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Original Article

Frequency of *Giardia duodenalis* infection and its genetic variability in dogs in Cuiabá, Midwest Brazil

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Abstract

Introduction: *Giardia duodenalis*, a unicellular, eukaryotic, and flagellated protozoan, presents two evolutionary forms in its life cycle, namely, trophozoites and cysts. It causes diarrhea in humans, dogs, cats, rodents, and ungulates. Despite being morphologically similar, the isolates of *G. duodenalis* are genetically diverse, affecting the stability and unanimity of taxonomic classification. Since different *Giardia* assemblages may occur within one isolate, multilocus genotyping is recommended for the genetic identification.

Methodology: To determine the frequency of *G. duodenalis* infections in domiciled dogs in Cuiabá Municipality (State of Mato Grosso, Midwestern Brazil) and characterize its genetic variability, fecal samples were collected from 147 dogs.

Results: Overall, 6.8% (10/147) of the samples presented cysts of *G. duodenalis*, which sequencing and genotypic characterization using *tpi* and *gluD* revealed assemblages C and A, genetic grouping of *G. duodenalis*. Only three samples amplified by *tpi* and one sample amplified by *gluD*.

Conclusions: The risk factors age, gender, breed, diet and the presence of other dogs in the same house were not correlationated with giardiasis. The host-specific and zoonotic genotype warns of the risk of inter and intraspecies transmission and it provides, for the first time, information about genetic characterization of *G. duodenalis* isolates in dogs in Cuiabá, Midwest region of Brazil.

Key words: molecular characterization; multilocus genotyping; waterborne.

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Introduction

Giardia duodenalis (syns. *Giardia intestinalis* and *Giardia lamblia*) is one of the most important waterborne protozoan pathogens in both developed and developing countries, causing diarrhea in many different species around the world [1]. It is genotypically divided into various assemblages (A-H) of G. duodenalis which include the sub genotypes AI, AII, BIII, BIV [2]. Assemblages C and D are most frequently found in dogs, but also can sporadically infect humans, whereas assemblages A and B are commonly reported in humans [3].

G. duodenalis infection occurs via the ingestion of its cysts in water and contaminated food, as well as via the fecal–oral route. In the stomach of the host, cysts rupture because of gastric acidity, releasing trophozoites that replicate in the intestine and cause watery diarrhea, abdominal pain, nausea, and vomiting. However, the attachment of their ventral disk to the epithelium of the intestine is responsible for pathophysiological mechanisms [4]. Most infections are self-limiting [3], depending on the characteristics of the parasite (strain, number of cysts ingested, ability to adhere and cause tissue damage, and virulence factors) and host (immune and nutritional status, gastric juice pH, and intestinal microbiota) [5].

Although *G. duodenalis* infection is global, there is a large difference in its prevalence between countries. In Brazil, its prevalence in canines ranges from 2% to 70% [1]. However, in the Midwest region of Brazil, there are only a few studies on the prevalence of giardiasis, especially in the State of Mato Grosso, except for reports of 72,7% of prevalence in children and 0,04% in vegetables [6,7].

Light microscopy is the most commonly used method for the identification of *G. duodenalis* cysts and trophozoites in fecal samples [8]. The sensitivity and specificity of antigen detection methods such as

enzymatic immunoassay and immunochromatography range from 26% to 100% and 79% to 100%, respectively [8]. In addition, polymerase chain reaction (PCR) has been used for the genotypic characterization of species [9,10] with different targets, such as, ribosomal subunit (SSU-rDNA), triose phosphate isomerase (TPI), β-giardin, glutamate dehydrogenase (GLUD), and internal regions of transcribers (ITS1-2) [11].

In the present study, we evaluated the frequency of *G. duodenalis* infection in dogs in Cuiabá Municipality, Midwest Brazil. In addition, this study aimed to genotyping cysts of *G.* duodenalis from dogs, using *tpi* and *gluD* genes.

Methodology

Study design

Fecal samples of dogs were collected at the Veterinary Hospital of Federal University of Mato Grosso, in the Cuiabá Municipality (15°35′56″S, 56°06′01″W) between January 2016 to December 2017. The owners of the 147 dogs from which the fecal samples were obtained answered a questionnaire based on closed-ended questions, with at least two questions to determine the profile of dogs and their households [age (≤ 1 year, puppy; > 1 year, adults), sex, breed, signs of diarrhea, other dogs in the house and diet].

Parasitological analysis

The samples were collected after defecation, stored in a plastic container for a maximum of 24 h under 4ºC refrigeration, and part of each sample was concentrated using zinc sulfate flotation technique with the final sediment being examined by just one trained veterinary medic using an light microscope to view of cysts [12].

Molecular tests

DNA was extracted from feces aliquots using the NucleoSpin Tissue Kit (Macherey-Nagel®, Düren, Germany). Briefly, the fecal samples with visible *G. duodenalis* cysts suspended in a sodium chloride solution were washed four times with Tris-EDTA solution (pH = 8.0), then subjected to thermal shock cycles to rupture the cysts [13]. The manufacturer's protocol was then followed to extract DNA.

For *G. duodenalis* genotyping, nested and seminested PCR amplification was performed as previously described [13,14] on 530 and 659 base pair (bp) fragments of *tpi* and *gluD*, respectively. Nuclease-free water and DNA from *G. duodenali*s obtained from naturally infected domestic dogs were used as the negative and positive controls, respectively. Primers used for PCR are presented in Table 1. The amplified products were analyzed by electrophoresis on a 1.5% agarose gel and visualized using the ChemiDoc® (Bio Rad, Berkeley, California) transilluminator. After purification using the GFX TM PCR DNA Purification Kit and Gel Band Kit (GE Healthcare, Chicago, Illinois), the samples were sequenced using the ABI-PRISM 3500 Genetic Analyzer (Applied Biosystems, Foster City, California) and subsequently analyzed using the BLAST NCBI program (http://www.ncbi.nlm.nih.gov/blast/Blast.cgi). The Neighbor-Joining method was used to build the phylogenetic tree with the concatenated *tpi* and *gluD* gene sequences after alignment with the Muscle method using MEGA program (Version 10.1.7).

Statistical Analysis

The frequency of infection was determined using the ratio of samples from dogs with cysts from the examined dogs. The association between sex, breed, age, diet, other animals in the house, and *G. duodenalis* infection was verified using the non-parametric Chi-square or Fischer's Exact test, if the cell values were less than five. These tests were performed in Epi Info® (CDC, EUA), with the significance level set to 5%.

Table 1. Genes used for genotyping *Giardia duodenalis*.

Gene	Sequence	Base pairs (number)
tpi [14]	First reaction: Forward: 5'AAATIATGCCTGCTCGTCG 3' Reverse: 5'CAAACCTTITCCGCAAACC 3' Second Reaction: Forward: 5'CCCTTCATCGGIGGTAACTT 3' Reverse: 5′ GTGGCCACCACICCCGTGCC 3′	605 bp 530 bp
gluD [13]	First reaction Forward: 5'AAYGAGGTYATGCGCTTCTGCCA 3' Reverse: 5'GATGTTYGCRCCCATCTGRTAGTTC 3' Second reaction Forward: 5'ACTTCCTBGAGGAGATGTGCAAGGA 3' Reverse: 5'-GATGTTYGCRCCCATCTGRTAGTTC 3'	890 bp 659 bp

Ethical statement

The study was approved by the Ethics Committee of the Federal University of Mato Grosso (CEUA-UFMT) with the approval number 23108.170944/2016-16.

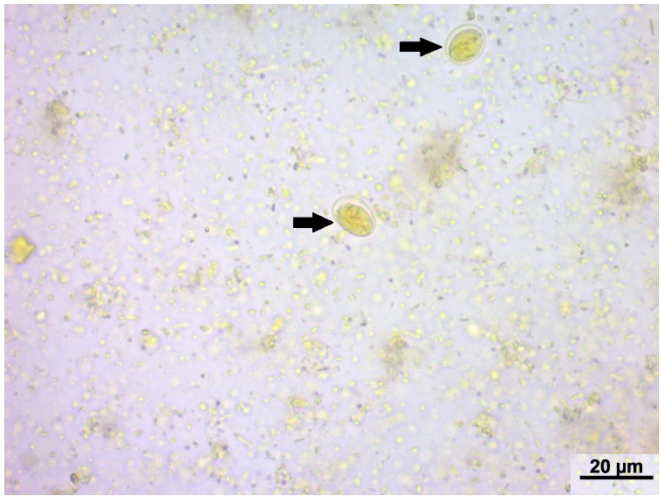
Results

Parasitological diagnostic

Among the 147 samples evaluated, *G. duodenalis* cysts were observed in 10 samples [6.8%; 95% confidence interval (CI) 3.3%–12.2%] (Figure 1). Among the dogs parasitized, 5 (50%) were undefined breeds, 2 (20%) were American Bullies, 1 (10%) was a Rottweiler, 1 (10%) was a Dalmatian, and 1 (10%) was a Cocker Spaniel. Regarding age, there was no significant difference between puppies and adults—7 dogs (70%) were under 12 months of age and 3 (30%) were adults. Similarly, there was no sexual predisposition, because six (60%) parasitized dogs were females and four (40%) were males. The clinical sign of diarrhea had no significant difference in dogs with *G. duodenalis* infection. Diet and the presence of other dogs in the same house were not significantly associated with *G. duodenalis* infection (Table 2).

Additionally, in the examination of 147 samples, using microscopy, 16 dogs were observed with helminth eggs and protozoan cysts, such as those of *Ancylostoma* spp. (4,8%; 95% CI 1.9%–9.6%), *Trichuris* spp. (1,4%; 95% CI 0.2% - 4.8%) and *Toxocara* spp. (0,7%; 95% CI 0 – 3,7%), in addition to *Cystoisospora* spp. (2,7%; 95% CI 0.7% - 6.8%) and *Entamoeba* spp. (1,4%; 95% CI 0.2% - 4.8%). However, coinfection with *G. duodenalis* was not observed.

Figure 1. *G. duodenalis* cysts visualized by light microscope using Lugol's iodine from fecal samples of dogs.



Molecular analysis

Of the total (n = 10) positive samples, by microscopy, 3 (30%) amplified fragments of the *tpi* gene, and 1 (10%) of the *gluD* gene.

Gene tpi

All isolates from dogs (CUIABA20, CUIABA164 and CUIABA165) were characterized as Assemblage C presenting homology with the LC437553.1 and MN270282.1 sequences stored at GenBank® witch were isolated from dogs.

Gene gluD

The isolated CUIABA165 was identified as assemblage A presenting 100% homology with sequences stored at GenBank® with accession numbers LC507405.1 witch was isolated from human.

Table 2. Frequency of dogs infected with *G. duodenalis* based on sex, age, breed, diet, and presence of other animals in the house.

Variable		Negatives	Positives	Total	P value
Sex	Male	61 (44.5%)	4 (40%)	65	0.52
	Female	76 (55.5%)	6 (60%)	82	
	Total	137	10	147	
Age	Puppy	54 (39.4%)	6 (60%)	60	0.17
	Adult	83 (60.6%)	4 (40%)	87	
	Total	137	10	147	
Breed	Mixed Breed	53 (38.7%)	5 (50%)	58	0.47
	Pure Breed	84 (61.3%)	5 (50%)	89	
	Total	137	10	147	
Animals contactants	More than one	79 (57.7%)	7 (70%)	86	0.33
	None	58 (42,3%)	3 (30%)	61	
	Total	137	10	147	
Diet	Commercial	129 (94.2%)	8 (80%)	137	0.13
	Homemade	8 (5,8%)	2 (20%)	10	
	Total	137	10	147	

Phylogenetic analysis

In order to determine the genetic relationship of the isolates, phylogenetic analysis was performed using concatenated data in the respective sequences of the *tpi* and *gluD* genes (Figure 2). The sequences of isolates deposited on GenBank with the specified assemblages were used for the construction of the phylogenetic tree (A - LC507554.1 and LC507405.1; B - LC507547.1 and LC507394.1; C - LC437503.1 and LC437380.1; D - LC437578.1 and LC437387.1; E - KY655482.1 and KY655480.1; F - KF993726.1 and KF993735.1). The isolate CUIABA165, from this study, remained in the same clade as *G. duodenalis* assemblage C.

Discussion

The frequency of *G. duodenalis* infection observed in dogs in this study was 6.8%, which is lower than that reported in Campo Grande, Mato Grosso do Sul (MS) (27.3%) [15] and higher than that reported in Goiânia, Goiás (GO) (1.6%), cities in the Midwest region of Brazil [16].

All dogs evaluated in this study originated from private households, unlike those in MS, which included dogs from a higher proportion of kennels, as well as, zoonoses control centers, which may have contributed to increased prevalence [15]. In GO, the protozoan was found only in domiciled dogs, and this can be attributed to the low prevalence in this group [16]. Therefore, despite the fact that the three cities are located in the Midwest region, other factors contribute to these different rates of prevalence, such as the type of environment in which the animals live and the presence of a population cluster [1].

Age, sex, and breed of infected dogs were not considered risk factors for infection [15,17]. However, some studies claim that puppies are more predisposed [18] due to immunosuppression and their habit of chewing objects [1,11]. In addition, male dogs have been reported to be at a higher risk because of activity in a greater territorial area [19].

The clinical sign of diarrhea was observed in positive and negative dogs, and did not increase the chance of finding *G. duodenalis* parasitism. This can occur because of the intermittent elimination of cysts [4] or because the variability of clinical manifestations depends on the hosts' immunocompetence [18]. In addition, most dogs show subclinical infection [20].

Regarding the non-association of food as a risk factor, it can be inferred that because they are domiciled dogs, the chance of consuming food contaminated with cysts is negligible when compared with stray dogs that live freely [17].

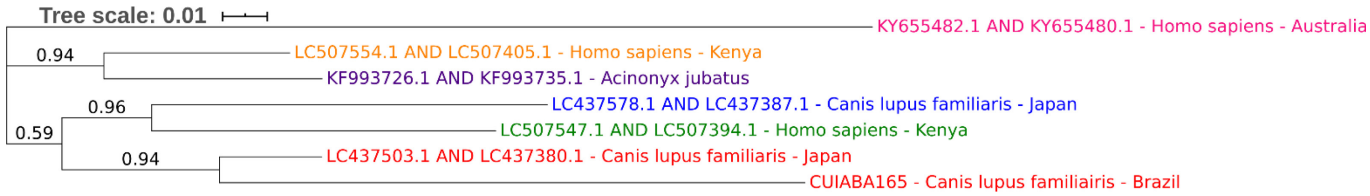
The presence of other dogs in the same household was not a significant factor for the occurrence of giardiasis in this study, which is consistent with previously reported findings [21], who reported that single dogs and the dogs living in the presence of more than one dog had a similar prevalence. Similarly, other research found a significant statistical difference of infection rates between shelter dogs and household pets [22]. This is probably due to the greater concentration of animals in a shelter and the greater ambient contamination to which they are exposed.

In Brazil, multilocus genotyping to characterize assemblages of *G. duodenalis* has been conducted only in the southern and southeastern regions with dogs, cats, sheep and cattle [23]. However, this study is the first to characterize *G. duodenalis* genotypes in dogs in the Midwest.

Concatenated analysis of the sequences showed that the *tpi* gene showed greater specificity when classifying the assemblage of *Giardia* species. This fact can be explained by mixed infection or by heterozygosis present in the sequence of *gluD* gene [24-26]. It is estimated that different loci of the same isolate can cause a discrepancy in the identification of assemblages in 15% of the cases [27]. This discrepancy has important implications for molecular studies that use only one gene to classify the assemblages of isolates [25,26].

Assemblage C identified in the sampled dogs has been described in several countries [28] including Brazil. Studies in São Paulo had a prevalence of 25.8% [29] and in Minas Gerais 18.7% [30]. Although this

Figure 2. Phylogenetic relationship of *G. duodenalis* isolate characterized by the sequencing of *tpi* and *gluD* genes. The phylogenetic tree was built using concatenated data from *tpi* and *gluD* genes by Neighbor-Joining analysis. Bootstrap values above 50% are shown in the figure. Assemblage A is represented by the color yellow, assemblage B by the color green, assemblage C by the color red, assemblage D by the color blue, assemblage E by the color pink and assemblage F by the color purple.



genotype is common in dogs, cats, coyotes, and wolves, it has been reported in human samples as well [31].

Another assemblage commonly found in dogs is type D [17,29]. Although assemblages C and D occur more commonly in dogs, assemblages A and B, which are common in humans, have been detected in dog feces in different continents, including South America [3]. Besides that, infection by assemblages C and D occurs as a result of species-specific transmission [32,33].

Moreover, the low rate of prevalence could be explained by the intermittent elimination of cysts and the low rate of DNA amplification can be explained by the small number of cysts in the sample, the loss of the cysts during their recovery, the small amount of DNA, the presence of fecal inhibitors in the samples, the small volume of the sample or the loss from washing the sample [13,30].

Two assemblages of *G. duodenalis*, assemblage A and C, were identified in the fecal sample of one of the examined dogs, however, when constructing the concatenated tree the sample showed greater proximity with assemblage C, these discrepancies between the loci of the same isolate may occur due to mixed infections or heterozygosis of the species [30].

In this study, *G. duodenalis* was most prevalent, followed by *Ancylostoma* spp. Similarly, other authors founded a high prevalence of *G. duodenalis* in the City of Medellín, Colombia [34] where dogs and cats had rates of *Giardia* infection higher than *Ancylostoma* spp.

Conclusions

The frequency of *G. duodenalis* infection in dogs was 6.8%, and age, gender, breed, diet and the presence of other dogs in the same house were not risk factors for the occurrence of giardiasis from several countries. Furthermore, the observation of assemblages A and C of *G. duodenalis* warns of the risk of inter and intraspecies transmission in the studied environment, especially for the zoonotic risk. It is suggested that further studies be carried out with other dogs and other mammals so that it is possible to characterize the genotypes that occur in several species of animals in the state of Mato Grosso, Midwest, Brazil.

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