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Original Article

Multilocus genotyping of *Giardia intestinalis* in pet dogs of Medellín Colombia

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ABSTRACT

According to a few parasitological and epidemiological studies, *Giardia* is the most prevalent parasitic infection among pet dogs in the city of Medellín, the second-largest city in Colombia. This study determined the assemblages of *Giardia* in the fecal samples of dogs obtained from 18 veterinary centers of Medellín. One hundred fecal samples of dogs diagnosed with *Giardia* using microscopy were analyzed via nested polymerase chain reaction (PCR) using three genes (*gdh, bg,* and *tpi*). The PCR products were purified and sequenced, and phylogenetic analyses were conducted using the maximum likelihood algorithm of the three loci. From the 100 samples analyzed, 47 were *Giardia*-positive via PCR. Genotypes C and D were detected in six samples, neither of which were associated with human infection. However, the zoonotic potential of *Giardia* cannot be ruled out because of the small number of samples that could be sequenced for assemblage assignation.

1. Introduction

Giardia intestinalis is an enteric zoonotic parasite distributed worldwide. The clinical spectrum of giardiosis varies from asymptomatic cases to acute diarrhea and malabsorption syndromes, and can affect humans and animals. Besides its clinical relevance, the zoonotic significance of giardiosis has been studied after numerous outbreaks were reported, with water contamination with animal feces as the main source of infection (Einarsson et al., 2016). Furthermore, pet animals have been associated with direct transmission to humans, particularly in endemic settings (Bouzid et al., 2015).

The life cycle of *Giardia* has two main stages: trophozoite (non-infective form) and cyst (infective form). Cysts ingested with water or contaminated food travel through the digestive track until the small intestine, and then excyst to liberate trophozoites that adhere to epithelial cells. Trophozoites divide via binary fission and may eventually migrate to the colon where they are released as cysts into the feces (Adam, 2001). Cysts can remain infectious for months in cool and damp areas, making the risk of infection a serious health concern (Olson et al., 1999).

The host specificity of *Giardia* is linked to genetic groups or assemblages. At present, there are eight recognized genetic assemblages named from A to H (Feng and Xiao, 2011). Assemblages A and B infect humans, livestock, and companion animals. Assemblages C and D have been found in domestic and wild canines, including dogs. Assemblage E has been found in domestic ruminants and pigs, assemblages F and G in cats and rodents, and assemblage H in seals and gulls (Lasek-Nesselquist et al., 2010).

The molecular characterization of *Giardia* involves the initial classification of species, followed by a genetic assemblage assignation. For the first step, in the *Giardia* genome, there are several multi-copy genes with enough polymorphisms to be used as markers for species differentiation [e.g., the small subunit ribosomal RNA gene (*ssuRNA*) and the elongation factor 1 gene (*ef1a*)] (Ballweber et al., 2010). For the assemblage and sub-assemblage assignation, the most common approach is multilocus genotyping (MLG). It entails an initial polymerase chain reaction (PCR) amplification of highly conserved target genes, such as glutamate dehydrogenase (*gdh*), triose phosphate isomerase (*tpi*), and beta giardin (*bg*) (Cacciò et al., 2008), and then the utilization of downstream procedures, such as restriction fragment length

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polymorphism or sequencing of PCR products. Because several studies have reported that *G. intestinalis* isolates can produce different results depending on the target locus used in the MLG (Feng and Xiao, 2011), MLG has been considered as one of the most informative approaches for genotyping this parasite.

In Colombia, according to the last national survey of intestinal parasitosis in 2012–2014, giardiosis has an infection rate of 15.4% in humans and mainly affects children (Ministerio de Salud y Protección Social, Universidad de Antioquia, 2015). Regarding the epidemiology of *Giardia* infections in dogs, a worldwide meta-analysis including 127 scientific publications and 4,309,451 animals has revealed a pooled prevalence of 15.2% (Bouzid et al., 2015). Meanwhile, some regional studies in Colombia have reported *Giardia* prevalence of 8.8% ($n = 68$) (Sierra-Cifuentes et al., 2015), 12.5% ($n = 104$) (Arroyo-Salgado et al., 2014), 13% ($n = 1111$) (López-Arias et al., 2019), 13.9% ($n = 187$) (Caraballo et al., 2007), and 14.2% ($n = 119$) (Rodríguez et al., 2014) among dogs. In the very few cases where genetic analysis was performed on these animals, only assemblages B, C, and D were observed (Arroyo-Salgado et al., 2014; Rodríguez et al., 2014).

The lack of data in this area of veterinary parasitology suggests a latent and unsolved problem for public health in Colombia regarding zoonotic transmission. The increasingly important role that pets play in our lives, added to sociocultural factors and sanitary conditions in some regions of the country may increase the risk of zoonosis. Therefore, to establish an epidemiological landscape of giardiosis in humans and animals in Colombia, further studies of prevalence and genotyping of *Giardia* are warranted. This study identified the *Giardia* genetic assemblages observed in dog fecal samples and the phylogenetic relationship between them.

2. Methods

2.1. Sample source

Between January 2018 and May 2018, dog fecal samples were collected from 18 veterinary clinics located in Medellín. After obtaining permission from the owners to include their pets in the study, dogs with or without gastrointestinal symptoms were selected for fecal sample collection immediately after natural defecation. In a previous work, we had performed microscopic analysis of these samples (López-Arias et al., 2019) using fecal smears (with Lugol staining) and flotation techniques with saturated sucrose solution and zinc sulfate (Deplazes et al., 2016). One aliquot from each sample was stored without preservatives at −20 °C. This study was approved by the ethics committee of the University of Antioquia–Colombia under the IACUC act 119 of 2018.

2.2. DNA extraction and quality control tests

Hundred *Giardia*-positive samples (identified via microscopy) were selected to perform molecular analysis. DNA was extracted from frozen samples using the QIAamp DNA Stool Mini Kit (Qiagen, Germany) according to the manufacturer's instructions with minor modifications described previously by Hernández et al. (2019). The DNA was eluted in 100 µL of the elution buffer (Qiagen) and stored at −20 °C until use.

To confirm the presence of DNA, the obtained extracts were evaluated via PCR using specific primers to amplify the 16S rRNA gene from Enterobacteriaceae (Yean et al., 2007; Dridi et al., 2009) (Table 1). The PCR reaction mixture contained 2 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primers, 0.5 U of DNA GoTaq® Flexi DNA Polymerase (Promega), and 2 µL of the template (DNA extracted from stool samples). The amplification program was as follows: initial denaturation at 95 °C for 5 min, followed by 45 cycles at 95 °C for 30 s, 50 °C for 30 s, and 60 °C for 30 s and a final extension at 72 °C for 5 min. DNA from *Enterococcus faecalis* strain ATCC 29212 was used as a positive control.

If the Enterobacteriaceae 16S rRNA gene was not amplified via PCR, a heterologous control DNA template was used in an independent reaction to detect PCR inhibitors. The internal amplification control used was a plasmid harboring the coding sequence of the Dengue Virus Nonstructural Protein 3 (pMAL_NS3403-599) (Morales et al., 2017). The PCR reaction included 1 mM $MgCl_2$, 0.2 mM dNTPs, 0.4 µM primers (Table 1), 0.5 U DNA GoTaq® Flexi DNA Polymerase (Promega), and 2 µL of template DNA (extracted from stool samples) plus 20 ng of plasmid DNA. The amplification program comprised an initial denaturation step at 95 °C for 3 min, followed by 45 cycles at 95 °C for 30 s, 51 °C for 30 s, and 72 °C for 30 s and a final extension at 72 °C for 5 min (Morales et al., 2017).

Table 1
Primers sequences used for PCR amplification for quality control of DNA samples.

	Enterobacteriaceae	Dengue virus (DENV2)
Target	16S rRNA	NS3
Forward primer	16SrRNA-F. 5'-TTACCGCGGCKGCTGGCAC-3'	NS3B_F1. 5'-CATATGGACTTTGTGGTCACGAC-3'
Reverse primer	Bact515R. 5'-TTACCGCGGCKGCTGGCAC-3'	NS3B_R1. 5'-GGATCCTAACCTAGCATCCAACC-3'
Product size	397 pb	606 pb
Reference	Yean et al. (2007), Dridi et al., 2009	Morales et al., 2017

2.3. Detection and genotyping of Giardia intestinalis via PCR

To identify *G. intestinalis*, nested PCR was performed using the method described by Appelbee et al. (2003). First, a 497-bp sequence was amplified using primers Gia2029 and Gia2150c (0.5 µM each). The second amplification reaction was performed using RH11 and RH4 (0.5 µM each) (Table 2). The cycling conditions for primary PCR comprised an initial denaturation step at 96 °C for 4 min, followed by 35 cycles at 96 °C for 30 s, 55 °C for 30 s, and 72 °C for 45 s and a final extension at 72 °C for 4 min. The same conditions were used for the secondary PCR, except that the annealing temperature was set at 59 °C. All PCR reactions included 2 mM $MgCl_2$, 200 µM dNTPs, 0.5 U of GoTaq DNA polymerase (Promega), and 5 µL of the extracted DNA (for primary PCR). For secondary PCR, 1 µL of the primary PCR product was used as the template. DNA from *G. intestinalis* strain WB-C6 for assemblage A was used as a positive control.

To identify *G. intestinalis* assemblages, a semi-nested PCR reaction was performed to amplify a *gdh* gene fragment (~432 bp) using the method described by Read et al. (2004). Amplification of *bg* (511 bp)

Table 2
Set of primers used for *Giardia intestinalis* PCR.

Target	Name	Sequence	Reference
ssuRNA	Gia2029	5'-AAGTGTGGTGCAGACGGACTC-3′	Appelbee et al., 2003
	Gia2150c	5'-CTGCTGCCGTCCTTGGATGT-3'	
	RH11	5'-CATCCGGTCGATCCTGCC-3'	
	RH4	5'-AGTCGAACCCTGATTCTCCGCCAGG-3'	
gdh	GDHeF:	5'-TCAACGTYAAYCGYGGYTTCCGT-3'	Read et al., 2004
	GDHiF:	5'-CAGTACAACTCYGCTCTCGG-3′	
	GDHiR:	5'-GTTRTCCTTGCACATCTCC-3′	
tpi	AL3543	5′-AAATIATGCCTGCTCGTCG-3′	Sulaiman et al., 2003 Lebbad et al., 2010
	AL3546	5′-CAAACCTTITCCGCAAACC-3′	
	TPIDF	5-CCGTTCATAGGTGGCAACTT-3	
	TPIDR	5-GTAGCCACTACACCAGTTCC-3	
bg	G7	5′-AAGCCCGACGACCTCACCCGCAGTGC-3′	Lalle et al., 2005
	G759	5′GAGGCCGCCCTGGATCTTCGAGACGAC-3′	
	GiarF	5′-GAACGAGATCGAGGTCCG-3′	
	GiarR	5′-CTCGACGAGCTTCGTGTT-3′	

and *tpi* (530 bp) fragments was performed via nested PCR using the method described by Lalle et al. (2005), Sulaiman et al. (2003), and Lebbad et al. (2010). All PCR conditions were as described by Hernández et al. (2019).

2.4. DNA sequencing and phylogenetic analysis

Secondary PCR products were purified, and both strands were sequenced by MACROGEN, (Korea). Chromatograms were analyzed and edited, and sequences were aligned using MEGA Version X (Kumar et al., 2018). Phylogenetic analyses of these sequences and reference sequences representing each assemblage (S1 Table) were performed using the maximum likelihood algorithm. The Tamura–Nei parameter model was used to construct phylogenetic trees with the highest likelihood logarithm value. The reliability of clusters was evaluated using a bootstrap of 1000 iterations. The WB reference strain of *G. intestinalis* was also used in the MLG assays. The nested-PCR products of *tpi*, *gdh,* and *bg* were sequenced, and these sequences were included in the phylogenetic analysis. Novel sequences obtained in this study were deposited in GenBank under accession numbers MT081971, MT081972, MT180091, MT180092, MT180093, MT188677–MT188685 (S2 Table).

3. Results

Among the 100 DNA samples extracted from dog feces, 83 were PCR-positive for Enterobacteriaceae. The remaining samples ($n = 17$) were analyzed again via PCR using primers targeting a heterologous amplification control. In this assay, products were obtained for only eight samples, suggesting the presence of PCR inhibitors in the other nine samples. DNA extraction was repeated for the eight positive samples; however, no PCR products were obtained, suggesting DNA degradation during transport or storage. Therefore, species classification and genetic assemblage assignment was performed for 83 samples. Fourty-seven samples were positive for at least one PCR for *Giardia*, whereas 36 samples did not give rise to any product in any PCR. The number of PCR-positive samples for *ssuRNA*, *tpi*, *gdh,* and *bg* were 47, 4, 5, and 5, respectively. Six samples were positive in at least one of the PCRs for *tpi*, *gdh,* and *bg,* and three of them were positive for all three assays simultaneously (Table 3). Assemblage assignation using the three loci revealed that 3 of 6 samples belonged to assemblage C and that the other three belonged to assemblage D (Table 3). Fig. 1 shows the phylogenetic relationship of the sequences of the three analyzed loci with reference sequences retrieved from NCBI. The obtained dendrograms differentiated assemblages with high bootstrap support. The phylogenetic analysis of *G. intestinalis* sequences obtained in the present study and those retrieved from GenBank were revealed the existence of different clades for the *bg, gdh* and *tpi* genes with in assemblages C and D. The obtained sequence samples of *bg* gene, CM002, CM094 and CM100 were closely clustered with assemblage D (accession number AY545647); but CM091 and CM023 were clustered with assemblage C (accession number AY545646). Similarly, sequence samples of *gdh* gene, CM002, CM094 and CM100 were also closely clustered with assemblage D (accession number U60986); but CM091 and CM010 were clustered with assemblage C (accession number U60982). The sequence samples of *tpi* gene, CM002 and CM100 were also closely clustered with assemblage D (accession number DQ246216); whereas the others sequences CM091 and CM023 were clustered with assemblage C (accession number AY228641).

Finally, heterogeneous positions (chromatograms showing overlapping nucleotide peaks at specific positions) and single nucleotide polymorphisms (SNP) at *tpi, gdh,* and *bg* loci are shown in Table 4. Samples sequences were compared with reference sequences obtained from GenBank.

Table 3
Multilocus genotyping results of *Giardia intestinalis* from dogs of Medellín.

Sample	Assemblage			MLG
	Gene marker			
	tpi	*gdh*	*bg*	
CM002	D	D	D	D
CM010	NA	C	NA	–
CM023	C	NA	C	–
CM091	C	C	C	C
CM094	NA	D	D	–
CM100	D	D	D	D

NA: non-amplified.

4. Discussion

In this study, we performed a genotyping analysis of *G. intestinalis* in dogs in Medellín, the second-largest city in Colombia, for the first time. in dogs, assemblages C and D are very common but they can also become infected with assemblages A or B, which are potentially zoonotic (Ballweber et al., 2010; Deng et al., 2017). Because dogs can harbor both zoonotic and host-specific assemblages, this study was conducted to examine the distribution of *Giardia intestinalis* genotypes among companion dogs in Medellín.

This study included 100 dog fecal samples previously confirmed as *Giardia*-positive by microscopy. However, only 47 samples were PCR-positive for the *Giardia* multi-copy ssuRNA gene; from these 47 samples, only 6 provided results in the MLG assay to identify genetic assemblages. Technical problems during DNA extraction were neglected based on the results of the PCR assay targeting the 16S rRNA gene from Enterobacteriaceae, which resulted in the detection of strong bands for 83 samples. For the remaining 17 samples with negative results, PCR targeting the heterologous control revealed that 9 samples had polymerase inhibitors and only 8 samples had no detectable DNA. Studies from Korea (Kim et al., 2019) and Germany (Sommer et al., 2018) using MLG analysis of three loci (*ssuRNA, gdh and bg or ssuRNA, gdh and tpi*) have shown similar results: the highest amplification was obtained using the *ssuRNA* locus, whereas those of the *gdh*, *bg,* and *tpi* loci were less sensitive. This lower PCR performance for genotype *Giardia* compared with that of the coproscopic method has been explained in other studies (Gelanew et al., 2007; Volotão et al., 2007) as well as differences in efficiency of PCR amplification between different genes (Ryan and Cacciò, 2013). For the present study, we believe that this discrepancy could also have been caused by long-time storage (6–12 months) before DNA extraction because some parasites are particularly vulnerable even in freezing conditions. This fragility can lead to *Giardia* structure degradation and DNA damage, thereby affecting the quantity and quality of the extracted nucleic acids (Flekna et al., 2007; Nechvatal et al., 2008; Wilke and Robertson, 2009). Another possible explanation for the false-negative results could be low DNA concentration in some samples related to a small number of cysts shredded in the feces. Unfortunately, although the ssuRNA gene is more sensitive than the *tpi*, *bg*, or *gdh* genes in detecting *Giardia*, it is less specific for assemblage determination. Genotypic discrimination cannot be performed using the ssuRNA gene because its SNPs content is very low.

A retrospective study of enteric parasites in 1111 dogs and 203 cats in Medellín is the most recent study for pet intestinal parasitosis in this city (López-Arias et al., 2019). Among the different parasites detected, *G. intestinalis* was the most frequent parasite in dogs (13%) and cats (20%). The present study used 100 fecal samples collected from dogs in the mentioned study and found assemblages C and D via DNA sequence analysis of three genes (*tpi, bg,* and *gdh*). Studies of the prevalence and molecular typing of *G. intestinalis* in dogs in Colombia are scarce. A study from the Caribbean coast in 2014 that sampled 13 *Giardia*-positive dogs could genotype four samples in the assemblage B (Arroyo-Salgado et al., 2014). Another study in the central area of the country (city of Ibagué) found *Giardia* in 17 samples; four of them were genotyped into C and D assemblages (Rodríguez et al., 2014). Recently, in a *Giardia* prevalence

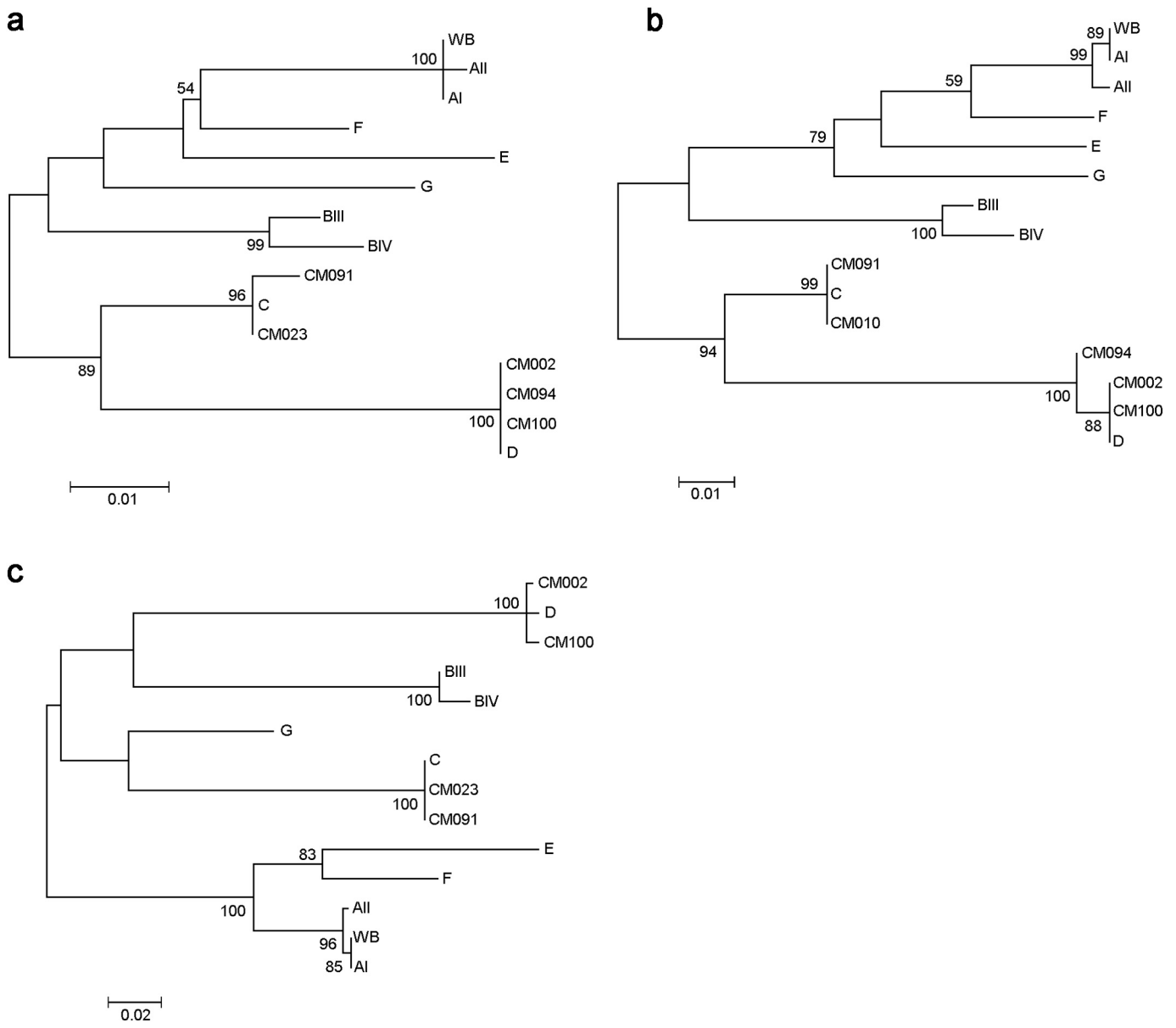

Fig. 1. Phylogenetic trees of *Giardia intestinalis* of canine samples based on nucleotide sequences of three gene loci. Sequences of the *bg* (a), *gdh* (b), and *tpi* (c) genes retrieved from samples obtained in this study were compared with reference sequences from GenBank (Supplementary Table S1 and S2). Bootstrap values >50% from 1000 interactions are indicated at nodes.

study in children and eight dogs in southwest Colombia (city of Popayán), a single dog *Giardia*-positive sample was analyzed and genotyped as sub-assemblage BIII (Villamizar et al., 2019). Similar to our study, in Brazil, genotypes C and D were found in naturally infected dogs from Sao Paulo (Souza et al., 2007). Likewise, a study in Vietnam found infected dogs with *Giardia* belonging to assemblages C and D exclusively (Nguyen et al., 2018), as well as studies from Korea, Israel and Poland (Kim et al., 2019; Salant et al., 2020; Piekara-Stępińska et al., 2020). A survey in Japan (Itoh et al., 2011) and several studies from China (Qi et al., 2016; Zhang et al., 2017) and Thailand (Tangtrongsup et al., 2018) have found similar results.

Human giardiosis is relatively frequent in Colombia, reaching a prevalence rate of 15.4% in children between 7 and 10 years old (Ministerio de Salud y Protección Social, Universidad de Antioquia, 2015). However, because the present study resulted in limited genotypic data, sufficient information is not available to establish whether there is a relationship between human and canine infections with *Giardia*.

The zoonotic potential of *Giardia* remains controversial as there is little evidence of animal-to-human transmission (Sprong et al., 2009; Cooper et al., 2010). In this sense, close contact between owners and their household dogs is assumed to promote canine *Giardia* infections with human assemblages A and B (Claerebout et al., 2009). On the other hand, the friendly nature of pet dogs facilitates close contact between them during encounters in public areas, leading to the distribution of dog-specific assemblages C and D (Wang et al., 2012). Although genotypes C and D have not been considered zoonotic for years, studies from Europe and China have shown human infections with *Giardia* from assemblage C, supporting the participation of dogs in human transmission (Sprong et al., 2009; Liu et al., 2014; Štrkolcová et al., 2015). Further studies with a larger population of dogs and their owners are warranted to establish which genotypes are circulating in this specific human and animal population.

Our results also suggest a moderate genetic diversity in the analyzed genes (Table 4) of these canine samples, with virtually all analyzed sequences showing different SNP frequency patterns, including well-defined point mutations and heterozygous (double peaks) sites. These

Table 4
Polymorphisms and heterogeneous positions at the *tpi*, gdh and *bg* loci of samples compared to reference sequences obtained from GenBank.

Gene marker	Assemblage	Reference sequence	Sample	Polymorphisms and heterogeneous positions[a]
tpi	C	AY228641[b]	CM023	G136K, T316Y, C369Y, A379M, G394R
			CM091	T316Y, C369Y, A379M
	D	DQ246216[c]	CM002	C204Y, G216R, C318Y, T337Y, **A339G**, C379Y
			CM100	C204Y, G216R, C318Y, T337Y, **A339G**
gdh	D	U60986[d]	CM002	None
			CM094	**T240C, T429C, G441A**
			CM100	G441R
	C	U60982[e]	CM010	None
			CM091	None
bg	C	AY545646[f]	CM023	None
			CM091	C217T, **G460A**
	D	AY545647[g]	CM002	A201R, C207M
			CM094	T276Y
			CM100	**A201G, C207A**

Standard mixbase definition: **Y:** C or T. **R:** A or G. **K:** T or G. **M:** A or C.

[a] Single nucleotide polymorphism (indicated in bold) and heterogeneous positions (overlapping nucleotide peaks at specific positions). Nucleotide position from start of reference gene.

[b] Isolate 2643.

[c] Isolate 6Mdog60.

[d] Isolate Ad-148.

[e] Isolate Ad-136.

[f] Isolate A29.

[g] Isolate A21.

findings are similar to several other studies worldwide including those from China (Li et al., 2015), Germany (Sommer et al., 2018) and Spain (Gil et al., 2017; Adell-Aledón et al., 2018), to give some examples. Further investigation of the impact of SNPs could provide valuable information for the classification of assemblages C and D into sub-assemblages, a field of *Giardia* genetics that remains unexplored.

5. Conclusions

Multilocus sequence analysis of *Giardia* in dog feces revealed assemblages C and D exclusively and moderate genetic diversity in the analyzed genes. The zoonotic potential of this parasite cannot be neglected due to the small number of samples that were successfully genotyped and even more to the fact that has been reported human infections with *Giardia* from dog specific assemblage C.

Ethical statement

This study was fully compliant with the ethical requirements for animal welfare. This study was approved by the ethics committee of the University of Antioquia–Colombia.

Declaration of Competing Interest

All authors declare that no competing interests exist.

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Appendix A. Supplementary data

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