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***Giardia duodenalis* infection in dogs affected by primary chronic enteropathy**

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Abstract

Background: Canine primary chronic enteropathy (CE) includes a heterogeneous group of diseases characterized by chronic gastrointestinal signs.

Aim: This study evaluated the occurrence of *Giardia duodenalis* infection in primary CE-affected dogs.

Methods: Forty-seven CE-affected dogs of different age and sex were enrolled in the study. For each dog, frequency of defecation, fecal consistency, and eventual fecal abnormalities were evaluated. A clinical scoring index of CE severity (clinical chronic enteropathy activity index) was also assessed, and the type of enteropathy was retrospectively classified. For parasitological analysis, fresh fecal samples collected from each dog were examined by fresh and Lugol stained smears, flotation test, and a rapid immunoassay. *Giardia duodenalis* genotypes were identified by molecular analysis. Differences of clinical parameters between *G. duodenalis* positive and *G. duodenalis* negative dogs were statistically evaluated.

Results: Among the CE canine patients, 16 out of 47 (34%) dogs were found positive for *G. duodenalis* and assemblages C and D were identified. No statistical differences emerged according to the types of CE between *G. duodenalis*-positive and *G. duodenalis*-negative dog groups. The clinical index of CE severity was indicative of significant less severe clinical forms in *G. duodenalis*-positive dogs ($p = 0.037$).

Conclusion: Results here obtained shows how *G. duodenalis* may be present in primary CE-affected dogs and further investigations are needed to clarify the real significance of mild clinical presentation in *G. duodenalis*-positive dogs affected by CE.

Keywords: Canine, Clinical forms, Genotypes, Giardiasis, Primary chronic enteropathy.

Introduction

Giardia duodenalis is a worldwide intestinal protozoan parasite that infects a wide range of hosts, including humans, domestic, and wild mammals (Monis *et al.*, 2009). The localization site of *G. duodenalis* is the small intestine, mainly duodenum and jejunum, and in the infected hosts, it may be responsible for gastrointestinal signs (Hawrelak, 2003; Tangtrongsup and Scorza, 2010). Based on genetic analysis, *G. duodenalis* is considered as a species complex, which includes at least eight distinct genetic groups or assemblages, from A to H (Ryan and Cacciò, 2013). Zoonotic assemblages A and B and canine-specific assemblages C and D have been reported in dogs (Ballweber *et al.*, 2010; Ryan and Cacciò, 2013; Sommer *et al.*, 2018). The prevalence of *G. duodenalis* infection in dogs may vary depending on the population examined and the diagnostic method used (Ballweber *et al.*, 2010; Epe *et al.*, 2010). Younger animals and some dog communities, as stray and kennels dogs, have a higher risk of *G. duodenalis* infection than other dog populations (Huber *et al.*, 2005; Tysnes *et al.*, 2014), while dogs kept as pets are less likely to be positive (Bouزيد *et al.*, 2014). The prevalence of *G. duodenalis* in pet dogs in Italy is about 4%–29%

(Riggio *et al.* 2013; Pipia *et al.*, 2014; Zanzani *et al.*, 2014), while it is about 25% in symptomatic dogs from Europe (Epe *et al.*, 2010). *Giardia duodenalis*-infected dogs may show a spectrum of clinical signs ranging from subclinical forms to acute and intermittent forms or chronic diarrhea (Epe *et al.*, 2010; Tangtrongsup and Scorza, 2010). Factors associated with the onset of the disease include the age and the clinical, nutritional, and immune status of the infected dog (Roxstrom-Lindquist *et al.*, 2006). Although some studies suggested a possible relation between *G. duodenalis* assemblage and the severity of clinical disease, other studies suggested the opposite (Tysnes *et al.*, 2014). The pathogenic mechanisms proposed for *G. duodenalis* infections include production of toxins, disruption of normal intestinal microbiota, inhibition of normal enterocyte enzymatic function, blunting of microvilli, intestinal motility disorders, intestinal epithelial cell apoptosis, and intestinal inflammation (Tangtrongsup and Scorza, 2010). To explain variations in signs both among dogs and over time within the same dog, it has been suggested that microbiota modifications may influence the pathogenicity of *G. duodenalis* (Tysnes *et al.*, 2014). Associations with bacterial pathogens have been

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also hypothesized (Tysnes *et al.*, 2014) although results from a recent study suggest that *G. duodenalis* may also protect against gastrointestinal disease induced by a co-infecting bacterial enteropathogen (Manko *et al.*, 2017).

In dogs, chronic enteropathy (CE) includes a heterogeneous group of diseases characterized by chronic gastrointestinal signs lasting for longer than 3 weeks, as diarrhea, vomiting, hyporexia, abdominal pain, weight loss, with the exclusion of extra-intestinal or intestinal diseases of other etiology (Dandrieux, 2016). At present, chronic enteropathies are classified by treatment response in food-responsive enteropathy (FRE), antibiotic-responsive enteropathy (ARE), immunosuppressant-responsive enteropathy (IRE), and non-responsive enteropathy (NRE). Moreover, to avoid misclassification, an early treatment with fenbendazole for *Giardia* is performed even if *G. duodenalis* is not detected by routinely laboratory tests (Hall and Day, 2017). However, because the complexity of interactions between dietary components, microbiota, intestinal epithelial integrity, and immune system is supposed to play the main role in maintaining chronic inflammation, the role of *G. duodenalis* in dogs with chronic enteropathy needs to be clarified.

The aim of this prospective study was to evaluate the occurrence of *G. duodenalis* infection in dogs affected by primary CE.

Materials and Methods

Animals

Forty-seven dogs presented at the Veterinary Teaching Hospital (VTH) “M. Modenato” of the University of Pisa from January 2016 to March 2017, were enrolled in the study.

At presentation to our VTH, parasitological examination for intestinal parasites was performed by using the same methods reported below. All dogs were treated then with fenbendazole (50 mg/kg once daily, for five consecutive days) before to be enrolled in the study even if there were negative for parasites. All 47 dogs had a history of recurrent gastrointestinal signs and were diagnosed with a primary CE before the enrollment in the study, since extra-intestinal or intestinal diseases of other etiology had been excluded by previous diagnostic and therapeutic work-up. More specifically, all dogs underwent a complete blood cell count, a complete biochemical profile, including serum trypsin-like immunoreactivity and an abdominal ultrasound. At the time of inclusion in the study, when the diagnosis of CE was made, a fresh fecal sample was newly obtained for the parasitological and molecular analysis. Written informed consent was obtained from all the owners of dogs enrolled in this study.

Clinical analysis

For each dog enrolled in the study, the frequency of defecation (normal ≤ 3 times/day) and fecal abnormalities, including the presence of blood and/

or mucus, were evaluated. In addition, the use of a fecal consistency scoring system (Purina Proplan Veterinary Diets. Fecal Scoring Chart. https://www.proplanveterinarydiets.ca/wp-content/uploads/2018/05/180107_PPPVD-Fecal-Scoring-Chart-UPDATE-EN-FINAL.pdf) allowed us to assign to each dog fecal sample a different score on a scale from 1 to 7. A clinical index of CE severity was also assessed based on the clinical chronic enteropathy activity index (CCECAI) (Allenspach *et al.*, 2007; 2016). Moreover, the type of enteropathy was retrospectively classified as FRE, ARE, or IRE. More specifically, FRE diagnosis was based on a positive response to a change of diet using an elimination diet. ARE was diagnosed when a positive clinical response to antibiotic treatment (tylosin 10 mg/kg/bid) was observed, intestinal signs reoccurred after the discontinuation of the treatment, and no other underlying etiology was identified. Finally, IRE was diagnosed in dogs with persistent or recurrent gastrointestinal signs who had a histopathological evidence of intestinal inflammation and all the possible causes of this condition were excluded (Hall and Day, 2017).

Based on serum albumin (Alb) concentrations, CE was further classified as protein-losing enteropathy (PLE; Alb < 2.0 g/dl) or non-protein-losing enteropathy.

Parasitological and molecular analysis

For parasitological analysis, individual fresh fecal samples collected from each dog were examined within 24 hours by fresh and Lugol stained fecal smears and by flotation test with a low-density solution (33% ZnSO₄ solution, specific gravity 1.18). A commercial rapid immunoassay for the search of *G. duodenalis* and *Cryptosporidium* spp. fecal antigens (RIDA QUICK® *Cryptosporidium/Giardia* Combi, R-Biopharm, Darmstadt, Germany) was also used. Positivity for *G. duodenalis* at least to one of these methods was assumed to indicate the positivity of examined samples. For molecular analysis, *G. duodenalis*-positive samples were processed by a commercial kit (QIAamp DNA Stool Mini Kit, QIAGEN, Valencia, CA) for DNA extraction. A nested PCR protocol was applied to amplify a fragment of the small subunit ribosomal RNA gene (Read *et al.*, 2002). Amplification products were run on 2% ethidium bromide agarose gel and visualized under ultraviolet light. Positive amplicons were purified using mi-PCR Purification Kit, Metabion International AG. Amplification products were sent to an external laboratory for sequencing (Bio-Fab Research, Rome, Italy); sequence multiple alignment was carried out by ClustalW to identify *G. duodenalis* assemblages. After performing all clinical and parasitological evaluations, all dogs that in the present study scored positive for endoparasites were treated with previously recommended antiparasitic protocols.

Statistical analysis

For all parameters considered in this study, i.e. age and sex, CCECAI, fecal score index (FSI), frequency

of defecation (FD), and type of enteropathy, including PLE, ARE, FRE, and IRE, possible statistical differences between *G. duodenalis* positive and *G. duodenalis* negative dogs were evaluated. Data analysis was performed using the statistical software GraphPad Prism 7 and data were analyzed by the Chi square test and the Fischer's exact test. The significance level was set at $p < 0.05$.

Ethical approval

In this study, only fecal samples were collected from dogs with primary chronic enteropathy, thus a formal ethics approval was not applicable.

Results

Animals included in this study were 20 females and 27 male dogs, ranging from 5 months to 14 years of age. Among the 47 dogs included in the study, 22/47 dogs (46.8%) were found affected by ARE, 18/47 dogs (38.3%) by FRE and 7/47 dogs (14.9%) by IRE. Moreover, PLE was diagnosed in 11/47 (23.4%) examined dogs.

At parasitological analysis, 16 out of the 47 examined dogs (34 %) were found positive for *G. duodenalis*. More specifically, all 16 dogs were positive at the immunoassay, while 12 and 14 dog fecal samples scored positive for *G. duodenalis* at the fresh fecal smear and at the remaining tests performed in the study, respectively. The mean age of the *G. duodenalis* positive group was 5.3 years, with a median of 3 years (6 months–14 years), and only three subjects were

younger than one year. In the *G. duodenalis* negative group, the mean age was 4.8 years with a median of 4 years (5 months–13 years).

Due to the small amount of fecal material available, PCR was performed only on 9/16 dog samples found positive for *G. duodenalis* at parasitological examination, while only for 6/9 positive dogs it was possible to identify also *G. duodenalis* assemblage. *G. duodenalis* assemblage D was identified in five of them and assemblage C in only one.

From the evaluation of possible differences between *G. duodenalis* positive and *G. duodenalis* negative dogs according to the FSI, a higher frequency of fecal consistency alteration was observed in the *G. duodenalis* negative dog group, although no significant differences emerged at statistical analysis (Table 1). Indeed, fecal consistency was greatly reduced (FSI 6 or 7) in the 77.4% (24/31) of *G. duodenalis* negative dogs and in the 44% (7/16) of the *G. duodenalis* positive dogs.

However, significant differences ($p = 0.037$) emerged at statistical analysis between *G. duodenalis* positive and *G. duodenalis* negative dogs according to the CCECAI index, since most of *Giardia*-negative dogs had a moderate/severe CCECAI score (20/31, 64.5%), while among *Giardia*-positive dogs the same index was mainly low/negligible (11/16, 68.8%) (Table 1).

No statistical difference was instead found between the two groups of dogs according to the types of enteropathies (PLE, FRE, ARE, and IRE) (Table 1) although a higher frequency of PLE was observed

Table 1. Comparison of clinical aspects in *Giardia duodenalis* positive and negative dogs affected by different type of chronic enteropathy.

Variable	<i>G. duodenalis</i> positive dogs (n. 16)	<i>G. duodenalis</i> negative dogs (n. 31)	p-values
CCECAI	MI+CI 11/16 (68.8%)	MI+CI 11/31 (35.5%)	$p = 0.037^{**}$
	S+M 5/16 (31.3%)	S+M 20/31 (64.5%)	
FSI	N 3/16 (18.8%)	N 2/31 (6.5%)	$p = 0.068^*$
	SA 6/16 (37.8%)	SA 5/31 (16.1%)	
	SVA 7/16 (43.8%)	SVA 24/31 (77.4%)	
MU and/or B	MU 4/16 (25%)	MU 12/31 (38.7%)	p (MU) = 0.517 ^{**}
	B 3/16 (18.8%)	B 10/31 (32.3%)	p (B) = 0.494 ^{**}
FD	N 9/16 (56.3%)	N 7/31 (22.6%)	$p = 0.056^{**}$
	I 7/16 (43.8%)	I 24/31 (77.4%)	
Type of enteropathy	FRE 5/16 (31.3%)	FRE 13/31 (41.9%)	$p = 0.647^*$
	ARE 9/16 (56.3%)	ARE 13/31 (41.9%)	
	IRE 2/16 (12.5%)	IRE 5/31 (16.1%)	
	PLE 2/16 (12.5%)	PLE 9/31 (29%)	

ARE, antibiotic-responsive enteropathy; B, presence of blood in feces; CCECAI, Canine Chronic Enteropathy Activity Index; CI, clinically insignificant; FD, frequency of defecation; FRE, food-responsive enteropathy; FSI, fecal score index; I, increased; IRE, immunosuppressant-responsive enteropathy; M, moderate; MI, mild; MU, presence of mucus; N, normal (FSI 2-4/7); PLE, protein losing enteropathy; S, severe; SA, slightly altered (FSI 5/7); SVA, severely altered (FSI 6-7/7). (*): Chi-square test; (**): Fisher's exact test.

among *G. duodenalis* negative dogs (9/31, 29%) in respect to that observed among *G. duodenalis* positive dogs (2/16, 12.5%).

Discussion

To date, this is the first study evaluating *G. duodenalis* in a selected population of dogs with a diagnosis of primary CE. Our findings are taken from a population which underwent on a fenbendazole treatment prior the inclusion.

The prevalence of *G. duodenalis* in primary CE found in the present study (34%) is much higher than data reported in recent studies in symptomatic dogs in Europe (Epe et al., 2010; Volkmann et al., 2017). Furthermore, it is higher than prevalence of *G. duodenalis* found in different dog populations in Italy (Pipia et al., 2014; Zanzani et al., 2014; Paoletti et al., 2015; De Liberato et al., 2018), including privately owned (about 4%) and kennel (about 5%) dogs of the same area (Riggio et al., 2013; Sauda et al., 2018).

In our opinion, this high prevalence which we found it is unlikely to be associated to a primary *G. duodenalis* infection. Although the reported efficacy of fenbendazole treatment is very high (Barr et al., 1994; O et al., 1998), a fenbendazole resistance may occur in dogs. Another possible explanation is that the risk of *G. duodenalis* infection seems to increase with high frequency of anthelmintic treatment. This may be due to the change of the intestinal niche caused by the anthelmintic therapy on major parasites (Bugg et al., 1999). However, it is not possible to exclude that the positive dogs may be newly infected due to incomplete animal and/or environmental disinfection (Raza et al., 2018). Finally, in our opinion, this result may be related to the dog population here considered because the intestinal alterations caused by chronic inflammation may predispose CE-affected dogs to the acquisition of *G. duodenalis* infections.

However, it is unlikely that *G. duodenalis* may have a significant role in the gastrointestinal signs in our dogs. Indeed, from the evaluation and scoring of clinical parameters, the presence of *G. duodenalis* was not associated with more severe clinical forms. On the contrary, the CCECAI score was indicative of significantly more severe clinical forms in *G. duodenalis* negative group. In addition, FSI and the presence of mucus or blood in stools were not statistically associated with *G. duodenalis* infection. No significant differences emerged also between *G. duodenalis*-positive and *G. duodenalis*-negative dogs according to the type of enteropathies (e.g., ARE, FRE, IRE, and PLE).

Giardia duodenalis infections can be asymptomatic, subclinical, and clinically evident forms may occur especially when other factors are also present, such as concurrent entero-pathogenic bacteria or parasites, food intolerance or decreased host defense ability and stress conditions (Ballweber et al., 2010; Tysnes et al., 2014). Interestingly, all clinical parameters used in this

study to score the severity of signs were indicative of less severe clinical forms in the group of *G. duodenalis*-infected dogs, especially the CCECAI score that gave statistically significant results.

To the best of our knowledge, no previous studies specifically investigated the prevalence of *G. duodenalis* in canine primary CE; its possible associations with the type of enteropathy and the severity of clinical presentation. In fact, only the stool consistence and the presence of diarrhea were previously evaluated in dogs infected by *G. duodenalis* (Epe et al., 2010; Upjohn et al., 2010; Pipia et al., 2014; Volkmann et al., 2017). However, it is also possible that some dogs considered negative in this study were instead positive, since only a single fecal sample per dog was examined. In fact, due to the inconstant shedding of the cysts into the feces, for the detection of *G. duodenalis* it is advisable to examine three samples collected in three non-consecutive days, while the examination of a single fecal sample has a lower sensitivity (Tangtrongsup and Scorza, 2010).

In this study, only the canine assemblages C and D were identified at genotyping of some dog fecal samples found positive for *G. duodenalis* at molecular analysis. These data seem to confirm the higher frequency of these canine-specific assemblages in privately owned dogs in Italy (Pipia et al., 2014; Paoletti et al., 2015; Simonato et al., 2017; Sauda et al., 2018). Nevertheless, it is not possible to exclude the presence of the zoonotic assemblages A and B in the remaining positive dog samples for which molecular analysis and genotyping were not possible. Indeed, the assemblages A and B have been also identified in owned dogs from Italy (Riggio et al., 2013; Zanzani et al., 2014; Simonato et al., 2017) and a high possibility of transmission of these zoonotic assemblages between dogs and humans has been supposed (Marangi et al., 2010; Feng and Xiao, 2011).

We acknowledge that this study has several limitations. First, the number of dogs examined in this study was low and follow-up for clinical conditions was not performed. Secondly, at inclusion in the study only a single fecal sample per dog to assess positivity or negativity for *G. duodenalis* was possible to analyze.

In conclusion, *G. duodenalis* may be present in primary CE-affected dogs and further investigations are needed to clarify the real significance of less severe clinical presentation observed in *G. duodenalis*-positive and CE-affected dogs and a possible higher diffusion of *G. duodenalis* in CE dog patients.

Conflict of interest

The authors declare that there is no conflict of interest.

Authors contribution

Stefania Perrucci and Veronica Marchetti conceived and designed the study. All authors contributed to perform research, interpreting the results, writing and critically revising the manuscript. All authors approved the final version of the manuscript.

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