First molecular characterisation of *Giardia duodenalis* infection in dairy goats in Brazil

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ABSTRACT: The aim of this study was to perform the first molecular genotyping of *Giardia duodenalis* from goats in Brazil, in order to assess the risk for zoonotic transmission. Samples were collected from two dairy goat farms (Saanen breed) located in the city of Niteroi in the state of Rio de Janeiro, Brazil. Goat faecal samples (n = 58) were collected directly from the rectums of all animals up to one year of age and were subjected to centrifuge-flotation in sugar saturate solution. Nested-PCR and sequencing using *β-giardin* and *tpi* gene targets was performed on positive samples. Seventeen out of fifty-eight (29.31%) faecal samples were positive for *Giardia duodenalis* cysts, all belonging to the same farm. Only eight isolates were successfully sequenced (eight samples for *β-giardin* and four samples for *tpi),* all belonging to genotype E. Two types of sequences were identified for each locus within isolates from the current study, which exhibited sequence heterogeneity with variable numbers of single nucleotide polymorphisms. The present study contributes to a better understanding of the molecular epidemiology of this parasite.

Keywords: goat; *Giardia duodenalis;* molecular characterisation; genotyping

Giardia duodenalis transmission occurs via cyst ingestion, especially from contaminated water and food (Adam 2001). The environment is contaminated when a large number of cysts are excreted in infected host faeces (Koudela and Vitovec 1998). Several animal species have reportedly been infected worldwide, and considerable economic losses in animal livestock are associated with the morbidity and mortality caused by *Giardia duodenalis* infection (Aloisio et al. 2006; Geurden et al. 2010; Sweeny et al. 2011).

Giardia duodenalis is subdivided into eight different genotypes (A-H), but only genotypes A, B and E have been reported in goats (Castro-Hermida et al. 2007; Geurden et al. 2008; Ruiz et al. 2008; Berrili et al. 2012; Lim et al. 2013).

Worldwide, *Giardia duodenalis* prevalence in goats varies from < 10% to > 40%, depending on animal age, geographical location, and diagnostic techniques (Robertson 2009). The role of goats in the zoonotic transmission of *Giardia duodenalis* is controversial because genotype E, which is not considered zoonotic, is more common than the zoonotic genotypes A and B. However, only a few molecular studies regarding *Giardia* prevalence in goats have been performed worldwide (Castro-Hermida et al. 2007; Geurden et al. 2008; Ruiz et al. 2008; Berrili et al. 2012; Lim et al. 2013), although a large number of studies have been conducted in other ruminant hosts (reviewed by Feng and Xiao 2011).

In Brazil, studies on the molecular epidemiology of *Giardia duodenalis* are scarce, but the genotyping of isolates has been reported in dogs, cats, cows, sheep, non-human primates, and exotic and wild animals (Volotao et al. 2007; Souza et al. 2007; Volotao et al. 2008; Volotao et al. 2011, Gomes et al. 2011; Soares et al. 2011; Paz e Silva et al. 2012a; Paz e Silva et al. 2012b; Fava et al. 2013).

To the best of our knowledge, molecular studies in goats in Brazil have not yet been published. Thus, the aim of this study was to perform the first

molecular genotyping of *Giardia duodenalis* from goats in the state of Rio de Janeiro, Brazil.

MATERIAL AND METHODS

This study was performed on two dairy goat farms (Saanen breed) located in the city of Niteroi in the state of Rio de Janeiro, Brazil. These farms are referred to as farms A and B. The farms were chosen based on convenience of location and their agreement to participate in the study.

Goat faecal samples (n = 58) were collected directly from the rectums of all animals up to one year of age. The faecal samples were transported in ice-cooled containers to the laboratory and processed according to Huber et al. (2007).

Following a light microscopy analysis, the faecal samples that were positive for *Giardia* were submitted to DNA extraction using a QIAamp® DNA Stool Mini Kit (Qiagen) with the modifications reported by Adamska et al. (2010). Briefly, 200µl of each sample were subjected to a freeze-thawing treatment before using the DNA extraction kit. The time and temperature of incubation in proteinase K was modified to 56 °C for 3 h.

The *β-giardin* and *tpi* gene targets were amplified using a Nested-PCR following published protocols with minor modifications (Sulaiman et al. 2003; Lalle et al. 2005; Caccio et al. 2008).

For *β-giardin* gene amplification, the PCR thermal cycles were as follows: denaturation step for 5 min at 94 °C; a set of 35 cycles at 94 °C for 30 s, 62 °C for 30 s, and 72 °C for 60 s; followed by a final extension step of 7 min at 72 °C. The nested-PCR thermal cycles were the same as described above with only a modification in the annealing step (55 °C for 30 s).

For *tpi* gene amplification, the PCR thermal cycles were defined as follows: denaturation step for 5 min at 94 °C; a set of 35 cycles at 94 °C for 45 s, 52 °C for 45 s, and 72 °C for 60 s; followed by a final extension step of 10 min at 72 °C. The nested-PCR thermal cycles were the same as those described above with only a modification in the annealing step (57 °C for 45 s).

All obtained products were electrophoresed on 1% agarose gels and stained with ethidium bromide. The DNA extracted from trophozoites of *Giardia duodenalis* WB strain (positive control) and a sample lacking a DNA template (negative control) were included in each PCR.

The nested-PCR products were purified using the Wizard® SV Gel and PCR Clean-up System (Promega), sequenced using the BigDye® Terminator v3.1 Cycle Sequencing Kit (Applied Biosystems) and analysed on an ABI 3130 Genetic Analyzer (Applied Biosystems).

The sequences were edited using Bioedit version 7.1 (Hall 1999) and aligned using the Clustal W algorithm (Larkin et al. 2007). The sequence identities were analysed with the DAMBE software version 5.3 (Xia and Xie 2001), and the isolates were compared with sequences in the Genbank database using BLAST analysis (http://www.ncbi.nlm.nih.gov/blast). Phylogenetic analyses were performed with the MEGA software version 5.0 (Tamura et al. 2011) using a neighbour-joining algorithm (Saitou and Nei 1987) with distance estimation calculated using a Kimura two-parameter model (Kimura 1980). Branch reliability was assessed using bootstrap analysis (1000 replicates).

The obtained nucleotide sequences were multiple-aligned against each other and against all genetic sequences obtained from goats deposited in GenBank for *β-giardin* and *tpi* loci (Tables 1 and 2). The sequences from genotypes A and B obtained in other hosts were also retrieved from GenBank for genetic comparison (Figures 1 and 2), as well as two *tpi* sequences from genotype E obtained from a human host in Egypt (Table 2). The sequences of the complete genome of the *Giardia duodenalis* isolates P15 (genotype E), WB (genotype A) and GS (genotype B) deposited in the *Giardia*-specific database (www.giardiadb.org) were used as reference sequences in the genetic analysis. The nucleotide sequence of *Giardia muris* was used as the outgroup.

RESULTS

Seventeen out of fifty-eight (29.31%) faecal samples were positive for *Giardia duodenalis* cysts after centrifuge-flotation in sugar saturate solution. All positive samples belonged to farm A and the infected animals were between two and three months of age. The presence of *Giardia* cysts in all 17 samples identified using microscopy was confirmed with nested-PCR in at least one of the gene targets. However, only samples with high cyst concentrations on microscopy yielded products with quantification exceeding 20 ng/µl for sequencing.

Sequencing generated good quality chromatograms in eight samples for *β-giardin* and four sam-

Table 1. Sequence analysis of the *β-giardin* gene from goat samples compared with the reference sequence from the complete genome of isolate P15 (genotype E), demonstrating single nucleotide substitutions

Isolate	Nucleotide position* and substitution																					
	7382	7384	7385	7404	7420	7422	7427	7434	7437	7445	7449	7500	7513	7524	7576	7611	7639	7650	7653	7665	7671	7674
P15 (GLP15_2766)	G	G	A	C	A	A	A	G	C	T	T	G	C	C	C	C	C	A	C	T	C	C
M20 (EU189370)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	A	–	G+	–	–	–	–
M28 (EU189374)	–	–	–	–	–	–	–	–	G	C	–	–	–	–	T+	–	A	G+	–	–	–	–
M25 (EU189372)	–	–	–	–	–	–	–	A+	–	–	–	–	–	–	–	–	–	G+	–	–	–	–
M9 (EU189364)	–	–	–	–	–	–	–	–	–	–	–	–	G	G	T+	–	–	G+	–	–	–	–
M5 (EU189360)	T	–	–	G	T+	–	–	–	–	–	–	–	–	–	T+	–	–	G+	–	–	–	–
M18 (EU189368)	–	T	C+	–	–	–	–	–	–	C	C	A+	–	–	T+	–	–	G+	–	–	–	–
M2 (EU189358)	–	–	–	–	–	–	C+	–	–	–	–	–	–	–	–	–	–	G+	–	–	–	–
M26 (EU189373)	–	_	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G+	–	–	–	G
M19 (EU189369)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	T+	–	–	G+	G+	–	–	G
M17 (EU189367)	T	–	–	–	–	–	–	–	–	C	–	–	–	–	T+	–	–	G+	–	–	–	–
M10 (EU189365)	–	–	–	–	–	–	–	A+	–	–	–	–	–	–	–	–	–	G+	–	–	–	–
M8 (EU189363)	–	–	–	–	–	–	–	A+	–	–	–	–	–	–	–	–	–	G+	–	–	A+	–
M1 (EU189357)	–	_	–	–	–	–	–	A+	–	–	–	–	–	–	–	–	–	G+	–	–	–	–
GS11(EU642899)	–	–	–	–	–	G+	–	–	–	–	–	–	–	–	–	–	–	G+	–	C+	–	–
GS21(EU642901)	–	–	–	–	–	–	–	–	–	–	–	–	–	T+	–	–	–	G+	–	–	–	–
C2[b] (JX876559)	–	–	–	–	–	–	–	A+	–	–	–	–	–	–	–	–	–	G+	–	–	–	–
C1[a] (JX876560)	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	–	G+	–	–	–	–

Nucleotide identity to isolate P15 represented by dots and deletions represented by dashes
*nucleotide position of polymorphic sites in the reference sequence (Giardiadb: GLP15_2766). Analysed sequence fragment: 7372–7711 (340 pb)
+synonymous nucleotide substitution
[b]sequence identical to isolates C15 and C12
[a]sequence identical to isolates C3, C4, C14 and C16

Table 2. Sequence analysis of the *tpi* gene from goat samples compared with the reference sequence from the complete genome of isolate P15 (genotype E), demonstrating single nucleotide substitutions

Isolate	Nucleotide position* and substitution																		
	14751	14788	14838	14968	15041	15042	15077	15081	15091	15093	15094	15095	15099	15106	15108	15111	15114	15123	15129
P15 (GLP15_4986)	C	A	A	T	G	G	A	G	A	C	C	G	C	G	A	C	T	T	G
M29[+] (EU189354)	–	G	–	–	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–
M16[+] (EU189341)	T	G	G	–	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–
GG-21 (JQ928717)	T	G	–	C	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–
4561 (JQ837808)	–	G	–	–	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–
Ma1 (EU272157)	T	G	G	–	A	T	G	–	–	–	–	–	–	A	–	–	–	–	–
D1 (EU272162)	T	G	G	–	A	T	–	–	G	–	–	–	–	–	–	–	–	–	–
D6 (EU272164)	T	G	G	–	A	T	–	A	–	T	A	A	T	–	G	T	C	C	T
C2[a]	T	G	G	–	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–
C3	T	G	G	–	A	T	–	–	–	–	–	–	–	–	–	–	–	–	–

Isolate	Nucleotide position* and substitution																		
	15132	15134	15135	15138	15142	15144	15147	15150	15153	15156	15157	15158	15160	15166	15168	15171	15172	15174	15177
P15 (GLP15_4986)	C	G	C	A	C	C	G	G	T	G	C	T	C	A	G	A	G	C	T
M29* (EU189354)	–	–	–	–	–	–	–	A	–	–	–	–	–	–	A	–	–	–	–
M16* (EU189341)	–	–	–	–	–	–	–	A	–	A	–	–	–	–	A	–	–	–	–
GG-21 (JQ928717)	–	–	–	–	–	–	A	A	–	–	–	–	–	–	A	–	–	–	–
4561 (JQ837808)	–	–	–	–	–	–	–	A	–	–	–	–	–	–	A	–	–	–	–
Ma1 (EU272157)	–	–	–	–	–	–	–	A	–	–	–	C	–	–	A	–	–	–	–
D1 (EU272162)	–	–	–	–	–	–	–	A	–	–	–	–	–	–	A	N	–	–	–
D6 (EU272164)	T	A	G	G	A	T	A	A	A	–	A	A	T	G	–	C	–	C	A
C2[b]	–	–	–	–	–	–	–	A	–	–	–	–	–	–	A	–	–	–	–
C3	–	–	–	–	–	–	–	A	–	–	–	–	–	–	A	–	A	–	–

Nucleotide identity to isolate P15 represented by dot

*nucleotide position of polymorphic sites in reference sequence (Giardiadb: GLP15_4986); analysed sequence fragment: 14744–15179 (436 pb)

[+]the complimentary reverse of the deposited sequence was used for comparison; identical sequences were represented only once

[b]sequence identical to isolates C12 and C14

ples for *tpi*, which facilitated the identification of the *Giardia duodenalis* genotype.

For the *β-giardin* gene target, eight samples were genotyped as assemblage E, which comprised two distinct sequence types. All sequences were deposited in GenBank under accession numbers KC122265, JX876559–JX876565. Isolates C2, C12 and C15 showed 100% identity to the published sequences from genotype E isolates obtained from goats in Spain (Ruiz et al. 2008). Similarly, isolates C1, C3, C4, C14 and C16 showed 100% identity to the published sequences from genotype E also obtained from goats in Spain (Ruiz et al. 2008). Moreover, a BLAST analysis showed 100% similarity between these isolates and genotype E samples obtained from sheep in Mexico (Di Giovanni et al. 2006) and in Spain (Gomez-Munoz et al. 2009).

For the *tpi* gene target, four samples were genotyped as assemblage E, which comprised two distinct sequence types. All sequences were deposited in GenBank under accession numbers KF483113–KF483115. Isolates C2, C12 and C14 showed 100%

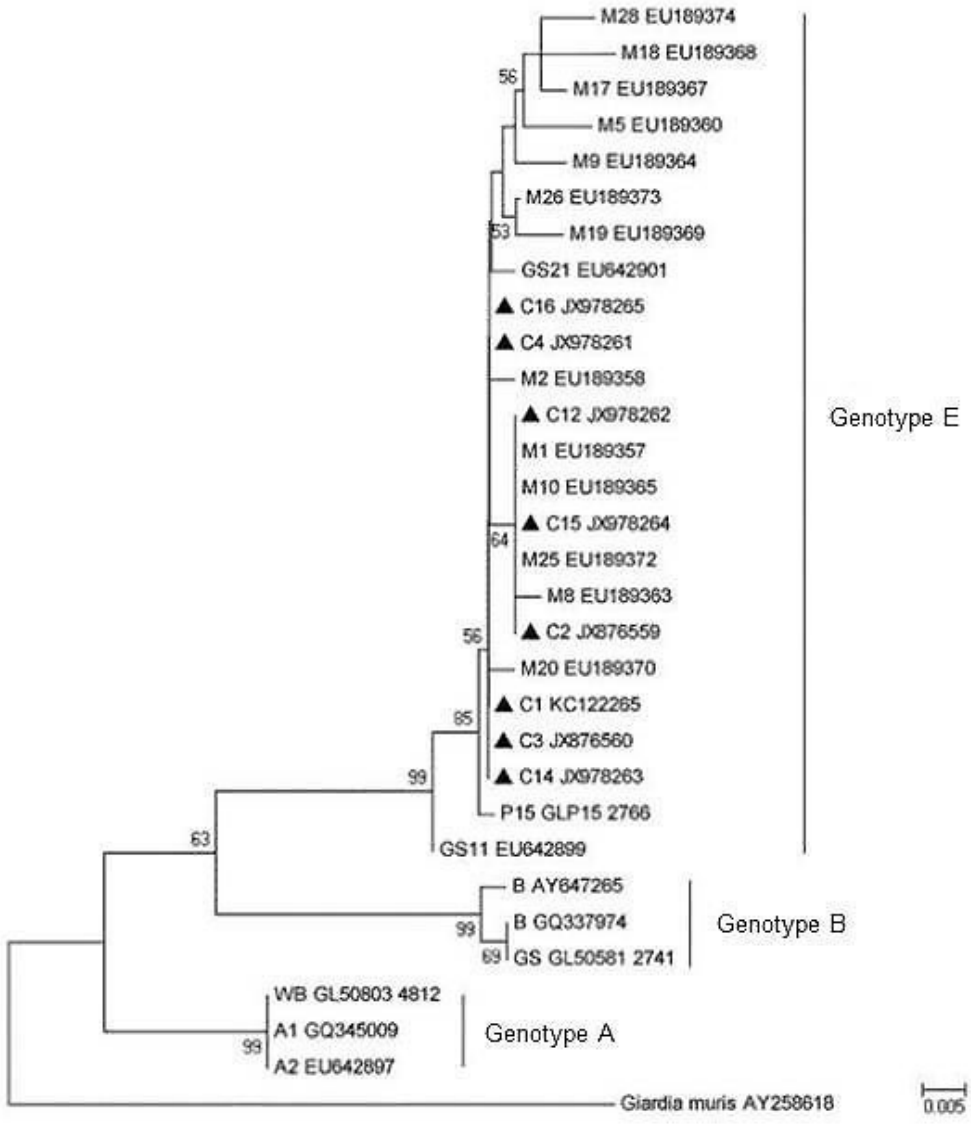

Figure 1. Genetic relationship among *Giardia* isolates inferred by a neighbour-joining algorithm using a Kimura two-parameter (1000 replicates) analysis based on the partial sequencing of the *β-giardin* gene (340 bp) from goats. The tree is rooted with *Giardia muris* [GenBank:AY258618]. Bootstraps above 50% are shown. Sequences obtained from GenBank are indicated by their access numbers. The following reference sequences from the complete genome obtained from www.giardiadb.org were used: GL50803_4812 (genotype A); GL50581_2741 (genotype B) and GLP15_2766 (genotype E). The following sequences are identical: [C2 = C15 = C12 = EU189362 = EU189371 = EU189359]; [C1 = C3 = C4 = C14 = C16 = EU189366 = EU189375 = EU189361] and indicate isolates obtained in the present study

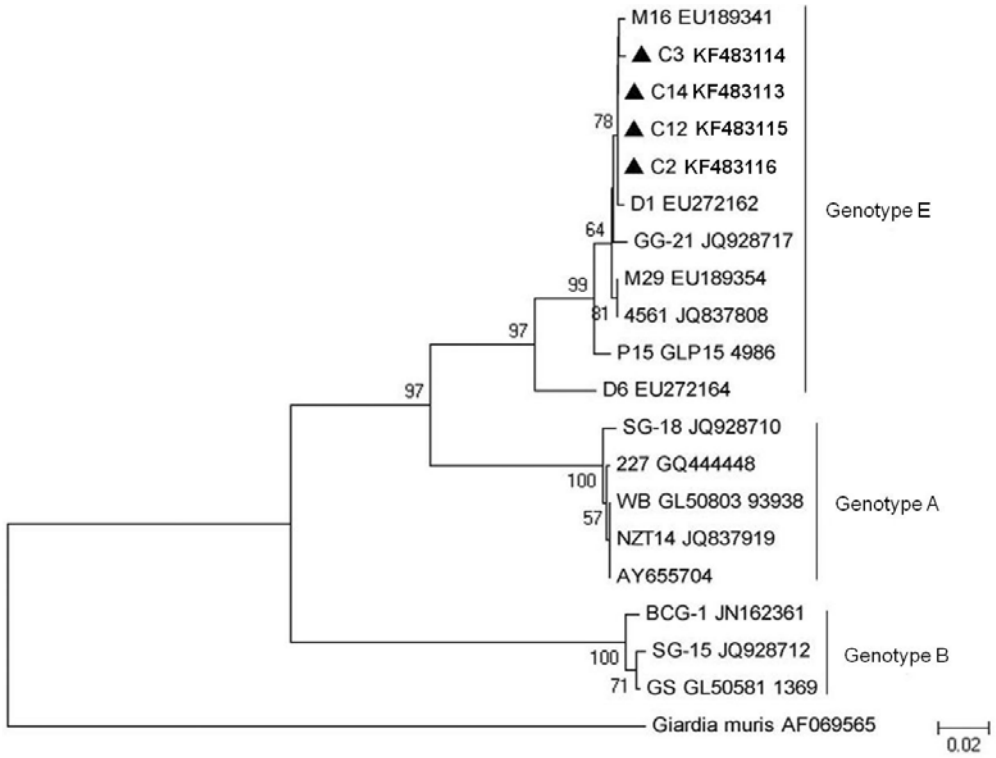

Figure 2. The genetic relationships among *Giardia* isolates inferred by a neighbour-joining algorithm using a Kimura two-parameter analysis (1000 replicates) based on the partial sequencing of the *tpi* gene (431 bp) from goats. The tree is rooted with *Giardia muris* [GenBank: AF069565]. Bootstraps above 50% are shown. Sequences obtained from GenBank are indicated by their access numbers. The following reference sequences from the complete genome obtained from www.giardiadb.org were used: GL50803_93938 (genotype A); GL50581_1369 (genotype B) and GLP15_4986 (genotype E). The following sequences are identical: [C2 = C12 = C14] and indicate isolates obtained in the present study

identity to the GenBank published sequences of genotype E obtained from goats in Spain (Ruiz et al. 2008), as well as one isolate obtained from a goat in Malaysia (HQ283233 – Lim et al. 2013). Moreover, a BLAST search showed a 100% identity match with genotype E isolate obtained from cows in the USA (Feng et al. 2008). Isolate C3 presented one nucleotide difference from the other isolates obtained in the present study and did not show 100% identity match with any sequence from GenBank.

The *β-giardin* sequence alignment of the obtained isolates, all goat isolates retrieved from GenBank, and the reference sequence from the complete genome of the P15 isolate (genotype E) showed intra-genotype heterogeneity (Table 1) with a variable number of single nucleotide polymorphisms (SNPs) per sequence. However, half of the observed SNPs represent synonymous nucleotide substitutions and thus do not generate different amino acids upon sequence translation. The obtained consensus tree (Figure 1) for the *β-giardin* gene target placed all isolates from this study in the same cluster as all genotype E isolates, with a high bootstrap value.

Likewise, the *tpi* sequence alignment of the four obtained isolates with genotype E, sequences from goats deposited in GenBank, two sequences assigned as genotype E obtained from human hosts, and the reference sequence from the complete genome of the P15 isolate (genotype E) showed intra-genotype heterogeneity (Table 2) with a variable number of single nucleotide polymorphisms (SNPs) per sequence. However, most observed SNPs represent synonymous nucleotide substitutions and thus do not generate different amino acids upon sequence translation. The obtained consensus tree (Figure 2) for the *tpi* gene target placed all isolates from this study in the same cluster as all genotype E isolates, including the two human isolates, with a high bootstrap value.

Regardless of the gene studied, the general conformation of both trees is maintained since all isolates are grouped in a single branch. However, in the *tpi* consensus tree the isolates C3 and M16 have one nucleotide difference from the other isolates

in the same branch, thus forming a separate cluster. Likewise, in the *β-giardin* consensus tree two clusters are formed in the same branch, with one nucleotide difference between them.

DISCUSSION

Most of the samples studied here contained a low quantity of cysts according to the microscopy examinations. This quantity was associated with a low concentration of DNA after extraction and led to a small amount of quantified product after amplification and purification. Other authors have also reported a failure to amplify and sequence samples using PCR that were positive upon microscopic examination (Castro-Hermida et al. 2007; Volotao et al. 2007; Lebbad et al. 2010; Paz e Silva et al. 2012a; Fava et al. 2013). According to Castro-Hermida et al. (2007), this feature can be observed when samples contain a small number of cysts (less than one cyst per field of view). Moreover, because the *β-giardin*-encoding gene is a single copy target, samples that contain a low quantity of cysts may yield negative or weak amplifications.

Lebbad et al. (2010) conducted a multilocus study (*gdh*, *tpi* and *β-giardin*) with samples from various hosts. They observed that some isolates that were not initially amplified exhibited nucleotide substitutions in the primer-annealing site, which was corrected by re-designing the primers. Specifically, the nested-PCR for the *tpi* gene target was less efficient than that of the *β-giardin* nested-PCR corroborating the findings of the present study. This difference could be attributed to the higher substitution rate of this gene as compared to *β-giardin* (Wielinga and Thompson 2007), which could interfere with the primer-annealing site.

The low genetic variability of isolates from the present study could be explained by the fact that all sequences were obtained from the same farm. Most of the sequences obtained for both gene targets (*β-giardin* and *tpi*) in the present study showed a 100% match to samples from other countries, suggesting a worldwide distribution of this genotype. Moreover, most dairy goat breeds raised in Brazil originated in Europe and Africa and were introduced to Brazil during colonisation. Furthermore, these breeds are currently still being imported to improve milk yield. Thus, these practices may have contributed to the introduction and dissemination of this genotype. However, studies on molecular epidemiology using samples from other states and other ruminant hosts, along with paleoparasitology studies of ruminant coprolites from the colonial period, need to be conducted to establish the origin, evolution, and epidemiology of *Giardia duodenalis* in Brazilian goats.

The genotyping results of both gene targets were both concordant with and differed from studies published by Lebbad et al. (2010) and Gomez-Munoz et al. (2012). These researchers reported genotyping inconsistencies, especially in cases of mixed infection by genotypes A and E. Caccio et al. (2008) also called attention to the importance of multilocus studies for an accurate genotyping of *Giardia duodenalis*.

Other authors have also observed intra-genotype heterogeneity in different hosts (Lalle et al. 2005; Volotao et al. 2007; Sprong et al. 2009; Gomez-Munoz et al. 2012; Fava et al. 2013), a phenomenon reviewed in Caccio and Ryan (2008). The *tpi* gene target was more variable, which was also reported by Gomez-Munoz et al. (2012) and Fava et al. (2013). However, because SNPs could represent sequencing artefacts, sequence heterozygosis, or mixed infections, these findings should be carefully evaluated and more studies are needed to determine the real value of these data.

Lastly, the heterogeneity observed in nucleotide sequences did not change the amino acid sequence of the protein, because a 100% identity match was found in a protein BLAST search. However, a nucleotide substitution, even by a synonymous nucleotide, could affect gene expression and delay protein translation. Thus, additional studies to determine the real importance of these alterations to the metabolism and pathogenesis of this parasite are needed.

A large number of *Giardia duodenalis* genotyping studies in ruminants report a higher occurrence of genotype E in these animals, with genotypes A and B being less frequent (Castro-Hermida et al. 2007; Geurden et al. 2008; Gomez-Munoz et al. 2009; Zhang et al. 2012). However, Geurden et al. (2008) and Berrilli et al. (2012) reported that zoonotic genotype A infected goats in Belgium and Côte d´Ivoire, respectively, and Lim et al. (2013) reported that genotypes A and B infected goats in Malaysia. These findings suggest that goats could be a potential source of zoonotic infection.

Even though zoonotic genotypes were not observed in the studied population, the E genotype has also been detected in humans living in close

contact with livestock, suggesting a potential for zoonotic transmission under certain circumstances (Foronda et al. 2008). Because goat farms in Brazil are generally small and most farm workers live inside or near the property, close contact with these animals could represent a risk for zoonotic transmission of this parasite.

Zoonotic genotypes A and/or B were reported in dogs, cats, non-human primates, cattle, sheep, exotic and wild animals in Brazil (Volotao et al. 2007, 2008, 2011; Souza et al. 2007; Gomes et al. 2011; Soares et al. 2011; Paz e Silva et al. 2012a,b; Fava et al. 2013), suggesting the potential for environmental contamination and a risk for goat infection.

This study represents the first molecular characterisation of *Giardia duodenalis* from goats in Brazil and contributes to parasite epidemiology in the country. However, additional molecular studies with samples from other geographical areas in the country, as well as other animal hosts and humans, are necessary to determine the role of these animals in zoonotic transmission.

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