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Original Article

Molecular characterization of *Giardia lamblia* and risk factors for giardiasis among immunocompromised patients in southern Brazil

Caracterização molecular de *Giardia lamblia* e fatores de risco para giardíase em pacientes imunocomprometidos no sul do Brasil

S. T. Jeske[a], M. R. P. Macedo[a], T. Bianchi[a], Í. F. Leon[a], N. B. Pinheiro[a]*, S. Borsuk[b] and M. Villela[a]

[a]Universidade Federal de Pelotas – UFPel, Departamento de Microbiologia e Parasitologia, Pelotas, RS, Brasil

[b]Universidade Federal de Pelotas – UFPel, Centro de Desenvolvimento Tecnológico, Laboratório de Biotecnologia Infecto-parasitária, Pelotas, RS, Brasil

Abstract

Acute Giardia infections often cause diarrhea and stomach upset. Chronic infections can lead to malnutrition, micronutrient deficiencies, malabsorption and weight loss. This study assessed the prevalence of *G. lambia* infection and assessed associated risk factors among immunocompomised patients undergoing chemotherapeutic treatment in southern Brazil. A total of 110 immunocompromised patients in Pelotas, RS, Brazil, consented to participate in this study and were recruited. Socioeconomic and epidemiological profile of patients was collected by questionnaire. The prevalence for *Giardia* were determined through microscopy by the centrifugation-flotation technique using stool samples of every patient. In addition, the genetic characterization of the parasite was carried out by amplifying and sequencing the glutamate dehydrogenase (*gdh*) gene. By microscopy, the prevalence of giardiasis was 17.3% (19/110). Furthermore, the DNA sequences revealed that 7 (36.8%) out of 19 isolates belonged to assemblage B, while 6 of them (31.6%) belonged to assemblage C, 5 (26.3%) to assemblage A and 1 (5.3%) to assemblage D. Risk factors ($p \leq 0.05$) for giardiasis were schooling level (OR=8.0 (1.02 – 62.91) sharing a house with more than three people (OR=14.1 (3.77 – 52.51), water sources (OR=38.9 (10.4 – 145.7), sewage treatment (OR=14.2 (3.1 – 65.5), waste destination (OR=7.44 (2.0 – 27.3), owning pets (OR=4.6 (1.0 – 21.2) and cultivating a vegetable garden (OR=4.2 (1.3 – 13.6). The prevalence of *G. lamblia* in immunocompromised patients was considered elevate with the identification of four assemblage of the parasite (A, B, C and D).

Keywords: giardiasis, genetic characterization, glutamate dehydrogenase gene, immunosuppression, risk factors.

Resumo

As infecções agudas por *Giardia* geralmente causam diarreia e dores de estômago. As infecções crônicas podem levar à desnutrição, deficiências de micronutrientes, má absorção e perda de peso. Este estudo avaliou a prevalência da infecção por *G. lambia* e os fatores de risco associados em pacientes imunocomprometidos em tratamento quimioterápico no sul do Brasil. Um total de 110 pacientes imunocomprometidos de Pelotas, RS, Brasil, consentiram em participar deste estudo e foram recrutados. O perfil socioeconômico e epidemiológico dos pacientes foi coletado por meio de questionário. A prevalência de *Giardia* foi determinada através de microscopia pela técnica de centrifugação-flutuação utilizando amostras de fezes de cada paciente. Além disso, a caracterização genética do parasita foi realizada pela amplificação e sequenciamento do gene da glutamato desidrogenase (*gdh*). À microscopia, a prevalência de giardíase foi de 17,3% (19/110). Além disso, as sequências de DNA revelaram que 7 (36,8%) dos 19 isolados pertenciam ao agrupamento B, enquanto 6 deles (31,6%) pertenciam ao agrupamento C, 5 (26,3%) ao agrupamento A e 1 (5,3%) ao agrupamento D. Os fatores de risco ($p \leq 0,05$) para giardíase foram, escolaridade (OR=8,0 (1,02 – 62,91), dividir casa com mais de três pessoas (OR=14,1 (3,77 – 52,51), fontes de água (OR=38,9 (10,4 – 145,7), tratamento de esgoto (OR=14,2 (3,1 – 65,5), destinação do lixo (OR=7,44 (2,0 – 27,3), possuir animais de estimação (OR=4,6 (1,0 – 21,2) e cultivar horta (OR=4,2 (1,3 – 13.6). A prevalência de *G. lamblia* em pacientes imunocomprometidos foi considerada elevada com a identificação de quatro conjuntos do parasito (A, B, C e D).

Palavras-chave: giardíase, caracterização genética, gene da glutamato desidrogenase, imunossupressão, fatores de risco.

*e-mail: nbernevet@gmail.com
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1. Introduction

Giardia lamblia (Kunstler, 1882), also known as *Giardia duodenalis* and *Giardia intestinalis*, is a flagellated protozoan which colonizes the gastrointestinal tract of vertebrate hosts, such as humans and domestic and wild animals (Einarsson et al., 2016; Gallo et al., 2020; Noor et al., 2021; Sadaf et al., 2021). This parasite has a worldwide distribution, and infects over 200 million people annually, a condition known as giardiasis. This disease has been categorized a serious public health issues, most especially among children and immunocompromised adults. The impact of the disease is greater in developing countries, where it is usually associated with precarious socioeconomic conditions which affect mainly children and/or the immunocompromised adults (Jeske et al., 2018; Ryan et al., 2019; Santos et al., 2012; Mmbaga and Houpt, 2017; Jeske et al., 2020), which usually get its most severe form of the disease.

In acute infections this parasite often causes diarrhea and stomach pain. Chronic infections can lead to malnutrition through micronutrient deficiencies, malabsorption and weight loss (Allain and Buret, 2020). Information on molecular epidemiology of the parasite is important to characterize the disease and set targets to control it (Breathnach et al., 2010; Cacciò and Sprong, 2010; Lebbad et al., 2011; Selcuk et al., 2022). Immunocompromised individuals are considered a risk group for giardiasis (Silva et al., 2011; Jeske et al., 2018), but in Rio Grande do Sul (RS), a state located in southern Brazil, there are not studies of genotypic diversity of *G. lamblia* in immunocompromised individuals.

Several genetic loci have been described for the investigation of *Giardia* assemblages and genotypes, the most commonly used are the small subunit ribosomal RNA (SSU rRNA), *bg* (β -giardin), *gdh* (glutamate dehydrogenase) and *tpi* (triose phosphate isomerase) gene (Lebbad et al., 2010). The b-giardin, *gdh* and *tpi* genes show more intra-assemblage variation, and sub-genotypes within assemblages A (AI and AII) and B (BIII and BIV) are used as discriminatory markers (Lebbad et al. 2010; Cacciò and Ryan, 2008). A systematic review shows that the most of Brazilian isolates sequences of genes of *G. lamblia* available on NCBI are from Glutamate dehydrogenase (*gdh*) gene (Coelho et al., 2017).

Therefore, this study aimed the determining the prevalence and risk factors of *G. lamblia* among immunocompromised patients undergoing chemotherapeutical treatment in southern Brazil, as well, provide phylogenetic information using the sequences of *gdh* gene from the *G. lamblia* isolates.

2. Material and Methods

2.1. Ethics statement

Ethics approval for this study was received from the Human Ethics Committee (no. 502,589), was carried out at the teaching hospital (TH) that belongs to the Universidade Federal de Pelotas (UFPel), in Pelotas, RS, Brazil, in the year of 2018.

2.2. Study area

The hospital in this study is a referral that assists patients from 23 municipalities in southern RS. Subjects of the study were oncological patients above 18 years old – with different malignant neoplasms – who were undergoing chemotherapeutic treatment. Patients with these characteristics were invited to participate in the research, and for that, the researchers explained the objectives, benefits and possible adverse effects arising from this study. Patients who agreed signed the Terms of Consent and the data collection started. It should be mentioned that test results were handed to the patients. When they were positive, data were also handed to the physician who was responsible for the patient so that adequate treatment could be prescribed.

2.3. Sample collection

A well structured pretested questionnaire was administered to study participants. The questionnaire assessed socioeconomic and demographic characteristics of the participants via the following variables; age (18-49/≥ 50 years old); gender (male/female); skin color (whit/black, brown); marital status (married/single, separated, widowed); place of residence (urban/rural); schooling (up to elementary school/more than elementary school); income (up to a minimum wage/≥ 1 minimum wage); number of people in the household (up to three people/more than three people); source of drinking water (treated water/other: wells and streams); sewage collection and treatment (yes/no); waste destination (public collection/burned, buried); presence of vegetable garden in the residence (yes/no); presence of pets (yes/no); use of antiparasitic drugs in the last 05 years (yes/no). Afterwards, they were given three identified disposable containers (universal sample collectors) and the right procedure to collect three stool samples, in alternate days, was explained to them. Samples were collected at the TH and taken to the Laboratory of Human Parasitology at UFPel where coproparasitological analysis were performed. Samples were stored at -20ºC up to DNA extraction.

2.4. Coproparasitological analysis of samples

In order to determine prevalence through microscopy, three stool samples of every patient were processed by the centrifugation-flotation technique in zinc sulfate at 33% with density of 1.18 g/mL (Faust et al., 1939). The samples were processed separately, and the results were considered positive when any of the three processed samples had parasites in the microscopic analysis.

2.5. Purification of Giardia lamblia cysts and DNA Extraction

The purification of *Giardia lamblia* cysts was performed as described previously (Faust et al., 1970). Briefly, about 5 g of every stool sample was washed with distilled water, filtered through folded gauze and centrifuged (at 203 g for 15 minutes) for concentration of *G. lamblia* cysts. The supernatant was discarded after every centrifugation process and distilled water was added. The procedure

was carried out at least three times, in order to obtain transparent supernatant. Afterwards, concentrated cysts were suspended in a solution of 70 µL SDS 10% and 6 µL proteinase K 20 mg/mL. It was incubated at 55-58ºC for about 2 hours. Tubes were manually inverted every 30 minutes. DNA extraction was carried out as recommended by the protocol of phenol-chloroform extraction described previously (Green and Sambrook, 2012). Negative controls (SDS solution + proteinase K + distilled water) were used in every extraction group. The DNA extraction was performed in all 110 samples.

Genomic DNA samples were evaluated, regarding their concentration and purity, by means of optical density (OD), by a NanoVue Plus® spectrophotometer (GE Healthcare Life Sciences).

2.6. Nested-PCR

Genetic characterization of *G. lamblia* was carried out by amplifying the glutamate dehydrogenase (*gdh*) gene through nested PCR. Primers GDHeF (TCAACGTYAAYCGYGGYTTCCGT), GDHiF (CAGTACAACTCYGCTCTCGG) and GDHiR (GTTRTCCTTGCACATCTCC) (Read et al., 2004) were used in the reactions. Both GDHeF and GDHiR were employed in the first reaction, while GDHiF and GDHiR were used in the secondary one, in agreement with the protocol previously described (Read et al., 2004).

Final volume of PCR's was made to 25 µL with 12.5 µL GoTaq® PCR Master Mix 1X (Promega Corporation), 0.4 mM of every primer, approximately 150 ng of DNA and ultrapure water. In the second reaction, the same concentrations of reagents and 1 µL of the PCR product of the first reaction were used. DNA samples of *G. lamblia* and nuclease-free distilled water were used as positive and negative controls, respectively, in all PCR reactions.

Sample amplification consisted in initial denaturation at 95ºC for 5 minutes, followed by 35 denaturation cycles at 95ºC for 1 minute, annealing at 56ºC for 1 minute, extension at 72ºC for 1.5 minutes and final extension at 72ºC for 3 minutes. Annealing took place at 59ºC for 30 seconds in the second reaction.

Products of nested PCR reactions were submitted to 2% agarose gel electrophoresis in 0.5X TBE buffer, with the use of Blue Green Loading Dye I® (LGC Biotecnologia). Amplicons were compared with 100-base pair DNA markers by an ultraviolet transilluminator; samples whose nested PCR amplified the base-pair (bp) fragment were considered positive.

2.7. Sequencing

Positive samples of nested PCR were purified by the QIAquick® Gel Extraction kit (Qiagen). Then, the PCR products were inserted into pCR4®-TOPOTA® cloning vector and transformed in the electrocompetent *Escherichia coli* strain DH5α. The characterized fragments were sequenced using an Applied Biosystems 3500 Genetic Analyzer® automatic sequencer (Life Technologies, United States) with the same primers used in nested PCR.

Consensus sequence alignment of fragments amplified by nested PCR was carried out by both Clustal W (Thompson et al., 1994) and BioEdit Sequence Alignment Editor (Hall, 1999). References were homologous sequences available in the Genbank. Analyses of similarity were conducted by the MEGA X software program (Kumar et al., 2018), while cladograms were constructed by both Neighbor-Joining (NJ) (Saitou and Nei, 1987) and Maximum Likelihood (ML) methods, with the use of Kimura 2 parameters (Kimura, 1980) the first model selected by Modeltest (Darriba et al., 2012). Firstly, isolates from immunocompromised patients (subjects of this study) with sequences of different genotypes isolated in Brazil – available in the GenBank – were compared by using the *Giardia muris* sequence (AY754879.1) as the external group. Afterwards, the sequence of *gdh* gene of 19 isolates of *G. lamblia* from this study, with sequences of different genotypes of the parasite found in analyses carried out in several countries, were compared; the species *G. agilis* (MF185954.1) was the outgroup in this comparison. Both analyses occurred with 1000-replicate bootstrap tests.

2.8. Statistical analysis

A database was constructed by the Excel 2016® software program to enable data analysis. Association between results of coproparasitological and molecular diagnoses and the epidemiological variables identified by the questionnaire were statistically analyzed by the MiniTab version XVIII® software program, with the use of the Chi-squared Test and the logistic regression. The associations whose significance values presented $p \leq 0.05$ were considered statistically significant.

3. Results

Nineteen (17.3%) out of 110 oncological patients who underwent chemotherapeutic treatment in our study were positive for *G. lamblia* by the coproparasitological test. All 110 stool samples were submitted to DNA extraction and to PCR for amplification of the fragment which corresponded to the *gdh* gene. The same 19 samples that tested positive by microscopy were also positive by PCR.

The analysis of sequences of products resulting from the amplification of the *gdh* gene showed that seven (36.8%) out of 19 isolates belonged to genotype B, while six (31.6%) belonged to genotype C, five (26.3%) to genotype A and one (5.3%) to genotype D.

Amplified products of seven patients (P03, P05, P08, P15, P16, P17 and P18), who were characterized as genotype B of *G. lamblia*, exhibited identity equal to or above 99%, by comparison with sequences from human isolates found in Brazil, as well as others countries (Australia, Japan, Egypt, Slovakia Czech Republic, Mexico, Jordan and Malaysia).

Isolates from six patients (P01, P04, P07, P09, P11 and P13) exhibited 100% identity with sequences of *G. lamblia* which belonged to genotype C found in isolates from dog stool samples from Brazil, China, Australia, India, Spain, Thailand and Japan. On the other hand, five isolates (P02, P06, P10, P12 and P14) aligned with genotype A of *G. lamblia*, with 100% similarity among them and sequences compared to genotype A from humans in Brazil, as well others countries as Iran, Mozambique, Canada, Ethiopia,

Australia, the United States, India, Zambia, Holland, Czech Republic, Slovakia and Norway. Finally, one of the amplified products (P19) exhibited identity above 99% with isolates of genotype D from the parasite of Brazil, Thailand, China, Japan, Mexico, Spain, and the United States.

Similarity cladograms resulting from the comparative analyzes of the *gdh* sequences from *G. lamblia* isolates found in this study and the ones of the same genotype from Brazil and other countries stored in GenBank are represented in Figure 1 and Figure 2. As expected, sequences assigned to assemblages A and B grouped in distinct clusters, as well assemblages C and D.

Regarding demographic, socioeconomic and sanitary parameters, which are significant in infection caused by *G. lamblia* (Table 1), the lowest level of patients' schooling was a risk factor, i. e., the ones who only attended Elementary School (8 years or less) were more likely to have an infection caused by *G. lamblia* (p=0.04). Concerning characteristics of general and sanitary infrastructure, patients that share their houses with more than three people exhibited higher positivity (p=0.0001) and are 14.1-fold more likely to be positive for the protozoan.

Regarding water, 15 (65.2%) out of 23 patients who drank non-treated water collected in wells and streams were positive for *G. lamblia*; thus, it is a statistically significant factor (p<0.0001). In terms of public services related to the sewage system and waste collection, those groups that did not have these services, were more likely to be positive for the protozoan (p= 0.0006).

Patients who care for a vegetable garden and consume its products showed significantly higher prevalence of giardiasis than the ones who do not have any (p=0.02). Statistically significant difference was also associated to the variable that dealt with pets. Those who had pets were more infected by *G. lamblia*, i. e., they were 4.6-fold more likely to be positive for the parasite. Other variables did not exhibit any statistical significance when they were correlated with occurrences of *G. lamblia* infection.

4. Discussion

Infection caused by *G. lamblia* was detected in 17.3% of patients undergone chemotherapeutic treatment that participated in this study. Although we did not use a control group of people without cancer treatment, the positivity of *G. lamblia* in the population of this study was high, compared to other studies in other populations carried out in Brazil. For example, in a study with a quilombola population, 2.4% were positive for *G. lamblia* (Damazio et al., 2013), and a level of 6.1% of positivity was found in waste pickers in state of Midwest Brazil (Higa et al., 2017).

In order to reach effective control and devise adequate prevention strategies, molecular epidemiology of giardiasis in a certain place, as well its propagation patterns and modes of transmission, must be well known (Breathnach et al., 2010). In this study we used the *gdh* gene in the molecular characterization of the parasite. This molecular marker

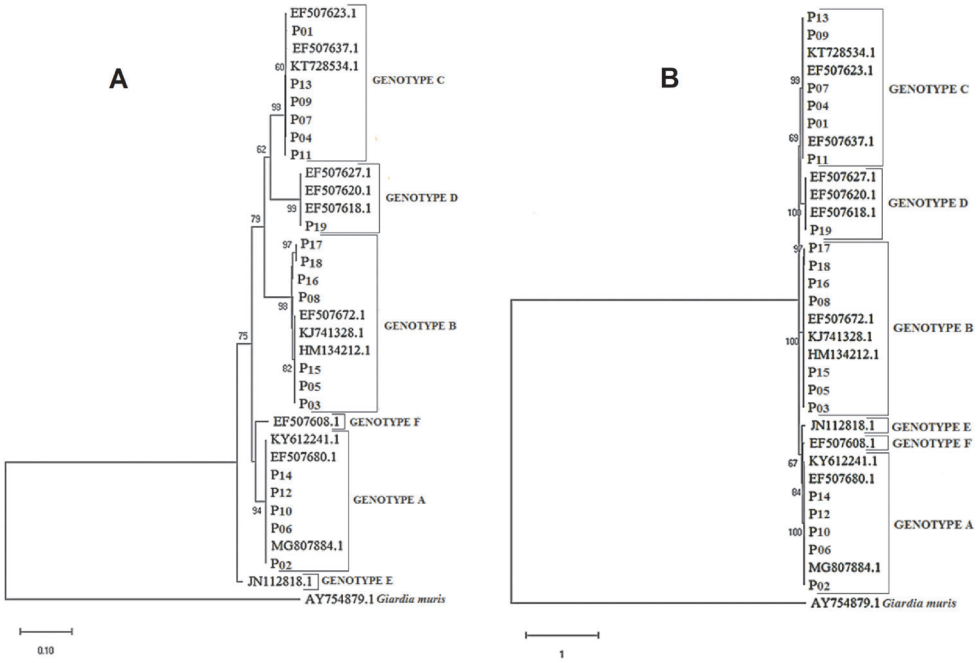

Figure 1. Similarity between sequences of the *gdh* gene of isolates from *G. lamblia* in immunocompromised patients in southern Brazil (P01 to P18) and sequences of the parasite found in Brazil and stored in the GenBank, by the Neighbor-Joining (NJ) method with 1000-replicate bootstrap (**A**) and by the Maximum Likelihood (ML) method with 1000-replicate bootstrap (**B**). Bootstrap values below 50 were omitted.

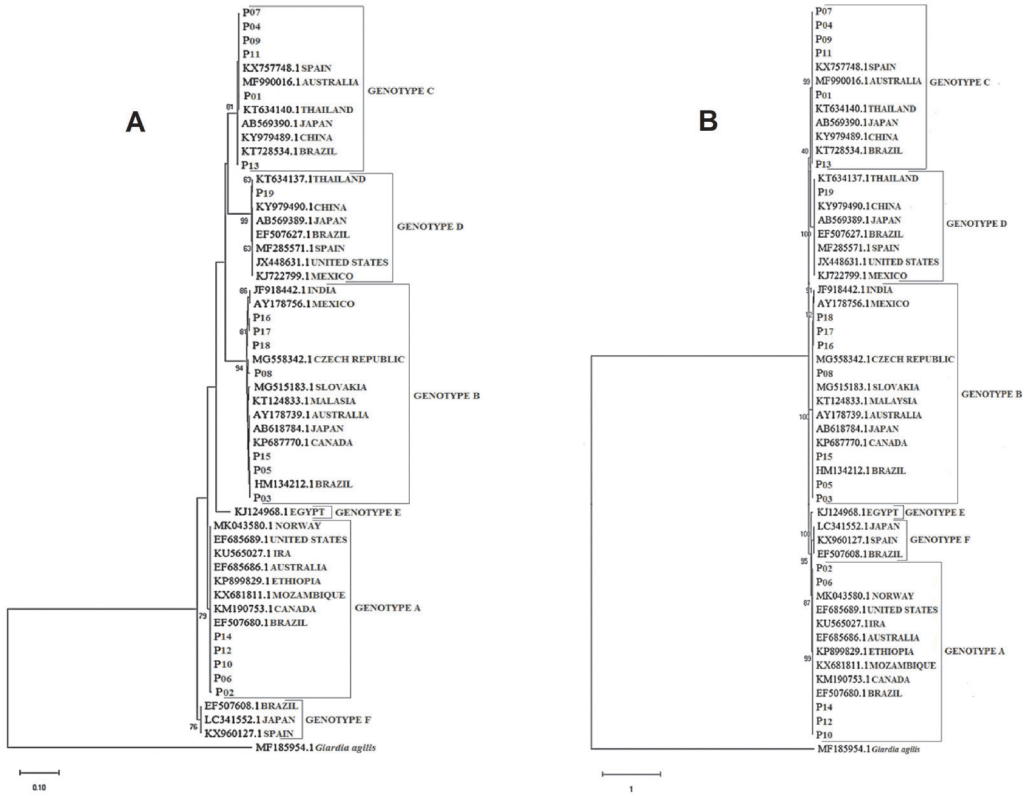

Figure 2. Similarity between sequences of the *gdh* gene of isolates from *G. lamblia* in immunocompromised patients in southern Brazil (P01 to P18) and sequences of the parasite in different countries and stored in the GenBank, by the Neighbor-Joining (NJ) method with 1000-replicate bootstrap (**A**) and by the Maximum Likelihood (ML) method with 1000-replicate bootstrap **(B**). Bootstrap values below 50 were omitted.

Table 1. Risk factors for infection caused by *G. lamblia* in immunocompromised patients in southern Brazil (n=19).

Variables	Total	+	%	*p value*	OR (95% CI)
Schooling level				0,04	8.0 (1.02 – 62.91)
Up to Elementary School	81	18	21.7		
Higher than Elementary School	29	1	3.4		
Number of people living in the house				0.0001	14.1 (3.77 – 52.51)
Up to three people	69	3	4.3		
More than three people	41	16	39.0		
Origin of drinking water				<0.0001	38.9 (10.4 – 145.7
Treated water	87	4	4.6		
Others (wells and streams)	23	15	65.2		
Sewage collection and treatment				0.0006	14.2 (3.1 – 65.5)
Yes	59	2	3.4		
No	51	17	33.3		
Waste destination				0.0025	7.44 (2.0 – 27.3)
Public garbage collection	56	3	5.3		
Others (burned, buried)	54	16	29.6		
Vegetable garden				0.02	4.2 (1.3 – 13.6)
Yes	58	15	25.9		
No	52	4	7.7		
Pet (s)				0.05	4.6 (1.0 – 21.2)
Yes	76	17	22.4		
No	34	2	5.9		

+: positive patients; OR: *odds ratio*; CI: confidence interval.

has polymorphic sequences that can differentiate genotypes of *G. lamblia* precisely (Read et al., 2004; van der Giessen et al., 2006). Since there are few studies of molecular epidemiology of *G. lamblia* in the south region of Brazil, this study highlights the importance of genetic characterization of the parasite in immunocompromised patients and can contribute with information about the parasite and the factors that predispose the population to infection by different genotypes.

Several studies have reported correlation between genotype B and symptomatic acute giardiasis, but both genotypes A and B are capable of producing symptoms (Einarsson et al., 2016; Pijnacker et al., 2016; Puebla et al., 2017). In this investigation, all patients that participated in this study were undergoing chemotherapy, which usually leads to intestinal disorders (Hauner et al., 2017), a fact that makes correlation between symptoms and *G. lamblia* more difficult.

Even though genotypes C and D are more frequent in dogs than in humans (Štrkolcová et al., 2015), both were found in 31.6% and 5.3% of oncological patients diagnosed with giardiasis, respectively in this study. Such fact had already been shown by previous studies which warned to the risk of human infection by *G. lamblia* from dogs that were found positive for genotypes with zoonotic potential (Durigan et al., 2014). However, domestic dogs do not seem to be suitable sources of human giardiosis in high-income countries (de Lucio et al., 2017). Our study showed that owning a pet increased 4.6-fold the chances of being positive for *G. lamblia*, a risk factor that was also highlighted by other studies (Jeske et al., 2018; Bowman and Lucio-Forster, 2010; Alemu et al., 2018; Shrestha et al., 2018).

G. lamblia propagation may occur through interpersonal contact with infected individuals, since cysts are eliminated in an infectious stage; this study showed that large families that live in a house are more prone to infections caused by this protozoan (Santos et al., 2012; Monteiro et al., 2018; Vaz Nery et al., 2019).

Consumption of non-treated water from wells and streams was found to be a risk factor for giardiasis not only by this study but also by others that also positively associated water sources with the acquisition of intestinal parasites, especially in immunocompromised patients (Ngui et al., 2011; Missaye et al., 2013; Gedle et al., 2017). Likewise, lack of sewage treatment services increased the likelihood of infection caused by *G. lamblia* (Visser et al., 2011; Bello et al., 2011; Júlio et al., 2012; Naz et al., 2018). Waste collection carried out by public services also contributed to low occurrence of *G. lamblia* in the population under study. This correlation was also reported by other studies (Visser et al., 2011; Zanotto et al., 2018); it may be explained by the fact that waste can contain infecting forms of parasites, such as disposable diapers and toilet paper, attract animals and insects and generate environmental pollution. It may even be related to parasitic contamination of vegetable gardens, which was another variable that exhibited relation with positive cases found in this study ($p=0.02$).

In conclusion the prevalence of *G. lamblia* in this study in the individuals with cancer is considered elevate and four assemblages of the parasite (A, B, C and D) were identified. Some Socioeconomic and environmental variable were considered risk factors for giardiasis. Thus, improvement in the population's hygienic-sanitary conditions is recommended. Moreover, this study recommends that isolates from *G. lamblia* should be collected and molecularly characterized in other human populations for understand the way of transmission of this protozoon.

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