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ORIGINAL ARTICLE

Giardia duodenalis infection in dogs from the metropolitan area of Lisbon, Portugal: prevalence, genotyping and associated risk factors

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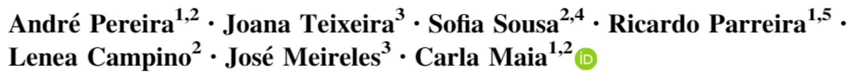

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Abstract *Giardia duodenalis* is a cosmopolitan enteric protozoan that affects a wide range of vertebrates, including humans and dogs. Genetic characterisation reveals eight different assemblages, with A and B having been found mainly in humans and several other animals, and thus considered potentially zoonotic, while C and D are adapted to infect dogs. This study aimed to determine the prevalence of *G. duodenalis*, their distribution into assemblages, and risk factors associated with their infection of dogs from the metropolitan area of Lisbon. *Giardia duodenalis* cysts were microscopically identified in 33.8% (27/80) of the faecal samples analysed. Multivariate logistic regression analysis revealed that dogs under 6 months of age and from both breeders and shelters, had a significantly higher risk of being infected with *G. duodenalis*. Based on phylogenetic analysis of the partial coding sequences for β-giardin, glutamate dehydrogenase, and triosephosphate isomerase, the parasites found in three dog isolates were typed as *G. duodenalis* assemblage C, 11 were typed as D, and four were typed as C or D, depending on the targeted genes. The risk to public health seems to be reduced, as no genotypes with zoonotic potential have been detected. Nevertheless, better health management towards a minimisation of the environmental faecal pollution, as well as an increase in the awareness of health professionals, dog owners, dog breeders and caregivers regarding the risks posed by this protozoan to the health of animals and humans, are recommended.

André Pereira, Joana Teixeira and Sofia Sousa have contributed equally to this work.

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Graphic abstract

Keywords Dogs · Epidemiology · *Giardia duodenalis* · Genotype · Portugal

Introduction

Giardia duodenalis (syn. *G. intestinalis*, *G. lamblia*) is a ubiquitous enteric parasite that affects a wide range of vertebrates, including humans and dogs. The life cycle of this protozoan is direct, and its infective stage, the cyst, is released in the environment through the hosts' faeces (Feng and Xiao 2011). Ingestion of cysts via contaminated water or food is the main transmission route to humans and other animals. In fact, *G. duodenalis* is a leading cause of water- and foodborne diarrheal disease worldwide (Feng and Xiao 2011; Buret et al. 2020) responsible for several waterborne outbreaks in the recent years (Baldursson and Karanis 2011). Apart from representing a major public health concern in both developing and developed countries, *G. duodenalis* infection and giardiosis are of veterinary importance affecting companion and farm animals (Feng and Xiao 2011).

This protozoan is currently regarded as a complex of eight different assemblages (A to H) and corresponding sub-assemblages. Assemblages A and B are mainly found in humans, but some sub-assemblages (e.g. AI, BIV) are considered potentially zoonotic as they have also been detected in domestic and wild animals (Sprong et al. 2009; Ballweber et al. 2010). On the other hand, assemblages C to H appear to infect a limited number of host species: C and D are found in canids, E in livestock, F in cats, G in rodents and H in marine vertebrates (Sprong et al. 2009; Feng and Xiao 2011). The detection of zoonotic sub-assemblages A and B in canine populations (Claerebout et al. 2009; Traub et al. 2009; Sprong et al. 2009), as well as canine-adapted C and D assemblages in humans (Traub et al. 2009; Sprong et al. 2009), poses the question whether dogs may play a role in the transmission of *G. duodenalis* infection to humans (Ballweber et al. 2010). As *G. duodenalis* assemblages are morphologically identical, molecular techniques including DNA amplification and sequencing of encoding genes, such as those encoding β-giardin (*bg*), triose phosphate isomerase (*tpi*) and glutamate dehydrogenase (*gdh*), have been largely used to evaluate the genetic variability of this protozoan (Cacciò et al. 2008; Feng and Xiao 2011; Heywoth 2016; Spotin et al. 2018).

In Portugal, studies on *Giardia* were mostly focused on the assessment of infection prevalence in humans (Lemos et al. 2005; Júlio et al. 2012; Ferreira et al. 2013), in animals (Ferreira et al. 2011; Ferreita et al. 2017; Neves et al. 2014), and water contamination (Lemos et al. 2005; Lobo et al. 2009; Almeida et al. 2010) with limited genotyping data. Prevalence of infection in humans ranged from 1.9% by direct examination (Júlio et al. 2012) to 6.8% by antigen detection by ELISA (Júlio et al. 2012), between 7.4 (Neves et al. 2014) and 23.0% by microscopy in animals (Ferreira et al. 2011), while in water samples the detection of parasite ranged between 5.1% by PCR-based methods (Lobo et al. 2009) and 63.3% by microscopy (Almeida et al. 2010). *Giardia duodenalis* assemblages A, B and E have been detected in cattle from northern Portugal (Mendonça et al. 2007), assemblages A to D in dogs and/or cats from the district of Évora (southern Portugal) (Ferreira et al. 2011), and assemblages A and B in human stool samples collected from the North and Centre of the country (Sousa et al. 2006), and children from Lisbon (Ferreira et al.

2013). To our knowledge, no previous study has been undertaken concerning the genetic characterisation of *G. duodenalis* and their zoonotic potential in dogs in the metropolitan area of Lisbon. Therefore, this cross-sectional study allowed for (1) an estimate of the prevalence of *G. duodenalis* infection in dogs from this region, (2) the identification of putative risk factors associated with *Giardia* infection in dogs through multivariate statistics; and (3) the characterisation of parasite assemblages using molecular data.

Methods

Faecal samples

From October 2014 to July 2016, 80 canine faecal samples (convenience sampling) were obtained from household dogs ($n = 33$), dogs from breeders ($n = 25$), and shelters ($n = 22$) from the metropolitan area of Lisbon. Whenever available, sex, age, breed and lifestyle (i.e. household, breeder or shelter) were recorded for each dog. Faecal samples were collected in plastic containers and numbered, and stored at 4 °C until further processing, which included microscopic analysis (within 24 h of sampling collection) and DNA extraction (after observation of cysts). Consent to process faecal samples was obtained from the owners of the dogs, breeders or the person in charge of the shelters.

Centrifugal faecal flotation and microscopy

Microscopic examination for the direct detection of *G. duodenalis* cysts was performed after 3–5 g of homogenised faeces were mixed with 33% zinc sulphate solution (specific gravity 1.18) and centrifuged as described previously (Zajac and Conboy 2012). Following centrifugation ($500 \times g$; 5 min), the flotation solution was added until a reverse meniscus is formed, and a microscopy coverslip placed over it. After 15 min, the coverslip was placed on a slide for examination under a microscope at 100× and 400× magnification.

DNA extraction, amplification and sequencing

DNA was extracted from faeces containing *Giardia* cysts using QIAamp DNA Stool Mini Kit (Qiagen GmbH., Germany) essentially following the manufacturer's recommended protocol. Exceptions to the experimental protocol suggested included increases of the lysis temperature (to 90 °C), and the incubation period of lysis (30 min), and a decrease of the volume of AE buffer used to elute DNA (down to 100 µL). For *G. duodenalis* molecular identification, three nested-PCR assays targeting a fragment of the coding sequences of *bg* (511 bp) *gdh* (530 bp), and *tpi* (530 bp) were performed as previously described (Sulaiman et al. 2003; Lalle et al. 2005; Cacciò et al. 2008) (Table 1). In all amplification reactions positive (*G. duodenalis* DNA) and negative (without DNA) controls were included. PCR products were visualised under UV illumination after electrophoresis on 1.5% agarose gels stained with GreenSafe Premium (Nzytech, Portugal). The obtained amplicons were purified and sequenced in one direction by Sanger's method (STABVida, Portugal), using as sequencing primers those used for DNA amplification.

Sequence analysis and phylogenetics

The search for homologous sequences was performed via BLASTn tool (https://blast.ncbi.nlm.nih.gov/Blast.cgi). Multiple sequence alignments of nucleotide datasets were carried out using the iterative G-INS-i refinement method as implemented in MAFFT v7 (Katoh and Standley 2013). The resulting alignments were treated via Gblocks (Castresana 2000) with the more permissive options selected, followed by their manual edition considering the correct open reading frame. The evolutionary histories of the sequences used in gene-specific datasets were inferred under the Maximum Likelihood (ML) optimisation criterium and a best-fitted evolutionary model, selected based on the Akaike Information Criterion, as implemented in jModelTest v2 (Darriba et al. 2012). The phylogenetic trees were constructed using Mega v6 (Tamura et al. 2013), and the stability of the obtained tree topologies was assessed by bootstrapping with 1000 resamplings of the original sequence data. The generated trees were edited for display using iTOL v4 (Letunic and Bork 2019). The representative sequences obtained during this study were deposited at the DNA Data Bank of Japan (DDBJ) (https://www.DDBJ.nig.ac.jp) under accession numbers LC552144–LC552146 (*bg*) LC552147–LC552163 (*gdh*) and LC552164–LC552169 (*tpi*).

Statistical analysis

Descriptive statistics were used to summarise and described the demographic characteristics of sampled dogs. Confidence intervals (95% CI) for proportions were obtained by the Wilson method. A stepwise multiple logistic regression analysis was applied to the data in order to assess, in an integrated way, the potential predictors associated with *Giardia* infection in dogs. The backward elimination method, based on the likelihood-ratio statistics, was employed as a criterium for variable selection. Multicollinearity was evaluated by checking both tolerance and variance inflation factor (VIF) values. In addition, the likelihood ratio test, the Hosmer and Lemeshow goodness-

Table 1 PCR protocols performed for detection and characterisation of *Giardia* DNA

Target gene	Primer sequence (5′–3′)	Amplicon size	Reaction setup	Thermocycling conditions	References
bg	1st PCR	753 bp	25 µl reaction: 2 µl of DNA; 0.4 µM of each primer; 12.5 µl of NZYTaq 2× Green Master Mix	95 °C—15 min; 35 cycles [95 °C—30 s; 65 °C—30 s; 72 °C—1 min]; 72 °C—7 min	Lalle et al. (2005)
	Fw: AAGCCCGACGACCTCACCCGCAGTGC				
	Rev: GAGGCCGCCCTGGATCTTCGAGACGAC				
	2nd PCR	511 bp	25 µl reaction: 2.5 µl of 1st PCR product; 0.4 µM of each primer; 12.5 µl of NZYTaq 2× Green Master Mix	95 °C—15 min; 35 cycles [95 °C—30 s; 55 °C—1 min; 72 °C—1 min]; 72 °C—7 min	
	Fw: GAACGAACGAGATCGAGGTCCG				
	Rev: CTCGACGAGCTTCGTGTT				
gdh	1st PCR	755 bp	25 µl reaction: 5 µl of DNA; 0.8 µM of each primer; 12.5 µl of NZYTaq 2× Green Master Mix	94 °C—2 min; 35 cycles [94 °C—30 s; 50 °C—30 s; 72 °C—1 min]; 72 °C—7 min	Cacciò et al. (2008)
	Fw: TTCCGTRTYCAGTACAACTC				
	Rev: ACCTCGTTCTGRGTGGCGCA				
	2nd PCR	530 bp	25 µl reaction: 5 µl of 1st PCR product; 0.4 µM of each primer; 12.5 µl of NZYTaq 2× Green Master Mix	94 °C—2 min; 35 cycles [94 °C—30 s; 50 °C—30 s; 72 °C—1 min]; 72 °C—7 min	
	Fw: ATGACYGAGCTYCAGAGGCACGT				
	Rev: GTGGCGCARGGCATGATGCA				
tpi	1st PCR	605 bp	25 µl reaction: 2 µl of DNA; 0.2 µM of each primer; 12.5 µl of NZYTaq 2× Green Master Mix	94 °C—5 min; 35 cycles [94 °C—45 s; 50 °C—45 s; 72 °C—60 s]; 72 °C—10 min	Sulaiman et al. (2003)
	Fw: AAATIATGCCTGCTCGTCG				
	Rev: CAAACCTTITCCGCAAACC				
	2nd PCR	530 bp	25 µl reaction: 2.5 µl of 1st PCR product; 0.2 µM of each primer; 12.5 µl of NZYTaq 2× Green Master Mix	94 °C—5 min; 35 cycles [94 °C—45 s; 50 °C—45 s; 72 °C—60 s]; 72 °C—10 min	
	Fw: CCCTTCATCGGIGGTAACTT				
	Rev: GTGGCCACCACICCCGTGCC				

bg, β-giardin; gdh, glutamate dehydrogenase; *tpi,* triosephospate isomerase; bp, base pars

of-fit test, and the determination of the area under the ROC curve were carried out to assess the final fitted model validity. Statistical significance was determined at $P \leq 0.05$. The statistical analysis as conducted using IBM® SPSS® Statistics v26.0 and OpenEpi v3.01 software.

Results

Giardia duodenalis cysts were microscopically identified in 27 (33.8%; $n = 80$) faecal samples (Table 2). Multivariate logistic regression analysis showed that the independent variables age group ($X^2_{\text{Wald}} = 13.724$; df = 2; $P = 0.001$) and lifestyle ($X^2_{\text{Wald}} = 8.002$; df = 2; $P = 0.018$) were significant predictors of *G. duodenalis* infection in dogs. Also, none of them presented tolerance and VIF values less than 0.2 and exceeding 10,

Table 2 Prevalence of *Giardia* infection in dogs from the metropolitan area of Lisbon: demographic characteristics of the study sample and multivariate logistic analysis of risk factors

Variable/categories	Tested, *n* (%)	Positive, *n* (%; 95% CI)	Multivariate statistics		
			X^2_{Wald}	*P* value	OR adjusted (95% CI)
Sex[a]	77				
Female	33 (42.9)	9 (27.3; 15.1–44.2)			
Male	44 (57.1)	15 (34.1; 21.9–48.9)			
Age group	80				
$\leq$ 6 months[b]	14 (17.5)	11 (78.6; 52.4–92.4)	13.724	0.001	
7–12 months	4 (5.0)	2 (50.0; 15.0–85.0)	2.545	0.111	0.096 (0.005–1.709)
> 12 months	62 (77.5)	14 (22.6; 14.0–34.4)	13.253	< 0.001	0.033 (0.005–0.207)
Breed[a]	80				
Defined	49 (61.3)	18 (36.7; 24.7–50.7)			
Mongrel	31 (38.8)	9 (29.0; 16.1–46.6)			
Lifestyle	80				
Household[b]	33 (41.3)	6 (18.2; 8.6–34.4)	8.002	0.018	
Breeder	22 (27.5)	11 (50.0; 30.7–69.3)	4.143	0.042	6.428 (1.071–38.563)
Shelter	25 (31.3)	10 (40.0; 23.4–59.3)	7.939	0.005	13.408 (2.204–81.574)
Total	80	27 (33.8; 24.4–44.6)			

CI confidence interval, X^2_{Wald} Wald chi-square test, *OR* odds ratio

[a]Variable not included in the fitted model

[b]Reference category

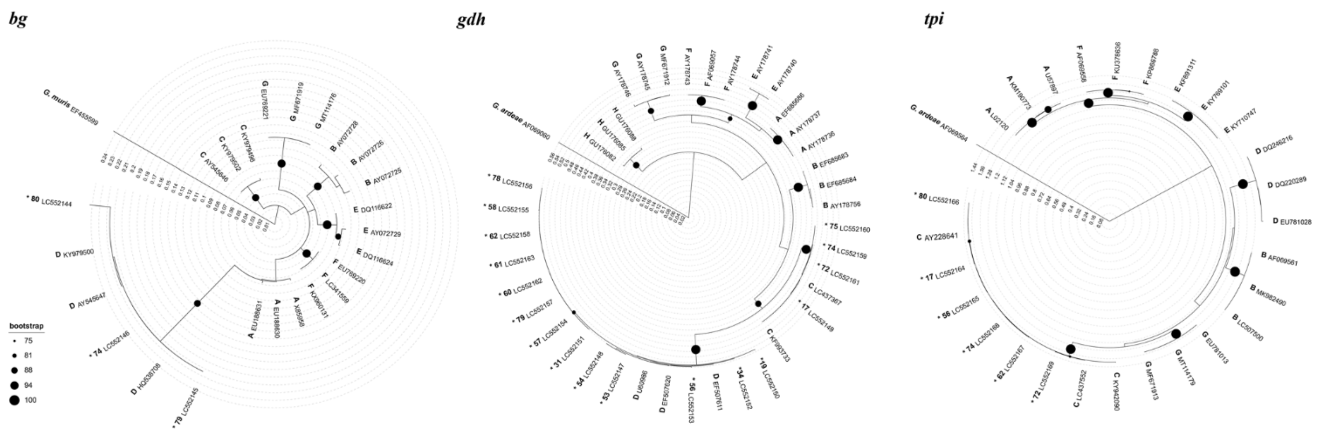

Fig. 1 Maximum likelihood phylogenetic trees based on unambiguous *β*-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose phosphate isomerase (*tpi*) sequences alignments. At specific branch nodes, bootstrap values (from 1000 random replicates of the original datasets) $\geq$ 75% are shown. The internal tree scale indicated the number of nucleotide substitutions per site. The trees were rooted using *G. muris* or *G. ardae* sequences (outgroup). The sequences obtained in this study are indicated with "*"

respectively, therefore revealing the absence of potential collinearity problems (Hosmer et al. 2013). According to the fitted model ($p = \frac{1}{1+e^{-[0.277-3.410\,Age\ group(>12\ months)+1.861\,Lifestyle\ (Breeder)+2.596\,Lifestyle\ (Shelter)]}}$), dogs older than 12 months had 0.033 lower odds (95% CI 0.005–0.207; $X^2_{Wald} = 13.253$; df = 1; $P < 0.001$) of being infected with *G. duodenalis* than those younger than 6 months. In contrast, dogs from breeders (adjusted OR = 6.428; 95% CI 1.071–38.563; $X^2_{Wald} = 4.143$; df = 1; $P = 0.042$) and shelters (adjusted OR = 13.408; 95% CI 2.204–81.574; $X^2_{Wald} = 7.939$; df = 1; $P = 0.005$) seem to have a significantly higher risk of being infected with this protozoan than household dogs. This model fit to the data well (Hosmer and Lemeshow test = 4.161, df = 3, $P = 0.245$), and showed an excellent discriminatory ability (AUC = 0.801; $P < 0.001$) (Hosmer et al. 2013).

Genetic data were gathered based on the analysis of 17 *gdh*, 6 *tpi,* and 3 *bg* sequences, obtained from 18 of the 27 stool samples (amplification success rate = 66.7%) where the presence of *G. duodenalis* cysts had been revealed by microscopy (Fig. 1). Phylogenetic inference analysis revealed that dogs had been infected with parasites typed as *G. duodenalis* included in assemblages C ($n = 3$) or D ($n = 11$), while in four animals parasite association to assemblages C or D depended on the target gene (Supplementary Table).

Discussion

In the course of this study, a prevalence of *G. duodenalis* infection of 33.8% was determined. This value is higher than that previously disclosed by Ferreira et al. (2017) in faecal samples collected from three dog parks situated in Lisbon (11.4%). This difference could be related to the fact that herein screening of *G. duodenalis* cysts was not restricted to the analysis of faecal samples from household dogs, which is the canine population that usually visits dogs' parks. In addition to the later, this study also included stool samples of dogs from breeders and housed at shelters which are normally more confined to a given area and, as a result, are more prone to dog-to-dog transmission of *G. duodenalis* cysts. Nevertheless, the infection rate reported here is probably an underestimation of the true prevalence as it was based on: (1) one single faecal sample per animal and it is known that *Giardia* cysts are intermittently shed; (2) conventional diagnostic methods which are known to have lower sensitivity than immunodiagnostic or molecular techniques, especially in light infections, where the number of cysts present in faeces is known/expected to be low (Traub et al. 2009; Bouzid et al. 2015).

When comparing the frequency of infection with dogs' lifestyle, the chance of a household dog being infected was significantly lower than that of dogs from breeders, and even lower when comparing with sheltered dogs. Previous reports have associated infection by *G. duodenalis* to be more common in places with a greater number of dogs, higher dog-turnover and less hygienic conditions, such as in kennels and shelters (Ballweber et al. 2010; Ferreira et al. 2011; Pipia et al. 2014; Sommer et al. 2018). Another reported trend seems to that infection with *G. duodenalis* is more common in young dogs (Claerebout et al. 2009; Pipia et al. 2014; Gil et al. 2017; Tangtrongsup et al. 2017; Adell-Aledón et al. 2018). In the present study, the presence of *Giardia* cysts was significantly higher in dogs younger than 6 months-old when comparing with those older than 12 months, which might be a result of acquired immunity decreasing the risk of infection in older dogs. Increased risk of infection in puppies by behavioural factors, such as higher tendency to scavenge and to lick surfaces contaminated with *Giardia* cysts, may also occur.

Beyond the risk to animal health, *G. duodenalis* infection is a potential public health concern; hence, determining the presence of this protozoan in dogs living in close contact to humans, and defining which assemblages they belong to is a priority. As zoonotic assemblages of *G. duodenalis* cannot be distinguished using morphological differentiation, the genetic characterisation needs to be undertaken. In the present study, the partial amplification of *bg, gdh* and *tpi G. duodenalis* genes was possible in 18 of the 27 samples where *Giardia* cysts were microscopically observed. The lower sensitivity of PCR-based methods over conventional microscopy has already been documented (Pipia et al. 2014; Tangtrongsup et al. 2017). Failure of nested-PCR assays to amplify the target sequences may result either from extracting DNA directly from faecal samples and not from samples used for microscopy, where cysts were concentrated, or due to the presence of PCR inhibitory substances in faecal samples (Tangtrongsup et al. 2017). Further, and although in the present study *G. duodenalis* load was not quantified, the performance of PCR-based techniques, especially those based on the amplification of single-copy genes, is compromised when the number of cysts is low (Gil et al. 2017; Sommer et al. 2018).

Of the three genes targeted in this study for *G. duodenalis* detection, the sensitivity of the nested-PCR assay employed for partial amplification of *gdh* ($n = 17$) was higher than those used for amplification of *tpi* ($n = 6$) and *bg* ($n = 3$), corroborating previous results (Tangtrongsup et al. 2017). The sequences from each of the three genes, obtained from the 18 *G. duodenalis* isolates, were assigned to the canine-specific assemblages C and D. In addition, for those DNA samples which were characterised at two or more loci, discrepant assignment to a specific assemblage was observed in four (22.2%) of them, as a result of the single-locus analysis. Inconsistent genotyping results using identical genetic markers to those used in in the present study have been reported (Sprong et al. 2009; Tangtrongsup et al. 2017; Zhang et al. 2017) and might be related with the occurrence of infections with parasites from multiple assemblages. Mixtures of genotypes in individual human and animals´ isolates have repeatedly been observed, and possible explanations for these findings include the uptake of genetically different *G. duodenalis* cysts by a host, subsequent infection of an already infected host or allelic sequence heterozygosity, which is reported to be higher in assemblages B, C and D (Sprong et al. 2009; Feng and Xiao 2011). The detection of canine-specific assemblages is in agreement with previous studies where most of the dogs housed in kennels or shelters were infected with *Giardia* from assemblages C or D

(Claerebout et al. 2009; Scaramozzino et al. 2009; Kim et al. 2019), reflecting that the transmission of dog-specific genotypes is likely favoured by the intense contact among dogs that live together (Traub et al. 2009). The potential role of domestic dogs in the transmission of *G. duodenalis* infections to humans remains unresolved as zoonotic assemblages, occasionally A, less frequently B, have been found, especially in household dog isolates (Lalle et al. 2005; Claerebout et al. 2009; Ballweber et al. 2010). As all *G. duodenalis* detected during this study were dog-adapted assemblages, the potential for zoonotic transmission of *Giardia* from dogs in the studied region seems to be low, reinforcing the negligible role of domestic dogs seem to play as sources of human giardiosis in European countries (de Lucio et al. 2017; Rehbein et al. 2019). However, further molecular epidemiological studies evaluating simultaneously parasite strains of human and animal origin will be required to confirm this result.

Conclusion

Infection with *G. duodenalis* is frequent in dogs from the metropolitan area of Lisbon, mainly in dogs from breeders and shelters. The risk to public health seems to be reduced, as no genotypes with zoonotic potential have been detected. However, it is important to note that anecdotical cases of human giardiasis caused by dog-specific assemblages have been documented. Therefore, the risk of infection by close contact among dogs and between dogs and humans should not be underestimated, highlighting that better health management towards a minimisation of the environmental faecal pollution with *Giardia* cysts, and a needed increase awareness of health professionals, dog owners and caregivers regarding the health risks posed by this parasite to the health of humans and other animals.

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Author contributions AP collected the faecal samples and information of each dog, performed microscopic, molecular, phylogenetic and statistical analyses, and revised the manuscript. JT collected the faecal samples and information of each dog and performed microscopic analysis. SS collected the faecal samples and information of each dog and performed microscopic examination and molecular analyses. RP supervised the phylogenetic analysis and critically reviewed the manuscript. LC critically reviewed the manuscript. JM supervised the study and critically reviewed the manuscript. CM supervised the study and wrote the manuscript.

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Compliance with ethical standards

Conflict of interest The authors declare that they have no competing interests.

Ethics approval Consent to process faecal samples was obtained from the owners of the dogs, breeders or the person in charge of the shelters. This study was ethically approved by the board of the Faculty of Veterinary Medicine (ULHT) as complying with the Portuguese legislation for the protection of animals (Law No. 92/1995).

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