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Original Article

Giardiasis in children and dogs, and the first report of assemblage E in dogs from northeastern Brazil

Giardíase em crianças e cães, e o primeiro relato de infecção por assemblage E em cães do nordeste brasileiro

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Abstract

Diagnosis is crucial for controlling giardiasis. We determined the prevalence and genetically characterize isolates of *Giardia duodenalis* of children and dogs from rural communities in northeastern Brazil. *G. duodenalis* cysts were concentrated by centrifugal flotation/sedimentation. Molecular characterization was carried out using the loci ssu-rRNA, *bg*, *tpi,* and *gdh*. By parasitological techniques, *Giardia* spp. infection was detected in 72/192 children (37.5%; 95% CI: 30.6%-44.7%) and 24/139 dogs (17.3%; 95% CI: 11.4%-24.6%). By molecular analysis, infection was detected in 60/141 children (42.5%; 95% CI: 34.3%-51.2%) and 26/92 dogs (28.3%; 95% CI: 19.4%-38.6%). The total prevalence of giardiasis was 54.9% in children (106/193; 95% CI: 47.1%-61.6%) and 32.9% in dogs (47/143; 95% CI: 25.2%-41.2%). Zoonotic assemblages A and B of *G. duodenalis* were detected in children, and assemblage E of *G. duodenalis* was detected in one child and two dogs. Parallel use of parasitological and molecular techniques proved to be a more effective strategy for detecting giardiasis in children and dogs from endemic areas.

Keywords: Diarrhea, dog population, parasitological diagnosis, protozoa, vulnerable communities, zoonoses.

Resumo

O diagnóstico é crucial para o controle da giardíase. Foram determinadas as prevalências e as características genéticas isoladas de *Giardia duodenalis*, em crianças e cães de comunidades rurais do nordeste brasileiro. Os cistos de *G. duodenalis* foram concentrados por centrífugo-flutuação/sedimentação. A caracterização molecular foi realizada utilizando-se os loci ssu-rRNA, *bg*, *tpi* e *gdh.* Pelas técnicas parasitológicas, a infecção por *Giardia* spp. foi detectada em 72/192 crianças (37,5%; IC 95%: 30,6%-44,7%) e 24/139 cães (17,3%; IC 95%: 11,4%-24,6%). Molecularmente, a infecção foi detectada em 60/141 crianças (42,5%; IC 95%: 34,3%-51,2%) e 26/92 cães (28,3%; IC 95%: 19,4%-38,6%). A prevalência total de giardíase foi de 54,9% em crianças (106/193; IC 95%: 47,1%-61,6%) e 32,9% em cães (47/143; IC 95%: 25,2%-41,2%). Os *assemblages* zoonóticos A e B de *G. duodenalis* foram detectados em crianças, e o *assemblage* E de *G. duodenalis* foi detectado nas duas populações. O uso paralelo de técnicas parasitológicas e moleculares mostra-se uma estratégia mais eficaz para detecção de giardíase em crianças e cães de áreas endêmicas.

Palavras-chave: Diarreia, população canina, diagnóstico parasitológico, protozoários, comunidades vulneráveis, zoonoses.

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Introduction

Giardia duodenalis is a zoonotic protozoa that causes diarrheal diseases in humans and animals (Buret et al., 2020). Globally, more than one billion cases of diarrhea in children aged up to 5 years have been reported, among which more than five hundred thousand deaths have occurred annually (WHO, 2017). Acute diarrheal diseases are important causes of morbidity and mortality among Brazilians and are mainly associated with economically vulnerable populations (Brasil, 2018; Bahia, 2019), with more than four million human cases registered annually (Bahia, 2019). In dogs, these infections are more severe in puppies and can lead to death (Thompson et al., 2008; Feng & Xiao, 2011).

Selection of diagnostic methods is essential for determining the correct prevalence that guides the planning of more effective surveillance and control actions for diarrheal diseases such as these infections (Soares & Tasca, 2016; Bahia, 2019). Among the diagnostic factors that can lead to underestimation of prevalence, as well as weaken the generation of reliable epidemiological data on these infections are the lack of diagnostic standardization in research of protozoa in human and animal populations, type and quality of the samples, the profile of the population studied, technical capacity of the microscopist, and the use of an isolated technique (Dantas-Torres & Otranto, 2014; Soares & Tasca, 2016).

In Brazil, giardiasis is diagnosed through parasitological tests, which can underestimate prevalence due to variation in the sensitivity of techniques, as well as variation in the protocols. Molecular diagnosis is still scarce in the country and is restricted to academic research due to its high cost (Katagiri & Oliveira-Sequeira, 2007; Dantas-Torres & Otranto, 2014; Bahia, 2019; Hooshyar et al., 2019).

The prevalence of giardiasis in Brazilian children ranges from 0.9% (Santos et al., 2014) to 31.2% (Mariano et al., 2015); in dogs, it ranges from 1% to 41% (Mundim et al., 2003; Campos et al., 2008). *G. duodenalis* is composed of a complex of multiple species, which includes zoonotic assemblages (A, B), and species-specific assemblages (C, D, E, F, G, and H), being C and D specific to domestic and wild canines (Feng & Xiao, 2011). Assemblages A, B, and E circulate among Brazilian children, with a higher occurrence of assemblage A. The majority of infected children are reported from daycare centers in urban and peripheral areas (Kohli et al., 2008; David et al., 2015; Quadros et al., 2016; Scalia et al., 2016; Figueiredo Pacheco et al., 2020). Brazilian dogs are infected with assemblages A, B, C, D, and E, among which assemblage A predominates (Fantinatti et al., 2020). This assemblage is also more prevalent in children and dogs from poor communities (Volotão et al., 2007; David et al., 2015; Quadros et al., 2016).

The aim of this study was to determine the prevalence and genetically characterize isolates of *G. duodenalis* from children and dogs from rural and semi-rural northeastern communities to identify priority areas to prevent and control diarrheal diseases in the rural area of the municipality of Ilhéus, Bahia, Brazil.

Material and Methods

Study area

The study was conducted in rural and semi-rural communities in the municipality of Ilhéus, State of Bahia, Brazil. Ilhéus has 184,236 inhabitants, of which 28,955 live in rural areas. The city has an average HDI (Human Development Index) of 0.69 and G (Gini Index) of 0.5; the infant mortality rate is 18.4/1000 live births, and the number of hospitalizations for diarrhea is 1.3/1000 inhabitants. These communities (Figure 1) are characterized by a high degree of social vulnerability, including the absence or low quality of health infrastructure, a high number of semi-restricted dogs, presence of farm animals on streets, frequent contact with wildlife, a high number of illiterates and people with incomplete primary education and low purchasing power. Only 65.9% of households are connected to the municipal sewage system (IBGE, 2010; Harvey et al., 2020).

Ilhéus is located in the Atlantic Rainforest Biome, with a tropical and humid climate. Annual rainfall ranges from 1000 mm to 2700 mm, and the mean annual compensated temperature is approximately 25°C. In rural communities, rivers are widely used for household chores, personal and animal hygiene, and leisure (Harvey et al., 2020). Most communities do not receive treated water (IBGE, 2010).

Study population and sample collection

Sample size calculation was based on an estimated proportion of 50% ± 5% accuracy with 95% confidence and population size of 3,851 for children and 5,348 for dogs. The estimated sample size was 350 for children and

Figure 1. A) Domestic activity on the Japu River; B) Presence of horses on a football field in the village of Castelo Novo. See the garbage dump near the camp; C) Example of a clay house present in several communities in Ilhéus; D) Black arrow indicating untreated water consumed in the community of Maria Jape; E) Cattle raised free in the community of Aritaguá. See the animal's interaction with open garbage. Source: Tatiani Harvey.

359 for dogs; the sampling was stratified by districts and systematized. Sample sizes were calculated using EpiTools (https://epitools.ausvet.com.au/oneproportion). The total number of collector flasks calculated for each population, for each type of analysis, was given to parents and dog owners. Between March and November 2016, fecal samples from all ten districts of Ilhéus, Bahia, Brazil, were delivered by only 193 children aged from 1 to 5 years, 192 for parasitological analysis and 141 for molecular analysis. Also, only 143 canine fecal samples were delivered by dog owners, 139 for parasitological analysis and 92 for molecular analysis. For parasitological analysis, three samples were collected per participant, on alternate days, using the TF-Test® Conventional commercial kit (Immunoassay Indústria e Comércio Ltda., Brazil). A fourth fecal sample was collected in a collector flask without preservatives, for molecular analysis. Health clinics, public schools, and volunteer residents received and stored the samples in cool boxes containing reusable ice packs on the day scheduled to collect samples.

Parasitological analysis

The samples collected were stored at 4 °C until processing in a maximum of 5 days. The analyses were made following the TF-Test™ kit protocol (Gomes et al., 2004; Coelho et al., 2013), which uses the sedimentation principle (TFC), and the Faust et al. (1938) method modified by Sloss et al. (1999), which is based on centrifugal-float method (CF). Kit samples were divided into two equivalent aliquots for further processing with each technique. All samples were analyzed by the same microscopist on slides containing a drop of Lugol solution and subsequently observed at 40X under optical microscopy to detect cysts or trophozoites of *G. duodenalis*.

DNA extraction, molecular analysis, and sequencing

The samples collected for molecular analysis were immediately homogenized, and a 2g- aliquot of feces was separated into a microtube and stored at -20 °C for approximately 1 year. DNA was extracted from 200 mg of feces, using the Purelink™ Microbiome DNA Purification kit (Invitrogen, Carlsbad, CA, USA), according to the manufacturer's recommendations, with the following adaptations: a) after resuspension in lysis buffer and lysis enhancer, the samples were incubated at 65 °C for 10 min; b) 5 cycles of freeze-thawing were applied by freezing samples in liquid nitrogen for 1 minute, followed by thawing at 65 °C; c) the recommended amount of the elution buffer was divided in half and samples processed according to the protocol. This step was repeated once more to

optimize DNA recovery. Genetic characterization of *G. duodenalis* was made by nested PCR (nPCR) targeting the small subunit ribosomal RNA, *ssu* rRNA (Hopkins et al., 1997; Appelbee et al., 2003), beta-giardin, *bg* (Lalle et al., 2005), glutamate dehydrogenase, *gdh* (Cacciò et al., 2008), and triosephosphate isomerase, *tpi* (Sulaiman et al., 2003) genes. The changes made to the protocols will be made available in a Supplementary Material (Suppl 01-Tables S1, S2, S3, and S4). nPCR amplicons were electrophoresed on 1 to 1.5% agarose, stained with SYBR® Safe DNA Gel Stain (Invitrogen, Carlsbad, CA, USA), and bands visualized using a UV transilluminator. Amplicons that produced strong bands were purified (Illustra™ GFX™ PCR DNA and Gel Band Purification Kit, Millipore Sigma) prior to Sanger-sequencing in both directions, with primers used for the secondary PCR. CodonCode Aligner software version 4.0.1 (CodonCode Corporation Dedham®, MA, USA) was used to determine a consensus sequence. Alignment was made using the Clustal W method (Thompson et al., 1997) in BioEdit (Hall, 1999), using homologous sequences downloaded from GenBank as references. Sequences were deposited in GenBank under accession numbers MW929203-MW929205 (*bg*) and MW929206-MW929208 (*tpi*).

Phylogenetic analysis

Phylogenetic analysis was made using the Neighbor-Joining method and the Kimura 2-parameter model (Kimura, 1980). The tree was constructed using MEGA X software. Evaluation of the reliability of clusters was confirmed using Bootstrap values from 1000 replicates.

Statistical analysis

The global prevalence of giardiasis was determined by the sum of positive cases in at least one method. Only samples analyzed by both methods were used in the comparison between microscopy and nPCR in both populations. The absolute and relative frequencies of amplification per gene and positive and negative cases in the parasitological diagnosis by the categorical variable are shown in Table 1. The agreement between methods was determined by the Kappa (*k*) index, statistically testing the consistency of *k* and ranking it as poor (0 to 0.20); low (0.21 to 0.40); moderate (0.41 to 0.60); substantial (0.61 to 0.80); and almost perfect (0.80 to 1.0) (Landis & Koch, 1977). All statistical analyses were conducted using Epi Info 7 (Centers for Disease Control and Prevention—CDC, Atlanta, USA).

Table 1. Results from parasitological screening of *Giardia* spp. and PCR protocols targeting the SSU-rRNA, bg, tpi, and gdh genes. Population: children aged up to 5 years (n= 141) and dogs (n= 92) from rural areas of Ilhéus, Bahia, Brazil, 2016.

Population	Genes		Parasitological Examination		
			Positive	Negative	Total
			n (%)	n (%)	n (%)
Children	SSU-rRNA†	Amplified n (%)	4 (7.3)	13 (15.1)	17 (12.1)
		Unamplified n (%)	51 (92.7)	73 (84.9)	124 (87.9)
	bg‡	Amplified n (%)	12 (21.8)	17 (19.8)	29 (20.6)
		Unamplified n (%)	43 (78.2)	69 (80.2)	112 (79.4)
	tpi §	Amplified n (%)	14 (25.5)	10 (11.6)	24 (17.0)
		Unamplified n (%)	41 (74.5)	76 (88.4)	117 (83.0)
	gdh†	Amplified n (%)	2 (3.6)	11 (12.8)	13 (9.2)
		Unamplified n (%)	53 (96.4)	75 (87.2)	128 (90.8)
Dogs	SSU-rRNA †	Amplified n (%)	2 (10.5)	11 (15.1)	13 (9.1)
		Unamplified n (%)	17 (89.5)	62 (84.9)	79 (90.9)
	bg †	Amplified n (%)	1 (5.3)	11 (15.1)	12 (8.4)
		Unamplified n (%)	18 (94.7)	62 (84.9)	80 (91.6)
	tpi †	Amplified n (%)	0 (0)	4 (5.5)	4 (2.8)
		Unamplified n (%)	19 (100)	69 (94.5)	88 (97.2)
	gdh †	Amplified n (%)	0 (0)	3 (4.1)	3 (2.1)
		Unamplified n (%)	19 (100)	70 (95.9)	89 (97.9)

† *Kappa* <0; ‡ *Kappa* = 0.02; § *Kappa* = 0.15.

Results

Although collector flasks were delivered to collect feces from 350 children and 359 dogs, we received samples from only 193 children and 143 dogs, considering samples for both parasitological and molecular analysis.

Giardiasis parasitological diagnosis

In the population of children, 52/192 (27.1%) positive samples were detected by the CF method and 51/192 positive samples (26.6%) by the TFC method. In the canine population, 20/139 positive samples (14.4%) were detected by the CF method and 12/139 positive samples (8.6%) by the TFC method. Considering the positivity in at least one test, giardiasis was detected in 72 children (37.5%; 95% CI: 30.6%-44.7%) and 24 dogs (17.3%; 95% CI: 11.4%-24.6%).

Giardia PCR results and sequencing

G. duodenalis DNA was detected in 60 fecal samples from children (60/141; 42.5%; 95% CI: 34.3%-51.2%), with different detection rates between genes (*bg*: 29/60, 48.3%; *tpi*: 24/60, 40%; *ssu* rRNA: 17/60, 28.3%; *gdh*: 13/60, 21.7%). Among the dogs, 26 samples were positive (26/92; 28.3%; 95% CI: 19.4%-38.6%), with also differences in detection rates (*ssu* rRNA: 13/16, 50%; *bg*: 12/ 26, 46.1%; *tpi*: 4/26, 15.4%; *gdh*: 3/26, 11.5%).

Only samples that had good DNA amplification (strong bands) were sequenced. Assemblages A, B, and E were identified in children and assemblage E in dogs (Table 2). Regarding *bg* sequences, three of them were identical to sequences obtained from cattle (KY769092, MH079430) and sheep (KY633473, MK573342) identified as assemblage E. One *bg* sequence, identified as assemblage A, was identical to sequences obtained from children (MG845549, MG845542) in Salvador, Brazil (Figueiredo Pacheco et al., 2020). All *tpi* sequences identified as assemblage A were identical to sequences obtained from humans (MH673818). In addition, two sequences identified as assemblage B were identical to sequences obtained from water (AY368171) in the USA and humans (KX468986) in Spain.

The evolutionary relationship of assemblages found in this study in both populations is shown in Figure 2.

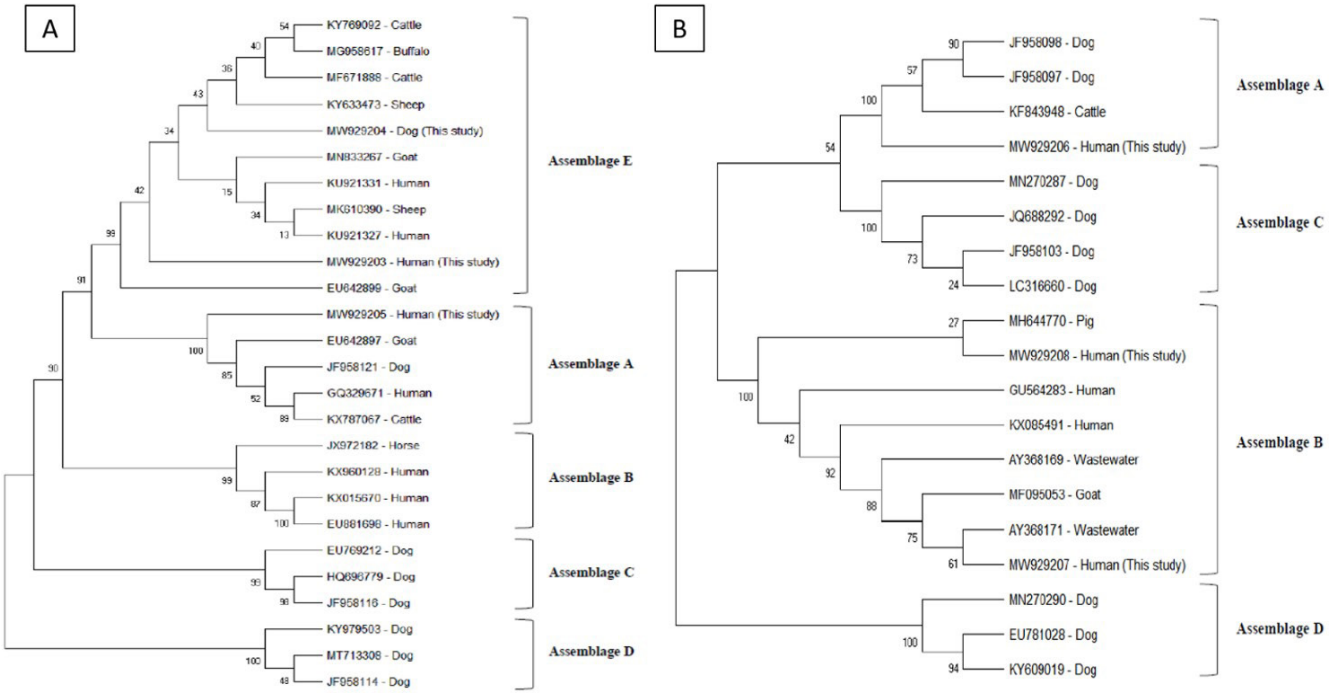

Figure 2. Phylogenetic tree between *Giardia duodenalis* obtained from this study and previous studies based on *bg* (A) and *tpi* (B) genes. The evolutionary history was inferred using the Neighbor-Joining method using the Kimura 2-parameter model. The percentage of replicate trees in which the associated taxa clustered together in the bootstrap test (1000 replicates) are shown next to the branches. Reference sequences with their accession numbers in GenBank are provided.

Table 2. Results from PCR and genetic sequencing and profile of clinical-epidemiological cases of infection by *Giardia duodenalis*.

Samples	Amplified genes PCR / Sequencing	Assemblage	Clinical Symptoms/Signs	Age (years)	Water source
Child					
55[†]	*bg, tpi / tpi*	A	Frequent diarrhea Weight loss Flatulence Inappetence Vomiting Pasty stools	3	well
64[†]	*bg, tpi / tpi*	B	Absent	3	spring river
95[†,‡]	SSU-rRNA, *Bg, tpi / bg*	E	Abdominal pain Diarrhea Pasty stools	4	well
111	*bg, tpi / tpi*	A	Frequent diarrhea Inappetence	5	well
145	*tpi / tpi*	A	Frequent diarrhea Inappetence Pasty stools	2	
150	*bg, tpi / tpi*	B	Abdominal pain Frequent diarrhea Anal itching Pasty stools	4	dam
162	*bg, tpi / bg, tpi* [§]	A	Frequent diarrhea Inappetence Anal itching Pasty stools	1	dam
182[‡]	*tpi / tpi*	A	Abdominal pain Inappetence Pasty stools	2	dam
Dog					
20[‡]	*bg*	E	Mucus in feces	>5	Treated water
40	*bg*	E	Absent	>5	spring well river

[†] = Children from the Lagoa Encantada, Rio do Engenho, and Jairi tourist communities, from the Municipality of Ilhéus, Bahia, Brazil; [‡] = Negative samples for *Giardia* in the parasitological analysis; § = Sample typed as Assemblage A by *tpi* generic primer. Epidemiological data presented in this table were collected through a questionnaire, from which risk factor analysis had been previously published (Harvey et al., 2020).

Clinical-epidemiological profile of children and dogs that had their infections confirmed by sequencing is described in Table 2. Children 145,150,162, and 182 were from the same community.

Comparison between parasitological and molecular diagnosis of *Giardia*

Considering positive samples in at least one parasitological method or loci, the overall prevalence was 54.9% in children (106/193; 95% CI: 47.1%-61.6%), and 32.9% in dogs (47/143; 95% CI: 25.2%-41.2%). Infection was present in all districts of Ilhéus (Figure 3).

In the population of children, twenty-six samples were positive with both parasitological and molecular techniques, but the agreement was poor (index k= 0.07). Of the 86 samples negative by microscopy, 34 were positive (39.5%) by PCR, and of the 81 samples negative by PCR, 29 were positive (35.8%) by microscopy. The agreement between the parasitological analysis and the *bg* (k= 0.02) and *tpi* (k= 0.15) genes was poor, and there was no agreement between the SSU-rRNA and gdh genes with the result of the parasitological analysis (k < 0).

In the population of dogs, three samples were positive with both techniques, which had no agreement between their results (k= -0.14). Of the 73 samples negative by microscopy, 23 (31.5%) were positive by PCR, and of the 66 samples negative by PCR, 16 (24.2%) were positive by microscopy. The agreement between the parasitological analysis and the SSU-rRNA gene (k= 0.09) was poor, and there was no agreement between the other genes with the result of the parasitological analysis (k < 0).

Kappa agreement between the techniques for both populations is shown in Table 3. The distribution of giardiasis cases detected by parasitological and molecular techniques is shown in Table 1.

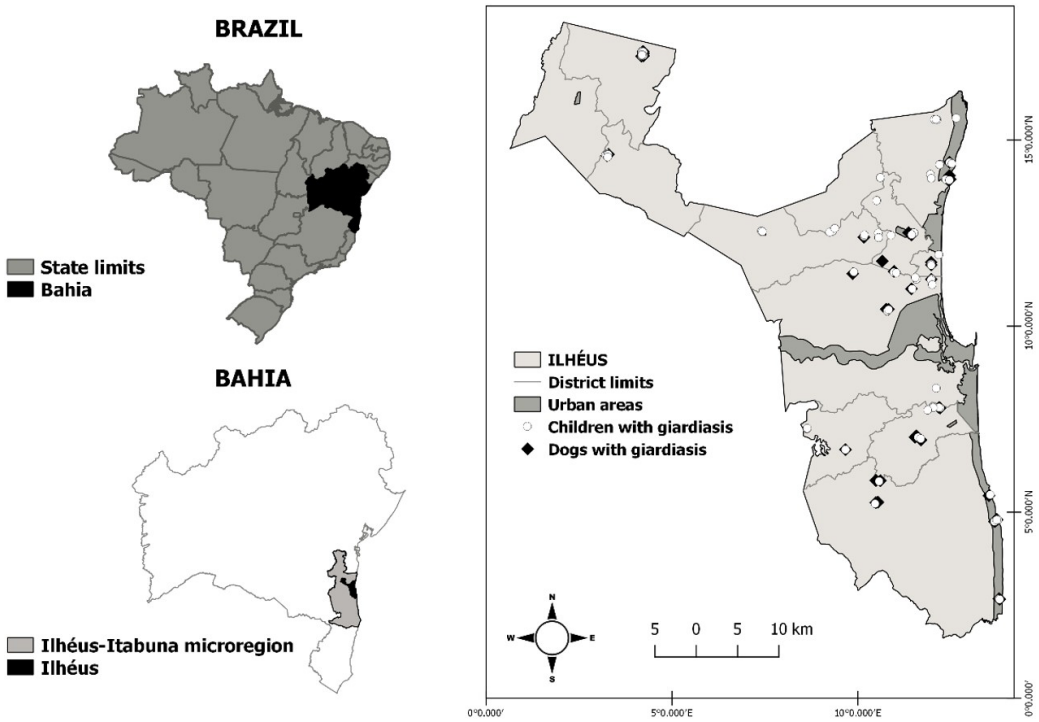

Figure 3. Location and distribution map of giardiasis cases in the Municipality of Ilhéus, State of Bahia, Brazil. This map was prepared using the QGIS program (version 2.18).

Table 3. Kappa agreement (*k*) between parasitological (OM) and molecular (Mol) methods used for diagnosis of *Giardia* infections.

Diagnostic methods	Children		Dogs	
	k	95% CI	*k*	95% CI
OM x SSU-rRNA*	< 0	-0.206 to 0.027	< 0	-0.233 to 0.130
OM x *bg**	0.02	-0.128 to 0.173	< 0	-0.259 to 0.032
OM x *tpi**	0.15	0.006 to 0.302	< 0	-0.143 to -0.012
OM x *gdh**	< 0	-0.205 to -0.007	< 0	-0.120 to 0.001
OM x Mol*	0.07	-0.089 to 0.241	< 0	-0.310 to 0.034

CI: Confidence Interval. *= p> 0.05. >0 = no agreement; 0 to 0.20 = poor; 0.21 to 0.40 = low; 0.41 to 0.60 = moderate; 0.61 to 0.80 = substantial; 0.80 to 1.0 = almost perfect.

Discussion

The greater number of cases of giardiasis detected from the combination of diagnostic techniques in both populations demonstrates and reinforces the need for the prevalence rates of this infection to be investigated using more than one parasitological technique or more than one genetic marker (Soares & Tasca, 2016; Hooshyar et al., 2019). In children, the overall prevalence of infection (54.4%) was higher than that reported in other Brazilian studies (Santos et al., 2012; David et al., 2015; Mariano et al., 2015; Nunes et al., 2016). In dogs, the prevalence was similar to previous reports (Mundim et al., 2003; Labruna et al., 2006; David et al., 2015; Coelho et al., 2017).

Within the scope of parasitological analysis, the individual performance of centrifugal-flotation and centrifugal-sedimentation was not as efficient as their parallel association in this study. In addition to reiterating the recommendations for the use of microscopy in the diagnosis of *Giardia* (Faria et al., 2016; Soares & Tasca, 2016; Hooshyar et al., 2019), our study reinforces the need for parallel use of parasitological techniques in protozoan research, especially in the screening of *Giardia* samples, prior to application of PCR (Volotão et al., 2007; David et al., 2011, 2015; Quadros et al., 2016; Faria et al., 2016). The presence of false-negative cases on microscopy was probably related to the low number of cysts observed in most of our samples, especially in canine fecal samples, in which the number of cysts was even lower (Katagiri & Oliveira-Sequeira, 2007; Soares & Tasca, 2016).

Several studies have shown greater sensitivity of molecular methods to detect *Giardia* (Bouzid et al., 2015; Ramírez et al., 2015). However just as the use of only one parasitological technique underestimated the prevalence of giardiasis in our study, the individual use of PCR can also underestimate cases in human and animal populations (Arroyo-Salgado et al., 2014; David et al., 2015). It should also be noted that the PCR technique can have varied sensitivity because, in addition to the presence of inhibitors, the extraction of DNA from the cysts is variable, with amplification rates from 36% to 85.7% (Babaei et al., 2011; Silva et al., 2022). Prevalence and diagnostic efficiency of molecular methods depend on the type and number of loci analyzed, and their applicability has been mainly restricted to the epidemiological purpose due to their high cost of execution (Feng & Xiao, 2011; Ryan & Cacciò, 2013). Ryan & Cacciò (2013) recommend using the loci *bg*, *tpi*, and gdh, at a minimum. The presence of false-negative cases in PCR, the difference between the amplification rates of the genes and the failure in the typing of the assemblages can result from many factors such as DNA degradation, ineffective DNA extraction, presence of PCR inhibitors, and the amount of non-specific amplified DNA (Arroyo-Salgado et al., 2014; Faria et al., 2016; Calegar et al., 2022). In this study, it should be considered that the DNA of the samples for PCR was extracted a few months after collection, which may have caused degradation. In addition, it is known that the deterioration of genetic material can limit the efficiency of PCR, especially for single-copy genes such as *bg*, *gdh*, and *tpi* (Peng et al., 2003). These factors may have determined the disagreement between the amplification rates of the ssu-rRNA, *bg*, *gdh*, and *tpi* loci in our study, which was also evidenced in other studies with populations of children (David et al., 2011, 2015; Ramírez et al., 2015; Scalia et al., 2016; Jerez Puebla et al., 2017) and dogs (Scorza et al., 2012; Gil et al., 2017). In this context, our study reinforces the need to use multiple genes for giardiasis diagnosis in human and animal populations (Faria et al., 2016; Soares & Tasca, 2016) to avoid diagnostic bias and the underestimation of prevalence. It is important to consider that in the absence of DNA degradation or inhibition during storage and/or processing, the use of a multicopy gene such as ssu-RNA would be expected to be sufficiently more sensitive than parasitological techniques or single-copy gene targeted PCRs. Thus, there would be no need for the parallel use of diagnostic techniques. However, in this study, it is reasonable to infer that the lack of sensitivity of the ssu-RNA gene was probably the result of DNA degradation, which may have significantly impacted the PCR sensitivity compared to the parasitological technique. We also emphasize the use of only one fecal sample per individual/dog in the molecular investigation of *Giardia* may also have underestimated the prevalence detected by this method (Scorza et al., 2012). Our finding also serves as a warning to researchers who usually use the ssu-rRNA gene in screening samples for *Giardia* before using other genes (Silva et al., 2012; Gasparinho et al., 2017).

Giardia genotyping studies in children and dogs have been restricted to both South and Southeast regions of Brazil (Volotão et al., 2007; David et al., 2015; Quadros et al., 2016; Scalia et al., 2016; Coelho et al., 2017). And in the Midwest and Northeast regions, which concentrate the largest series of acute diarrheal diseases in the country (Brasil, 2018), the epidemiology of this infection has still been poorly understood. Zoonotic assemblages A and B were detected in Northeastern children in a resource-poor community, in Ceará (Kohli et al., 2008) and in daycare centers in Bahia (Figueiredo Pacheco et al., 2020). Our study brings the first report of infection by these assemblages in children from rural areas, including tourist communities, in this region. We observed the presence of diarrhea in the infection with both assemblages. In contrast, Figueiredo Pacheco et al. (2020) detected cases of diarrhea only in children infected with the AI sub-assemblage. They also found that 91.8% of children with giardiasis had no diarrhea or any other relevant symptoms. Unfortunately, it was not possible to investigate the association between these assemblages and clinical signs due to the low number of samples that had their assemblages detected by sequencing. However, Kohli et al. (2008) did not find an association between diarrhea and assemblages A and B in single or mixed infections in children from Ceará.

Despite being considered livestock-specific, assemblage E has been detected inside and outside Brazil in humans (Foronda et al., 2008; Helmy et al., 2014; Abdel-Moein & Saeed, 2016; Fantinatti et al., 2016; Zahedi et al., 2017) and dogs (Feng & Xiao, 2011; Fava et al., 2016) that interact with environments frequented by farm animals, as we also observed. At the clinical level, as reported in a few other studies (Helmy et al., 2014; Abdel-Moein & Saeed,

2016; Zahedi et al., 2017), we detected assemblage E in samples of diarrheal children, suggesting the existence of a risk of zoonotic transmission. To the best of our knowledge, this is the first report of giardiasis by assemblage E in Northeastern children and dogs.

In these communities, the high prevalence of giardiasis in children probably results from social and behavioral factors. Among them, it could be included inappropriate habits of food and personal hygiene, use and consumption of untreated water, inappropriate handling of pets and farm animals, and contact with environments contaminated with dog and farm animal feces. All these factors can favor regular interaction with different transmission cycles (Santos et al., 2010; Thompson & Ash, 2016; Ryan et al., 2019; Calegar et al., 2022).

We understand that the parallel use of parasitological techniques for the detection and screening of fecal samples of *Giardia* is a viable option for maintaining the diagnostic efficiency of giardiasis, both in private laboratories and in research. Likewise, when feasible, we reinforce the recommendation of using multiple genetic markers, especially in the context of research, avoiding the bias of prevalence that underpins the elaboration of plans for the prevention of diarrheal diseases. It is a fact that the association of methods makes the diagnostic process more expensive. However, the cost-effectiveness of these associations for controlling these diseases must be rationally evaluated. Accurate information on prevalence is essential for controlling giardiasis in highly endemic tourist areas, such as the municipality of Ilhéus, which has become a focus for the spread of diarrheal diseases, also beyond its borders.

Conclusion

The complementarity of parasitological and molecular methods proves to be a more effective strategy for detecting and controlling giardiasis in highly endemic areas, such as rural and semi-rural communities in the municipality of Ilhéus. However, given the high cost of implementing molecular techniques, especially when it is necessary to use several genetic markers, we recommend that the diagnosis of *Giardia* infection be made through the parallel use of parasitological techniques that use the principle of fluctuation. The zoonotic potential of assemblage E should be further investigated in this region.

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Ethics declaration

This study was approved by the Ethics Committee for the Use of Animals under protocol no 023/2015 and by the Ethics Committee for Research with Humans of the State University of Santa Cruz, Bahia, under protocol CAAE 51181915.6.0000.5526.

Conflict of interest

There is no conflict of interest.

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Supplementary Material

Supplementary material accompanies this paper.

Suppl 01. Modifications made to established gene amplification protocols used in molecular research on *Giardia*.

Table S1. Modifications of the bg gene amplification protocol established by Cacciò et al. (2002) (1st reaction) and by Lalle et al. (2005) (2nd reaction) for the molecular research of *Giardia lamblia*.

Table S2. Modifications of the gdh gene amplification protocol established by Cacciò et al. (2008) (1st and 2nd reactions) for the molecular research of *Giardia lamblia*.

Table S3. Modifications of the ssu-rRNA gene amplification protocol established by Appelbee et al. (2003) (1st reaction) and by Hopkins et al. (1997) (2nd reaction) for the molecular research of *Giardia lamblia*.

Table S4. Modifications of the tpi gene amplification protocol established by Sulaiman et al. (2003) (1st and 2nd reactions) for the molecular research of *Giardia lamblia*.

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