of piped water and sewage system, presence of kitchen garden), food hygiene habits (washing raw foods), personal hygiene (washing hands before meals and after using the toilet) and domestic animals in the home. The second was a questionnaire for the daycare center staff containing questions related to the establishment's sanitary structure and conditions (number of staff and children, the children's routine, and the presence of piped water, sewage system and domestic animals). The third was a questionnaire for the food handlers, containing questions relating to their demographic and social data, personal hygiene, hygiene in food preparation and use of individual protection equipment (IPE).

2.3. Processing of fecal material

Fecal samples were collected from all children whose parents or guardians consented to participate in the study. Three fecal samples from each child whose parents consented to their participation in the study were collected on alternate days due to the intermittent elimination pattern of *G. duodenalis* cysts. The identified collection jars containing fecal material were taken to the Laboratory of Parasitology at the Federal University of Uberlândia to be processed within a maximum period of 24 h. Each fresh stool sample was processed by the centrifugal float-sink technique in a 33% zinc sulfate solution.[7] According to work carried out by Thompson et al.,[8] the centrifugal flotation method in 33% zinc sulfate and the PCR technique show consistent results in the diagnosis of giardiasis. The slides were examined by two trained professionals to improve the accuracy of the results. The results of the stool tests were written up and the positive cases sent to the community health service.

When a child tested positive, fecal samples were also collected from the food handlers at the daycare center attended by the child, family members and their pets. The number of samples collected and processing of the stools was carried out following the methodology described above.

2.4. Molecular characterization

For the molecular characterization, 30 samples that tested positive for *Giardia* were selected, 29 from children and 1 from a food handler. These samples were selected for the PCR due to the presence of numerous cysts. PCRs performed with fewer cysts (less than one per microscope field) lead to unsuccessful amplification due to the scantiness of genetic material extracted.[9]

Floated material was transferred to the slide and examined by light microscopy. When cysts of *G. duodenalis* were observed, the slides were washed with 1 mL TE (10 mM Tris-HCL pH 8.0; 1 mM EDTA pH 8.0) in sterile Petri dishes. The cysts were then transferred to 1.5 mL microtubes and

washed twice with TE. After the last wash, the supernatant was discarded and the pellet was re-suspended in 500 μL of the lysis buffer (10 mM Tris-HCL pH 8.0; 25 mM EDTA pH 8.0; 100 mM NaCL; 1% SDS) and incubated at 37 °C overnight.[10]

DNA extraction of the cysts, nested-PCR amplification and sequencing reactions of PCR products were performed exactly as described previously,[11] except for the use of two sense primers (GDH-FI: AAYGAGGTYATGCGCTTCT-GCCA and GDH-FII: CTTCCTBGAGGAGATGTGCAAGGA) and one antisense primer (#579II: GATGTTYGCRCCCATCT-GRTAGTTC). The expected size of the nested fragment was 600 nucleotides long. The fragments of the nested-PCR were visualized by electrophoresis in 1.5% agarose gel immersed in ethidium bromide 0.5 μg/mL. The nested-PCR products were excised from the gel using the kit GFX ILUSTRA™ (GE Healthcare, formerly Amersham Biosciences, Buckinghamsire, UK), and sequenced using the original primers and the Big Dye chemistry (Applied Biosystems, Foster City, California, USA). Sequencing products were analyzed on an ABI377 automated sequencer. Both strands of each PCR products were sequenced at least four times in both directions to increase the confidence of sequencing. DNA sequence of each sample was aligned manually using the Clustal X v.1.83[12] and BioEdit Sequence Alignment Editor[13] programs and compared with the sequences from GenBank. The following reference sequences were used in the analysis: AD1, Portland1 (AY178735, M84604, assemblage AI), Bris 136 (AY178737, assemblage AII), Ad-45 (AY178739 assemblage B), P15 (AY178741, assemblage E), Ad-136 (U60982, assemblage C), Ad-148 (U60986, assemblage D) and Ad-23 (AF069057, assemblage F). Phylogenies were reconstructed with distance NJ using the model of Maximum Composite Likelihood (implemented in MEGA4). Data were bootstrapped with 1000 replicates.[11]

2.5. Statistical analysis

The data were stored in the EpiData version 3.1 program (EpiData Association, Odense, Denmark) and analyzed with the EPI INFO 3.3.2 program (CDC, Atlanta, Georgia, USA). Fisher's exact test and the χ^2 test were used for comparisons of the two proportions. The Student's t test and the Wilcoxon test were used for comparison of the two means and two medians, respectively. To determine the possible risk factors associated to *G. duodenalis* infection, the OR was used with a 95% CI.

3. Results

A total of 735 fecal samples were taken from the 245 children and examined. Altogether, 127 children (51.8%) were infected with *G. duodenalis*, and all the daycare centers had children who tested positive for *G. duodenalis*. Of the 127 *G. duodenalis* positive children, 61(48%) were boys and 66 (52%) were girls with a mean age of 2.85 ± 1.36 years. The highest prevalence occurred in 1–3-year-olds, although it was not statistically significant. Most of the parents (70.9%) had a basic education level and low family income (Table 1). All the homes had piped water. However,

Table 1

Sociodemographic profile of children positive and negative for *Giardia duodenalis* from 13 public daycare centers

Characteristic	Positive		Negative	
	n	%	n	%
Sex				
Male	61	48.8	64	51.2
Female	66	55.0	54	45.0
Age (y)				
1–3	80	51.2	76	48.8
4–5	47	52.8	42	47.2
Mean (SD)	2.85 (1.37)		3.05 (37)	
Parents' education level				
Basic education	90	51.7	84	48.3
Intermediate education	34	52.3	31	47.7
Higher education	3	50.0	3	50.0
Family income				
≤1 minimum salary[a]	76	55.9	60	44.1
2–3 minimum salaries	46	46.0	54	54.0
≥4 minimum salary	5	55.6	4	44.4

[a] Minimum salary in Brazil = US$270.00.

only 68.3% were connected to the public sewage system. The average number of people per home was 4.42 ± 1.30 and the average number of people per bedroom was 2.35 ± 1.21. No statistically significant differences were observed between the variables.

In the analysis of the other variables related to risk factors, drinking unfiltered and unboiled water (OR 2.12, 1.26–3.69) and washing hands only with water (OR 2.14, 1.19–4.04)) were considered a twofold higher risk for *Giardia* infection and were statistically significant (p<0.001). Drinking filtered water (OR 0.5, 0.27–0.98) and washing hands with soap and water (OR 0.44, 0.22–0.86) were significant and considered protection factors against the disease (Table 2).

With regard to healthcare variables (Table 3), periodic stool examinations proved to be a protection factor against parasitosis (OR 0.40, 0.22–0.70) and was significant (p = 0.0009).

In the analysis of the questionnaires for the daycare centers, it was found that each center had an average of 56 children who were cared for by 8 employees. All the daycare centers had piped water and 76.9% were connected to the public sewage system. The daycare centers without a sewage system discharged their wastewater into cesspits. The food consumed at the daycare centers was supplied by government agencies and prepared by food handlers. No statistical differences (p>0.001) were found for these variables.

Each daycare had one food handler, giving a total of 13 individuals. All were women, with an average age of 45.46 ± 8.32 years and low to middle education and income level. All lived in homes which had piped water and were connected to the city's sewer system and drank filtered water and stated they washed raw foods with water and hypochlorite. Washing hands before preparing food and after using the toilet, visiting the doctor, making periodic stool exams and using IPE were reported as routine in this population.

Table 2
Variables analyzed for comparison of risk factors associated with infection by *Giardia duodenalis*

Variables	Infected		Uninfected		OR (95% CI)	P-value[a]
	n	%	n	%		
Piped water						
Yes	121	51.3	115	48.7	0.53 (0.08–2.54)	NS
No	6	66.7	3	33.3		
Sewage system						
Yes	86	50.6	84	49.4	0.87 (0.49–1.56)	NS
No	40	54.1	34	45.9		
Filtered water						
Yes	92	48.2	99	51.8	0.5 (0.25–0.98)	<0.001
No	35	64.8	19	35.2		
Unfiltered and/or unboiled water						
Yes	65	62.5	39	37.5	2.12 (1.22–3.69)	<0.001
No	62	44.0	79	56.0		
Hands washed only with water						
Yes	43	65.2	23	34.8	2.14 (1.15–4.04)	<0.001
No	83	46.6	95	53.4		
Hands washed with soap and water						
Yes	88	47.1	99	52.9	0.44 (0.22–0.86)	<0.001
No	38	66.7	19	33.3		
Hands washed before eating						
Yes	75	51.0	72	49.0	0.94 (0.54–1.62)	NS
No	51	52.6	46	47.4		
Hands washed after using the toilet						
Yes	55	56.7	42	43.3	1.40 (0.81–2.43)	NS
No	71	48.3	76	51.7		
Nails clipped and kept clean						
Yes	69	47.3	77	52.7	0.64 (0.37–1.11)	NS
No	57	58.2	41	41.8		
Food washed only with water						
Yes	62	58.5	44	41.5	1.60 (0.93–2.76)	NS
No	65	46.8	74	53.2		
Food washed with soap and water						
Yes	60	49.6	61	50.4	0.84 (0.49–1.42)	NS
No	67	54.0	57	46.0		
House with vegetable garden						
Yes	19	55.9	15	44.1	1.21 (0.54–2.70)	NS
No	108	51.2	103	48.8		

NS: not significant.

[a] Fisher's exact test.

At least one infected child was found at each daycare center and, therefore, fecal examinations were performed on all 13 food handlers. Two (15.4%) were diagnosed positive only for *G. duodenalis* and one of these reported feeling abdominal pain. No association was found between the food handlers' positivity and the positive children in the same daycare center (p > 0.001).

Although 127 children tested positive, only 66 families agreed to provide stool samples from other family members (100 samples from parents and siblings) and pets

Table 3
Variables related to health care, analyzed for comparison of risk factors associated with infection by *G. duodenalis*

Variable	Infected		Uninfected		OR (CI 95%)	P-value[a]
	n	%	n	%		
Regular visits to the doctor (1× per year)						
Yes	53	55.2	43	44.8	1.25 (0.75–2.16)	0.47
No	74	49.7	75	50.3		
Fecal examinations carried out?						
Yes	61	43.0	81	57.0	0.40 (0.22–0.70)	<0.001
No	63	65.6	33	34.4		

[a] Fisher's exact test.

(20 samples from dogs, 8 from cats and 1 from rabbit) giving a total of 129 samples, all of which were negative.

In addition to *G. duodenalis*, samples were also positive for other endoparasites, such as *Entamoeba coli* (44; 18.0%), *Ascaris lumbricoides* (19; 7.8%), *Enterobius vermicularis* (4; 1.6%), *Trichuris trichiura* (5; 1.6%), *Cryptosporidium* spp. (3; 1.2%), *Ancylostoma duodenale* egg (1; 0.4%) and *Strongyloides stercoralis* larva (1; 0.4%).

Partial *gdh* sequences of *G. duodenalis* were obtained from the human samples. The nucleotides of each sample were sequenced between positions 706 and 1220 of the *gdh* gene. Of the 30 samples selected, all belonged to the genotype (assemblage) B. A phylogenetic inference for the molecular identification of the isolates was not necessary, since all the samples presented a nucleotide identity of more than 99.5% against standard samples.

4. Discussion

The results of this study demonstrated that *G. duodenalis* was the most prevalent parasite in the children and food handlers investigated. The presence of other endoparasites was expected, due to the profile of the individuals of this study. The association of parasitic infections in young individuals living in crowded conditions with low levels of personal hygiene and poor standards of basic sanitation has been reported previously.[14,15] The high prevalence of *G. duodenalis* found in this study suggests the children's poor hygiene and crowded living conditions facilitated contamination. The prevalence of 51.8% found for *G. duodenalis* was higher than that found in previous studies conducted in several regions in Brazil, which reported positivity of 8–29%.[16,17] According to Thompson,[2] the high prevalence at daycare centers suggests an infection pattern similar to that of enteric bacterial infections, which are introduced by a single child, spread rapidly, remain in the environment and serve as a source of future infections. Worldwide prevalence studies indicate that the parasite is cosmopolitan, reaching rates of 2–5% in industrialized countries and 20–30% in developing countries.[2] Nunez et al.,[18] Ostan et al.,[15] Mohammed-Mahdy et al.[4] and Haghighi et al.[19] found prevalence rates of 9–41% in studies in several regions around the world. The difference between prevalence rates found in different regions in Brazil and in the world may be associated with the number of sample collections, the methodology used, the sanitary standard of children and environmental differences. It should also be noted that prevalence values may be underestimated due to the intermittent excretory pattern of cysts in feces; in fact, Cartwright et al.[20] reported a pattern of false negatives of 10–50%. In the present study, three samples were collected from each child on alternate days in an attempt to minimize false negatives. This contributed to the high prevalence found and also increased the reliability of the results.

The prevalence of *Giardia* is strongly associated with a variety of risk factors related to the host, such as sociodemographic, environmental and zoonotic conditions.[1] Most studies of this parasite have found that young children are the most infected, but no correlation between giardiasis and age was found in this investigation. This finding does not corroborate the results reported by Quihui et al.[14] and Ostan et al.,[15] who found a correlation. Although no statistical significance was found, the most prevalent age range was 0–3 years, corroborating the findings of Fraser et al.[21] and Newman et al.[22]

Giardiasis is a waterborne disease, so drinking water plays a major role in *Giardia* transmission.[18] The quality of water is essential in studies of risk factors, since studies conducted worldwide have found strong evidence that contaminated water is a risk factor for giardiasis.[23] In this study, children who drink unfiltered and unboiled water showed increased risk of contracting giardiasis, while drinking filtered water was considered a protection factor. The consumption of contaminated drinking water is responsible for outbreaks of giardiasis and conventional treatment processes do not ensure the complete removal or destruction of cysts.[23] Although filtration does not prevent the risk of infection completely, it should be used because it can reduce the number of cysts present in water.

Another aspect analyzed in this study was washing hands, and the use of soap and water was found to be a protective factor against giardiasis. Most studies on hand hygiene are related to intestinal parasites in general, without any specific work on giardiasis. The role of hand contamination in the fecal–oral transmission of diseases has been mentioned in developing countries. Ostan et al.,[15] in Turkey, reported that people who never washed their hands or who only washed them sporadically and without soap were at greater risk of contracting any intestinal disease.

In this study, annual fecal parasitology tests were considered a factor in protecting against *G. duodenalis*. Periodic fecal exams of children, especially in the high-risk age group, are essential for the prophylaxis and control of diseases. The early detection of *Giardia* in feces prevents the dissemination of the protozoon, particularly in places where environmental sanitation is deficient.

Human to human transmission of *Giardia* can occur indirectly through the accidental ingestion of cysts in contaminated water or food, or directly in environments where hygiene levels may be compromised, such as daycare centers.[3] Contamination risks may be associated with susceptible people who are in greater contact with children, caregivers of children, food handlers and contaminated family members, who may disseminate the infection. Moreover, one must consider the zoonotic transmission of the parasite, since animals, including dogs and farm animals, may be reservoirs of infection.[3] In the present study, 2 of the 13 food handlers tested positive for *Giardia*. Food handlers may play a fundamental role in the transmission of giardiasis, since they are responsible for preparing the food at daycare centers and can be sources of transmission when they present inadequate hygiene habits. In this study, the presence of cysts in the feces of these professionals was not indicative of the association between the food handlers and the children suffering from giardiasis, leading to conjectures that other factors were more important in the dissemination and transmission of the parasite in the children. As the fecal samples from parents, siblings and pets all yielded negative results, it seems safe to suspect that the focus of infection was also not to be found

in the home environment, suggesting that transmission occurs mainly among the children through direct contact. Some authors claim that person to person transmission is the most important determinant of infection.[15]

Although giardiasis is very common in Brazil, its genetic characterization has rarely been documented.[24,25] The analyses of the sequences of 30 samples revealed that all belonged to assemblage B, corroborating the findings of Souza et al.[11] and Yang et al.[26] in other parts of the world. However, Volotão et al.,[23] in Brazil, did not find assemblage B to be the most prevalent. The prevalence of each assemblage varies from country to country, and the genotype B seems more common overall, even though no strong conclusion can be drawn from current data.[3,27] Sprong et al.[28] stated that *Giardia* assemblage B occurs predominantly but not exclusively in humans. In their study, they found assemblage B in other animals such as cats, bovines, sheep and pigs.

A recent study reported that assemblage B has a high cyst excretion pattern and that, allied to oral-fecal transmission, it may contribute to make genotype B the most prevalent and with the highest dispersion.[29] Several studies have been conducted using 18S rRNA markers. However, although it is an excellent marker, *gdh* has higher variability and allows for the subtyping within assemblages.[3] All the samples in this study presented higher than 99.5% nucleotide identity with the respective standard samples. Values of molecular identity of this magnitude between two sequences are sufficient to classify them as belonging to the same genotype.[10]

The prevalence of *G. duodenalis* found in this study should be considered carefully and deserves greater attention from the public health authorities in Brazil. Risk factors for a population should always be evaluated, since they are important in determining the disease and may be the target of preventive measures. The molecular characterization of the isolate affecting the population is fundamental for an understanding of its cycle, its transmission and its behavior.

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