

Canine giardiasis in Sardinia Island, Italy: prevalence, molecular characterization, and risk factors

Anna Paola Pipia¹, Antonio Varcasia¹, Claudia Tamponi¹, Giuliana Sanna¹, Mara Soda¹, Barbara Paoletti², Donato Traversa², Antonio Scala¹

¹ Parasitology, Veterinary Medicine Department, University of Sassari, Sassari, Italy

² Faculty of Veterinary Medicine, University of Teramo, Teramo, Italy

Abstract

Introduction: The flagellate protozoan *Giardia duodenalis* causes infection in humans and in various animals. Eight distinct assemblages (A-H) have been identified within *G. duodenalis*; assemblages A and B are those specific to humans and animals, and assemblages C to H are restricted to animal hosts.

Methodology: The present study estimated the prevalence of *G. duodenalis* assemblages in dogs living in the Sardinia region and evaluated the related risk factors. Individual fecal samples were collected from 655 dogs between January 2007 and December 2010, and a form was filled out for each animal to analyze historic data that were available at the time of sampling. Fecal samples were subjected to microscopic and genetic investigations.

Results: Cysts of *G. duodenalis* were found in 172 (26.3%) samples, with significant values in puppies between three and nine months of age, and in kennelled and hunting dogs. The molecular characterization showed the presence of assemblages D (49%), C (36.1%), and subtype A2 (4.2%).

Conclusion: The present survey contributes to the knowledge of the occurrence of canine giardiasis in Italy in a region with a high number of dogs and numerous animal movements, which is especially relevant for touristic reasons.

Key words: dogs; *Giardia duodenalis*; risk factors; zoonosis; assemblages; Sardinia

J Infect Dev Ctries 2014; 8(5):655-660. doi:10.3855/jidc.4255

(Received 23 September 2013 – Accepted 30 January 2014)

Copyright © 2014 Pipia *et al.* This is an open-access article distributed under the Creative Commons Attribution License, which permits unrestricted use, distribution, and reproduction in any medium, provided the original work is properly cited.

Introduction

Giardia duodenalis is a flagellate protozoan infecting the intestine of a wide range of animals (*e.g.*, canids, felids, primates, rodents, ruminants, ungulates) and human beings [1]. The characterization of morphologically identical isolates of *G. duodenalis* has demonstrated the genetic heterogeneity of the parasite. Eight distinct assemblages (A-H) have been identified within *G. duodenalis*, with assemblages A and B infecting humans and animals, and assemblages C to H being restricted to animal hosts [2].

Giardiasis is transmitted via the fecal-oral route and may be associated with gastrointestinal disorders both in animals and humans [3,4]. Most concern has been directed to the public health significance of giardiasis [5-7], although recent findings have suggested that the zoonotic transmission occurs more rarely than was previously believed [8].

Infection with *G. duodenalis* has been described in both household and kennelled dogs worldwide, with varying prevalence rates from 5% in privately owned

dogs to up to 100% in kennelled animals [9-13]. In Italy, infection rates of kennelled and private dogs have been found to be 14%-74% and 4%-19%, respectively [14-20]. Also, molecular studies have reported that dogs from Italy may harbor the canine-specific assemblages C and D, the zoonotic assemblage A, and also mixed infections with more than one assemblage [15].

About 350,000 dogs live in Sardinia, an island in the middle of the Mediterranean Sea with a population of about 1.5 million people. Of these dogs, 269,289, were enlisted in 2012 in the regional canine registries, and included sheepdogs, pets, hunting, and guard animals. Additionally, a non-quantifiable number of dogs arrives with their owners in Sardinia in tourist seasons. Despite a considerable autochthonous dog population and the extensive movements of animals, no information is available on the local prevalence of *Giardia* spp. in dogs. The only data on giardiasis in Sardinia is from an old study carried out in the 90s, which revealed the presence of the protozoan in

primary school children (15.5%) and in adult patients (52.2%) with gastro-enteric symptoms [21]. Thus, the aim of the present study was to investigate the potential epidemiological and zoonotic significance of *G. duodenalis* in dogs living in Sardinia Italy, by molecular characterization and analysis of associated risk factors.

Methodology

Sampling and copromicroscopic analysis

Given that no previous data on *Giardia* spp. in dogs was available in the literature for Sardinia, the minimum sample size ($n = 600$) was calculated based on the population of dogs of 269,289 (inscribed to regional registries and electronically tagged), an estimated prevalence for *Giardia* of 50% with a maximum sampling error of 4%, and a confidence interval (CI) of 95%.

From January 2007 to December 2010, individual fecal samples were collected from 655 dogs (356 privately owned dogs and 299 kennelled dogs) by vet practitioners from all the seven provinces of Sardinia (39°13' 0" N; 9°7'0" E). The study population included 286 female and 369 male dogs, ranging from 1 month to 16 years (mean = 37.00 ± 40.62 months) of age. For each sample, when possible, a form with the following information was completed: sex, age, use of dog, stool consistency, and co-habitation with other dogs.

However, the information on age, use, and co-habitation of 78, 213, and 480 dogs, respectively, was not available, as it was not possible to determine the historical deworming treatment of dogs.

When available, data on age (in months) were recorded based on information reported on the vaccination records of sampled dogs and/or by teeth examination. Fecal samples were taken directly from rectums (in accordance with animal welfare guidelines) or from the ground immediately after defecation and then classified for consistency as normal (well-formed feces), pasty (soft feces, not well formed) or diarrheic (liquid feces).

Samples were stored at 4°C until examination, which was performed within 24 hours of collection.

All samples were subjected to a copromicroscopic examination with a centrifugation flotation (2,000 rpm for 10 minutes) test using a zinc sulphate solution ($ZnSO_4$, specific gravity 1,200) [22].

Positive samples were classified according to the number of cysts found in a single microscopic field: weakly positive (≤ 20 cysts), medium positive (> 20 and < 50 cysts), or highly positive (> 50 cysts). Other parasitological findings were recorded.

A statistical analysis was performed to correlate the copromicroscopic findings with the historic data of the dogs (Table 1) in order to evaluate risk factors associated with *Giardia* spp. infection using the Chi-square test. The odds ratio (OR) values for each risk factor were evaluated using EPI INFO software, version 6.4, and differences were considered statistically significant when $p < 0.05$.

Biomolecular characterization

Forty-seven samples (17 from kennelled and 30 from privately owned dogs) copromicroscopically positive for *Giardia* were selected according to geographical origin (*i.e.*, from all the seven different provinces of Sardinia) and subjected to the technique previously described [23-25] to concentrate the protozoan cysts.

An aliquot of 200 μ L of suspension from each sample was subjected to genomic DNA extraction using the ZR Fecal DNA kit (Zymo Research, Orange, CA, USA). All samples were examined with two different PCR protocols specific for *G. duodenalis* assemblages. The detection of major genotypes was based on a PCR specific for the gene encoding for the *Giardia* small subunit ribosomal DNA (SSU-rRNA). Subsequently, samples genotyped as zoonotic assemblages were typed by a PCR specific to the gene encoding for the β -giardin protein. Briefly, a ~145 bp fragment of SSU-rRNA gene and ~511 bp fragment of the β -giardin gene were amplified by nested PCRs with slight modifications applied to the protocols previously described [23-25].

In the second step, PCR products for both PCR protocols were resolved by electrophoresis in a 1.8% agarose gel stained with Gel Red (Biotium, Hayward, CA, USA). Amplicons were then purified over minicolumns (Ultrafree-DA, Millipore, Milan, Italy) and sequenced using a Taq Dye Deoxy Terminator cycle sequencing kit in an ABI PRISM model 377 sequencer (Perkin Elmer Applied Biosystems, Warrington, UK). Sequence accuracy was ensured by two-directional sequencing, and all electropherograms were manually checked and edited. Nucleotide sequences obtained were searched against the GenBank database of available *Giardia* spp. sequences using the software Basic Local Alignment Search Tool [26].

Table 1. Dogs examined (T) and positive (P) for *Giardia* spp. infection in Sardinia, Italy. Rates of positivity (R) are presented in relation to epidemiological variables.

Variables	T	P	R	95% CI	Statistical data	OR
Total	655	172	26.3	22.9-29.7		
Sex						
Male	369	83	22.5	18.3-26.7	p = 0.0128 $\chi^2 = 6.19$	1.00
Female	286	89	31.1	25.8-36.4		1.53
*Age in months (m)						
≤ 3 m	112	31	27.7	19.4-36.0	p = 0.007 χ^2 for linear trend = 13.733	1.00
> 3 ≤ 6 m	72	34	47.2	35.7-58.7		2.34
>6 ≤ 9 m	35	19	54.3	37.8-70.8		3.10
> 9 ≤ 12 m	39	8	20.5	7.8-33.2		0.67
>12 ≤ 24 m	72	21	29.2	18.7-39.7		1.08
> 24 m	247	46	18.6	13.8-23.4		0.60
Total	577	159				
*Use						
Kennel	299	107	35.8	30.4-41.2	p = 0.0064 χ^2 with three degrees of freedom = 12.29	1.00
Hunting	23	7	30.4	11.6-49.2		0.78
Guard	25	5	20.0	4.3-35.7		0.45
Pets	95	17	17.9	10.2-25.6		0.39
Total	442	136				
Stool consistency						
Normal	220	48	18.3	13.1-23.5	p = 0.0469 χ^2 with two degrees of freedom = 6.12	1.00
Pasty	315	83	26.3	21.4-31.2		1.28
Diarrheic	120	41	34.2	25.7-42.7		1.86
Total	655	172				
Co-habitat**						
Yes	102	27	26.5	18.0-35.0	p = 0.89 $\chi^2 = 0.02$	
No	73	20	27.4	17.2-37.6		

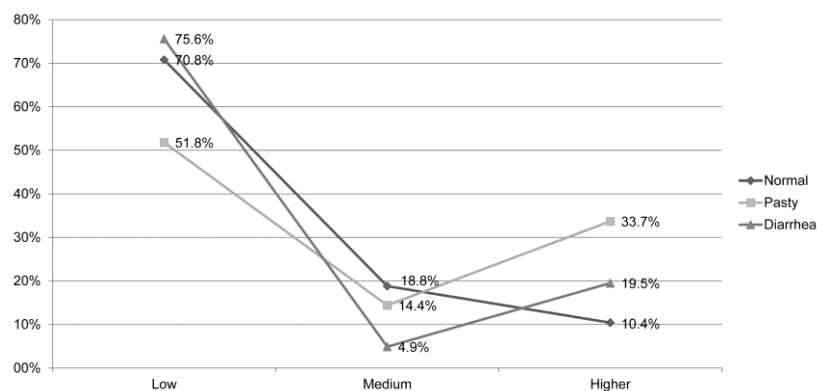
*This information was not available for all the dogs.

**This information was analysed only for privately owned dogs.

Table 2. Parasites present in 61 dogs infected by *Giardia* spp. in Sardinia, Italy

Parasites	Dogs	Prevalence (%)
Ancylostomatidae	31	18
Ascarids	28	16.3
<i>Trichuris vulpis</i>	7	4.1
Isospora spp.	6	3.5
Taeniidae	1	0.6
<i>Dipylidium caninum</i>	1	0.6
Total	74	

Figure 1. Correlation between the degree of *Giardia* cyst shedding and stool consistency in 172 infected dogs from Sardinia, Italy



Results

Copromicroscopic analysis

Prevalence rates for *Giardia* spp. infection according to the epidemiological factors are reported in Table 1.

Cysts of *G. duodenalis* were found in 26.3% of dogs examined (172/655). Of these animals, 22.5% were males (83/369) and 31.1% were females (89/286) ($\chi^2 = 6.19$; $p = 0.0128$) (OR = 0.64). Differences between prevalence rates in the six examined groups of age were statistically significant (χ^2 for linear trend = 13.733; $p < 0.0002$) (Table 1). In particular, the highest infection rate was detected in dogs between six and nine months and between three and six months of age.

The following prevalence rates were found according to the dogs' habitat/use: 35.8% for kennelled dogs (107/299), 30.4% for hunting dogs (7/23), 20% for guard dogs (5/25), and 17.9% for pets (17/95) (χ^2 with three degrees of freedom = 12.3; $p = 0.007$).

The highest value of positivity was found in diarrheic stool samples (34.2%; 41/120), followed by pasty (26.3%; 83/115) and normal samples (18.3%; 48/220). In most (62.8%) of the positive cases (108/172), the degree of cyst elimination was low, while percentages of samples with medium and high degrees of cyst shedding were 13.4% (23/172) and 23.8% (41/172). The degree of cyst shedding in feces of different consistency differed significantly in comparison with the values obtained for the samples having the same consistency ($p < 0.0001$) (Figure 1). No significant differences (χ^2 Yates corrected = 2.05; $p = 0.15237$) were found in positive pasty and diarrheic consistency samples with a high degree of cyst shedding. Positive pasty feces showed OR values double those of diarrheic samples (OR = 2.10).

Other endoparasites were found in 35.5% (61/172) of samples positive for *Giardia*; the ones most frequently found were Ancylostomatidae (hookworms) and Ascarididae (roundworms) in 18% and 16.3% of positive animals (Table 2), respectively. The difference between prevalence rates in samples positive either for only *Giardia* spp. or for co-infections was statistically significant ($\chi^2 = 29.07$; $p < 0.05$). With regard to mixed infections, significant values were found only for the co-existence of *G. duodenalis* with roundworms and *Trichuris vulpis*. Specifically, the highest prevalence of *G. duodenalis* was found in dogs infected at the same time with roundworms (16.3% vs. 7.2%; $\chi^2 = 11.90$; $p = 0.0005$), while the lowest values were recorded in dogs

parasitized by *T. vulpis* (4.1% vs. 11.6%; $\chi^2 = 11.6$; $p = 0.004$).

Genetic characterization

Of the 47 samples genetically examined, 42 were PCR positive for *Giardia* spp. (84%), while 5 did not produce any amplicons. The sequence analysis of the SSU gene showed that 17, 23, and 2 samples were positive for assemblages C, D, and A, with 98%-100% homology with GenBank sequences AB569372.1, AB569371.1, and FJ668859.1, respectively. The genotyping of the β -giardin amplicons showed that the two assemblage A isolates had 100% homology with the subtype A2 (GenBank Accession Numbers EU642896.1).

Discussion

The results herein presented indicate that *G. duodenalis* infection in dogs is present and circulates in Sardinia. Indeed, the prevalence rate found in Sardinia is consistent with rates (19.3%-26.6%) recently found in similar surveys carried out in continental Italy [14,27,28] and with values (16.4%-28.47%) recently reported from different European countries as well [4]. The similarity of the results found in Sardinia and continental Italy could be due to the same risk factors, which could nurture the dispersion of the infection in these two territories.

The age of the animals was found to be positively correlated with the occurrence of *G. duodenalis*, thus corroborating the findings of previous studies, which demonstrated that dogs under six months of age are more susceptible [29] than adult dogs [30] to infection.

The spread of *Giardia* spp. in kennels has been associated with the stressful situation in such environments, which can lead to the impairment of immunological responses in the intestine [29]. The same driver could also account for the high prevalence found in hunting dogs.

The present data on the correlation between degree of cyst shedding and fecal consistency confirm that diarrhea could be an indicative sign of infection with *G. duodenalis* as also described in other surveys [30], although it has also been reported that diarrheic samples are present mainly in human giardiasis rather than in animal infections [9]. Interestingly, the present study showed that stools of pasty consistency may be twice as likely as diarrheic samples to have a higher number of cysts. The results of the present survey regarding the correlation between fecal consistency (diarrhea vs. pasty/normal feces) and cyst excretion are consistent with those reported in similar investigations

[9], and suggest that, in clinical practice, *Giardia* spp. should not be suspected or investigated only in animals with diarrhea.

The higher prevalence of giardiasis in dogs infected with roundworms than in dogs with whipworm infections (positive for roundworms, mean = 19.01 months; positive for *Trichuris*, mean = 55.44 months; Student's $t = 0.003877$) is likely due to the age of the animals; young animals are more susceptible to *Giardia* and roundworms, while *T. vulpis* is more prevalent in adult dogs who, at the same time, are less susceptible to giardiasis. In fact, the average age of dogs positive for ascarids and *T. vulpis* was 1.6 and 4.6 years, respectively. The molecular results are consistent with those found in a previous study from Italy; specific dog-genotypes D and C were the most prevalent, though the zoonotic assemblage A subgenotype A2 was found as well [15].

Indeed, the highest zoonotic potential is displayed by the subgenotype A1 [25] and, interestingly, a recent study reported the simultaneous presence of this subgenotype in both dogs and children living in the same Rom community [31]. Assemblage A1 was also detected with a high prevalence (15.9%) in dogs in Spain [32]. The presence of the zoonotic subgenotype A2 in the animals herein examined suggests that they may be considered potential reservoirs of infection for humans, especially considering that the dispersion of cysts is not constantly accompanied by clinical symptoms, thus impairing prompt diagnosis and treatment. On the other hand, few studies have evaluated of the actual zoonotic potential of *G. duodenalis* isolates from dogs [31,33-34], thus the role of these animals in the transmission of the infection to humans is a subject of debate [1].

Conclusion

The present survey contributes to the knowledge of the occurrence of canine giardiasis in Italy, specifically in a region with a high number of dogs and numerous animal movements, due mainly to tourism. Statistical data on tourism income in Sardinia estimated more than 1.9 million arrivals in 2012 and highlighted also that since 2000, the number of foreign people coming to Sardinia for holidays dramatically increased from 24% to 42% [35]. Although dogs are checked at the entrance to the island with a control of their vaccination records (specifically rabies), these data are not recorded in an official registry.

Dogs entering Sardinia with tourists may be at significant risk of infection, but, on the other hand,

they can also be considered sources of infection for the local population.

Further studies are warranted for definitive molecular and epidemiological evidence of the zoonotic transmission of canine giardiasis and about epidemiological patterns and drivers influencing the potential parasite dynamics between autochthonous dogs, local populations, and tourist dogs and people, both of which can contribute to the design and implementation of appropriate control programs.

Acknowledgements

The research was funded by a grant of the Regional Government of Sardinia, prot. CRP2 134 (L.R. 7, 2007).

References

- Cacciò SM, Ryan U (2008) Molecular epidemiology of giardiasis. *Mol Biochem Parasit* 160: 75-80.
- Feng Y, Xiao L (2011) Zoonotic potential and molecular epidemiology of *Giardia* species and giardiasis. *Clin Microbiol Rev* 24: 110-140.
- Barutzki D, Thompson RCA, Wielinga C, Parker U, Schaper R (2007) Observations on *Giardia* infection in dogs from veterinary clinics in Germany. *Parasitol Res* 101: 153-156.
- Epe C, Rehker G, Lorentzen L (2010) *Giardia* in symptomatic dogs and cats in Europe – results of a European study. *Vet Parasitol* 173: 32-38.
- Leonhard S, Pfister K, Beelitz P, Thompson RCA (2007) The molecular characterisation of *Giardia* from dogs in southern Germany. *Vet Parasitol* 150: 33-38.
- Sprong H, Cacciò SM, van der Giessen JWB (2009) Identification of zoonotic genotypes of *Giardia duodenalis*. *Plos Neglect Trop Dis* 3: 558.
- Ballweber LR, Xiao L, Bowman DD, Kahn G, Cama VA (2010) Giardiasis in dogs and cats: update on epidemiology and public health significance. *Trends Parasitol* 26: 180-189.
- Monis PT, Cacciò SM, Thompson RC (2009) Variation in *Giardia*: towards a taxonomic revision of the genus. *Trends Parasitol* 25: 93-100.
- Upjohn M, Cobb C, Monger J, Geurden T, Claerebout E, Fox M (2010) Prevalence, molecular typing and risk factor analysis for *Giardia duodenalis* infections in dogs in a central London rescue shelter. *Vet Parasitol* 172: 341-346.
- Barr SC, Bowman DD (1994) Giardiasis in dogs and cats. *Comp Cont Educ Pract Vet* 16: 603-614.
- Bugg RJ, Robertson ID, Elliot AD, Thompson RCA (1999) Gastrointestinal parasites of urban dogs in Perth, Western Australia. *Vet J* 157: 295-301.
- Barutzky D and Schaper R (2003) Endoparasites in dogs and cats in Germany 1999–2002. *Parasitol Res* 90: 48-50.
- Dubná S, Langrová I, Nápravník J, Jankovská I, Vadlejch, J, Pekár S, Fechtner J (2007) The prevalence of intestinal parasites in dogs from Prague, rural areas, and shelters of the Czech Republic. *Vet Parasitol* 145: 120-128.
- Gianguaspero A, Paoletti B, Traversa D, Iorio R (2002) *Giardia* spp. in dogs and humans in Abruzzo region (Central Italy). *Parassitologia* 44: 81.
- Gianguaspero A, Berrilli F, Brandonisio O (2007) *Giardia* and *Cryptosporidium* and public health: the epidemiological scenario from the Italian perspective. *Parasitol Res* 101: 1169-1182.

16. Capelli G, Paoletti B, Iorio R, Frangipane di Regalbono A, Pietrobelli M, Bianciardi P, Giangaspero A (2003) Prevalence of *Giardia* spp. in dogs and humans in Northern and Central Italy. *Parasitol Res* 90: 154-155.
17. Capelli G, Frangipane di Regalbono A, Iorio R, Pietrobelli M, Paoletti B, Giangaspero A (2006) *Giardia* species and other intestinal parasites in dogs in North-East and Central Italy. *Vet Rec* 159: 422-424.
18. Berrilli F, Di Cave D, De Liberato C, Franco A, Scaramozzino P, Orecchia P (2004) Genotype characterisation of *Giardia duodenalis* isolates from domestic and farm animals by SSU-rRNA gene sequencing. *Vet Parasitol* 122: 193-199.
19. Bianciardi P, Papini R, Giuliani G, Cardini G (2004) Prevalence of *Giardia* antigen in stool samples from dogs and cats. *Rev Med Vet-Toulouse* 155: 417-421.
20. Papini R, Gorini G, Spaziani A, Cardini G (2005) Survey on giardiasis in shelter dog populations. *Vet Parasitol* 31: 333-339.
21. Garippa G, Sanna ML, Mura MS, Arru E (1995) Infezioni da protozoi gastrointestinali nella città di Sassari. *Riv Parassitol* 12: 205.
22. Zajak AM, Johnson J, King SE (2002) Evaluation of the importance of centrifugation as component of zinc sulfate fecal flotation examinations. *J Am Anim Hosp Assoc* 38: 221-224.
23. Read C, Walters J, Robertson ID, Thompson RCA (2002) Correlation between genotype of *Giardia duodenalis* and diarrhoea. *Int J Parasitol* 32: 229-231.
24. Cacciò SM, De Giacomo M, Pozio E (2002) Sequence analysis of the beta-giardin gene and development of a polymerase chain reaction-restriction fragment length polymorphism assay to genotype *Giardia duodenalis* cysts from human faecal samples. *Int J Parasitol* 32: 1023-1030.
25. Lalle M, Pozio E, Capelli G, Bruschi F, Crotti D, Cacciò SM (2005) Genetic heterogeneity at the beta-giardin locus among human and animal isolates of *Giardia duodenalis* and identification of potentially zoonotic subgenotypes. *Int J Parasitol* 35: 207-213.
26. Altschul SF, Madden TL, Schaffer AA, Zhang J, Zhang Z, Miller W, Lipman DJ (1997) Gapped BLAST and PSI-BLAST: a new generation of protein database search programs. *Nucl Ac Res* 25: 3389-3402.
27. Paoletti B, Iorio R, Capelli G, Sparagano OAE, Giangaspero A (2008) Epidemiological Scenario of Giardiasis in Dogs from Central Italy. *Ann Ny Acad Sci* 1149: 371-374.
28. Scaramozzino P, Di Cave D, Berrilli F, D'Orazi C, Spaziani A, Mazzanti S, Scholl F, De Liberato C (2009) A study of the prevalence and genotypes of *Giardia duodenalis* infecting kennelled dogs. *Vet J* 182: 231-234.
29. Gates MC, Nolan T (2009) Endoparasite prevalence and recurrence across different age groups of dogs and cats. *Vet Parasitol* 166: 153-158.
30. Li J, Zhang P, Wang P, Alsarakibi M, Zhu H, Liu Y, Meng X, Li J, Guo J, Li G (2012) Genotype identification and prevalence of *Giardia duodenalis* in pet dogs of Guangzhou, Southern China. *Vet Parasitol* 188: 368-371.
31. Marangi M, Berrilli F, Otranto D, Giangaspero A (2010) Genotyping of *Giardia duodenalis* among children and dogs in a closed socially deprived community from Italy. *Zoonoses Public Hlth* 57: 54-58.
32. Dado D, Montoya A, Alejandra MB, Mirò G, Saugar JM, Bailo B, Fuentes I (2012) Prevalence and genotypes of *Giardia duodenalis* from dogs in Spain: possible zoonotic transmission and public health importance. *Vet Parasitol* 111: 2419-2422.
33. Traub RJ, Monis PT, Robertson I, Irwin P, Mencke N, Thompson RC (2004) Epidemiological and molecular evidence supports the zoonotic transmission of *Giardia* among humans and dogs living in the same community. *Parasitol* 128: 253-262.
34. Volotão AC, Costa-Macedo LM, Haddad FS, Brandão A, Peralta JM, Fernandes O (2007) Genotyping of *Giardia duodenalis* from human and animal samples from Brazil using beta-giardin gene: a phylogenetic analysis. *Acta Tro* 102: 10-19.
35. Istituto Nazionale di Statistica ISTAT (2012) Servizio Statistica Regionale Sardegna. Available on <http://www.istat.it/it/archivio/sardegna>. Accessed on May 2, 2014.

Corresponding author

Antonio Varcasia
Parasitology, Veterinary Medicine Department
University of Sassari
Via Vienna 2 – 07100 Sassari, Italy
Phone: +39079229467
Email: varcasia@uniss.it

Conflict of interests: No conflict of interests is declared.