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Seasonal distributions and other risk factors for *Giardia duodenalis* and *Cryptosporidium* spp. infections in dogs and cats in Chiang Mai, Thailand

Sahatchai Tangtrongsup[a,b,c,*], A. Valeria Scorza[d], John S. Reif[e], Lora R. Ballweber[f], Michael R. Lappin[d], Mo D. Salman[c]

[a] Department of Companion Animal and Wildlife Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Muang Chiang Mai 50100, Thailand
[b] Research Center of Producing and Development of Products and Innovations for Animal Health and Production, Chiang Mai University, Muang Chiang Mai 50100, Thailand
[c] Animal Population Health Institute, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA
[d] Center for Companion Animal Studies, Department of Clinical Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA
[e] Department of Environmental and Radiological Health Sciences, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA
[f] Department of Microbiology, Immunology and Pathology, College of Veterinary Medicine and Biomedical Sciences, Colorado State University, Fort Collins, CO 80523, USA

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ABSTRACT

The objectives of this study were to explore risk factors associated with *Giardia* and *Cryptosporidium* infections in dogs and cats in Chiang Mai, Thailand, to describe the seasonal distributions of *Giardia* and *Cryptosporidium* prevalence, and to determine the potential for zoonotic transmission through genetic characterization of isolates. Fecal samples from 301 dogs and 66 cats were collected between August 2009 and February 2010. The presence of *Giardia* cysts and *Cryptosporidium* oocysts was determined using zinc sulfate centrifugal flotation and immunofluorescent assay (IFA). Genotype/species were determined by DNA sequence analyses of PCR products from *Giardia* glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triosephosphateisomerase (*tpi*) and *Cryptosporidium* heat shock protein 70KDa (*hsp70*) and small subunit-rRNA (*SSU-rRNA*) genes. Information related to specific risk factors was collected from owners of each animal using a questionnaire. The risk factor data were analyzed for associations with *Giardia* and *Cryptosporidium* infections using logistic regression.

The overall estimated prevalence of *Giardia* and *Cryptosporidium* in dogs was 25.2% and 7.6%, respectively and in cats, 27.3% and 12.1%, respectively. The estimated prevalence of *Giardia* infection in dogs in the rainy season (31.7%) was significantly higher than in the drier, winter season (17.2%) ($p < 0.01$). The estimated prevalence of *Cryptosporidium* infection in dogs and of *Giardia* and *Cryptosporidium* infections in cats was not associated with season ($p > 0.05$). Multivariable analysis indicated that *Giardia* cysts were more likely to be detected in fecal samples of dogs that resided in high-density environments, drank untreated water, were shedding *Cryptosporidium* oocysts, were having acute diarrhea or a history of chronic diarrhea, and were collected in the rainy season. All 19 *Giardia* PCR positive samples typed as *G. duodenalis* canine adapted genotypes (assemblages C or D). In cats, of six *Giardia* PCR positive samples, five typed as dog assemblages and one typed as assemblage AI. Of ten dogs with *Cryptosporidium* PCR positive samples, eight typed as *C. canis,* one as *C. parvum* (a zoonotic species) and one had both *C. canis* and *C. parvum*. Of three *Cryptosporidium* PCR positive samples in cats, one typed as *C. felis* and two typed as *C. parvum*.

The presence of zoonotic *G. duodenalis* assemblage AI in a cat, and *C. parvum* in feces of dogs and cats suggests a potential role for a reservoir for zoonotic transmission. Whether or not these presences were from exposure to other animal or human hosts or environment are needed to be confirmed.

* Corresponding author at: Department of Companion Animal and Wildlife Clinic, Faculty of Veterinary Medicine, Chiang Mai University, Muang, Chiang Mai 50100, Thailand.
E-mail address: sahatchai.t@cmu.ac.th (S. Tangtrongsup).

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1. Introduction

Giardia duodenalis and *Cryptosporidium* spp. are common causes of diarrhea in mammalian hosts including humans, dogs, and cats (Feng and Xiao, 2011; Thompson and Smith, 2011; Ryan and Caccio, 2013). *Giardia duodenalis* is comprised of eight genotypes or assemblages (A–H) (Ballweber, 2015). Assemblages A and B, considered to be the primary zoonotic genotypes, are most commonly detected in humans and may also be detected in a range of other hosts including domestic animals and wildlife. Globally, dogs and cats tend to be infected with species-adapted assemblages with assemblages C and D occurring in dogs and assemblage F in cats. However, other assemblages, including assemblages A and B in both dogs and cats, and assemblages C, D, and E in cats, have also been reported (Feng and Xiao, 2011; Scorza et al., 2012). The genus *Cryptosporidium* contains at least 27 distinct species (Ryan and Hijjawi, 2015). *Cryptosporidium hominis* and *C. parvum* are most commonly detected species in humans but *C. meleagridis, C. canis* and *C. felis* have also been documented (Xiao, 2010). The most frequently reported species from dogs is *C. canis*, although *C. parvum, C. muris*, and *C. meleagridis* have occasionally been documented. In cats, *C. felis* occurs most frequently with rare reports of *C. muris* (Santin, 2013).

Because it is not possible to morphologically differentiate species within *Giardia* and *Cryptosporidium* genera, infections in dogs and cats are generally reported at the genus level only. The detection of *Giardia* cysts and *Cryptosporidium* oocysts in dogs and cats is often assumed to represent a potential zoonotic hazard. Molecular techniques have been used to identify these pathogens at the species level (Caccio et al., 2005). There were several target genes for polymerase chain reaction (PCR) assays that have been reported for these pathogens. Glutamate dehydrogenase (*gdh*), beta-giardin (*bg*) and triosephosphate isomerase (*tpi*) genes have been widely accepted for multilocus genotype determination of *Giardia duodenalis* and small subunit ribosomal RNA (*SSU-rRNA*) and heat-shock protein 70 kDa (*hsp70*) for species determination of *Cryptosporidium* spp. With DNA sequence analysis after PCR, the potential zoonotic hazard for transmission to humans, could be better determined.

Several risk factors have been reported to be associated with infections by these parasites. The risks associated with *Giardia* infection were age of less than one year, having diarrhea and living in a crowded setting like shelters, kennels, cattery, breeding farms or stray dogs (Kirkpatrick and Laczak, 1985; Ponce-Macotela et al., 2005; Mundim et al., 2007; Solarczyk and Majewska, 2010). Other risk factors included breed of dog (Gates and Nolan, 2009), visiting a dog park (Wang et al., 2012), presence of other enteric parasites (Vasilopulos et al., 2006; Ballweber et al., 2009), and season of the year (Fontanarrosa et al., 2006; Batchelor et al., 2008).

In a previous study of fecal samples from 109 dogs from Chiang Mai, Thailand, the prevalence of infection was 45.9% for *G. duodenalis* and 31.2% for *Cryptosporidium* spp. (Tangtrongsup et al., 2017). Chiang Mai city was selected for this study to represent a continuation of that research in a newly defined population for the following reasons: 1) it has a tropical environment with a hybrid landscape of rural and urban city development, agricultural, industrial, and tourism areas; 2) preliminary data from a previously conducted study in dogs from this area were available (Tangtrongsup et al., 2017); and 3) disseminating information on the prevalence of infection and predominant species of these parasites in dogs and cats to Thai veterinarians could increase awareness of these protozoal infections in the region. Therefore, the aims of the study reported here were to extend the scope and results of the previous study by 1) determining risk factors associated with *Giardia* and *Cryptosporidium* infections in dogs and cats from this area; 2) exploring the seasonal distributions of *Giardia* and *Cryptosporidium* prevalence in dogs and cats, and 3) genetically characterizing the genotype/species of these organisms in dogs and cats to gather further data on the prevalence of genotypes/species with potential for zoonotic transmission.

2. Materials and methods

2.1. Study location

Chiang Mai is located in the northern part of the country. This province is the second largest of Thailand and covers an area of 20,107.057 sq. km. Approximately 70% of the area is covered with forest, 13% is agricultural land, and 17% is for housing and other uses. There are three seasons: rainy from mid-May through October; winter from November to mid-February; and summer from mid-February to mid-May (Chiang Mai Provincial Office, 2012). The average temperature (average minimum to maximum) in rainy, winter, and summer are 27.7 °C (22.4 °C–36.0 °C), 23.9 °C (14.4 °C–34.35 °C), and 28.5 °C (20.1–39.1), respectively. Average rainfall in rainy, winter, and summer is 16.0 cm, 1.8 cm, and 4.5 cm respectively (Thai Meteorological Department: Northern Meteorological Center, 2018).

2.2. Sample collection

Dog and cat populations in Chiang Mai province, Thailand include owned-, stray-, and shelter animals. However, there is no pet registry which could have been used as a sampling frame. Therefore, a cross-sectional study was conducted in order to estimate the prevalence of *Giardia* and *Cryptosporidium* infection by collecting samples from dogs and cats brought to the Small Animal Veterinary Teaching Hospital of the Faculty of Veterinary Medicine, Chiang Mai University, and those housed by shelters and breeders in the province. A sample size of at least 325 fecal samples for dogs and at least 97 fecal samples for cats was targeted for collection from August 2009 to February 2010. This sample size was calculated based on the earlier estimate of the prevalence of 30.3% for *Giardia* in dogs (Tangtrongsup et al., 2017), alpha of 0.05, error rate of 5% and power of 0.80 (Fleiss, 2003). For cats, a prevalence of 50% was used for the sample size calculation with the same settings as for dogs, but with an error rate of 10% due to an expected lower participation rate.

The samples were collected on a volunteer basis regardless of the health status of the animal, sex or age. All dogs and cats visiting the outpatient department of the veterinary teaching hospital during the study period were eligible and the owners were asked to participate in the study. When the owner agreed to participate, fecal samples were obtained within one week. Owners or caregivers of the animals were asked to complete a written questionnaire with information regarding geographic and demographic data, a number of animals in the home or at the site, details of housing and living conditions, health status, owners' or caregivers' socioeconomic status. The fecal samples were collected in a plastic cup, stored on ice and transported to the laboratory within 24 h of collection.

2.3. Diagnostic methods

Fecal consistency was estimated upon receipt of the sample at the Small Animal Veterinary Teaching Hospital using a standardized scoring system (Nestle-Purina Pet Food Co, St Louis, MO, USA). Fecal scores of 1–3 were considered normal with scores of 4–7 classified as diarrheic. Fecal samples were stored in closed plastic containers at 4 °C until screened.

Microscopic examination of fecal samples was performed after zinc sulfate (s.g. 1.18) centrifugal flotation at 650 × *g* for 5 min (Zajac and Conboy, 2012) to determine the presence of *Giardia* cysts and other intestinal parasitic infection. The fecal sample was collected once from each animal and the fecal examination was done within 5 days of collection. Fecal samples were then stored at −20 °C until shipped to Colorado State University for immunofluorescent assay (IFA) and molecular analysis. Fecal samples were shipped to the USA on dry ice and stored at −70 °C upon receipt.

Fecal samples were evaluated for *Giardia* cysts and *Cryptosporidium*

oocysts using a commercially available IFA (Merifluor® *Cryptosporidium/Giardia* IFA kit, Meridian Diagnostic Corporation, Cincinnati, OH). Prior to IFA, all fecal samples were thawed at room temperature and concentrated using sugar concentration techniques as previously described (O'Handley et al., 2000). Fifteen microliters of fecal concentrate were smeared on glass slides and the IFA was performed following the manufacturer's instructions.

A sample was considered positive for *Giardia* if cysts were detected on either zinc sulfate centrifugal flotation or IFA evaluation; samples were considered positive for *Cryptosporidium* based on IFA results only. Animals were grouped by age (< 1 yr, 1–7 yrs, and > 7 yrs). Fecal samples collected during August to October 2009 were grouped as collected during the rainy season while those collected during November 2009 to February 2010 were grouped as winter samples. In dogs, housing was categorized into a household group and a shelter/breeder/temple group, the latter representing a high-density environment.

2.4. Molecular analysis

Genomic DNA was extracted from fecal samples containing *Giardia* cysts or *Cryptosporidium* oocysts following an established protocol (Da Silva et al., 1999) and stored at −20 °C until subsequent PCR assays. PCR assays targeting *Giardia* glutamate dehydrogenase (*gdh*), beta-giardin (*bg*), and triosephosphate isomerase (*tpi*) genes and *Cryptosporidium* heat-shocked protein (*hsp70*), and small subunit ribosomal RNA (*SSU-rRNA*) were used for molecular identification of the respective pathogens. Previously described PCR protocols were used (Morgan et al., 1997; Xiao et al., 1999; Lebbad et al., 2001; Morgan et al., 2001; Caccio et al., 2002; Read et al., 2004; Sulaiman et al., 2004) with the following modification; all PCR reactions were performed in 25-µl reaction using HotStarTaq Master Mix (Qiagen, Valencia, CA) with 10 pmol of each primer, 1.5 mM of $MgCl_2$, and 1 µl of template DNA.

The PCR products were analyzed by nucleotide sequencing using a commercially available service (Proteomics and Metabolomics Facility, Colorado State University). The obtained sequences were compared with nucleotide sequences from the nucleotide database, GenBank, by BLAST analysis (http://blast.ncbi.nlm.nih.gov/Blast.cgi).

2.5. Statistical analyses

Questionnaire data were entered into a spreadsheet and all statistical analyses were performed using Stata statistical software release 10.1 (Stata Corp., College Station, Texas, USA). The overall prevalence and 95% confidence intervals (95% CI) were calculated. Odds ratios (OR) and 95% CI were estimated initially to measure the strength of association using univariable logistic regression. Variables found to be associated with *Giardia* or *Cryptosporidium* infection ($p \leq 0.1$) were included in a multivariable logistic regression analysis. A multivariable logistic regression model was constructed using a backward stepwise elimination procedure, against either protozoal test result (Hosmer and Lemeshow, 2000). Variables were retained in the model based on the likelihood ratio χ^2 statistic, at $p < 0.05$. *P* values < 0.05 were considered statistically significant.

3. Results

A total of 301 canine and 66 feline individual fecal samples were obtained for this study. Of 301 canine fecal samples, 93.4% were from households and 6.6% were from high-density environments (shelter/breeding farm/temple). For cat fecal samples, 80.3% were from households and 19.7% were from a breeding farm. The questionnaires were answered or returned by 92% of dog owners and 71.2% of cat owners or caregivers. For dogs, the number of fecal samples collected in the rainy and winter seasons were 167 (55.5%) and 134 (44.5%), respectively. In cats, the number of samples collected in the rainy and winter seasons were 24 (36.4%) and 42 (63.6%), respectively. Among the canine samples, 67 (22.3%), 161 (53.5%), and 73 (24.3%) were from dogs less than 1 year of age, 1–7 years of age, and more than 7 years of age, respectively. Among feline samples, 20 (30.3%), 40 (60.6%), and 6 (9.1%) were from cats age less than 1 year of age, 1–7 years of age, and more than 7 years of age, respectively. Among the dogs, 48.5% were male (146/301) whereas 57% (38/66) of the cats were male.

Table 1
Samples testing positive for intestinal parasites in dogs in Chiang Mai, Thailand (n = 301).

Parasite	No. positive (%)
At least one infection	115 (38.2)
Single infection	
Giardia duodenalis	**38 (12.6)**
Hookworm	19 (6.3)
Cystoisospora spp.	5 (1.7)
Trichuris vulpis	2 (0.7)
Cryptosporidium spp.	1 (0.3)
Strongyloides spp.	1 (0.3)
Toxocara canis	1 (0.3)
Dual infection	
***G. duodenalis* - *Cryptosporidium* spp.**	**18 (6.0)**
G. duodenalis – *Cystoisospora* spp.	8 (2.7)
G. duodenalis - Hookworm	4 (1.3)
Hookworm - *T. vulpis*	4 (1.3)
G. duodenalis - *T. canis*	3 (1.0)
Hookworm - *Cystoisospora* spp.	2 (0.7)
Hookworm - *Strongyloides* spp.	1 (0.3)
Hookworm - *T. canis*	1 (0.3)
T. canis - *Cystoisospora* spp.	1 (0.3)
Triple infection	
***G. duodenalis* - *Cryptosporidium* spp. - Hookworm**	**3 (1.0)**
G. duodenalis - *Cryptosporidium* spp. - *Cystoisospora* spp.	1 (0.3)
Quadruple infection	
G. duodenalis - Hookworm - *T. canis* - *T. leonina*	1 (0.3)
Hookworm - *T. canis* - *T. leonina* - *Cystoisospora* spp.	1 (0.3)

The overall estimated prevalence rates of all intestinal parasitic infections were 38.2% (95% CI: 32.7–43.7) and 45.5% (95% CI: 33.4–57.5) in dog and cat fecal samples, respectively. The estimated prevalence rates of *Giardia* infections in dogs and cats were 25.2% (95% CI: 20.3–30.2) and 27.3% (95% CI: 16.5–38.0), respectively. Overall estimated prevalence rates of *Cryptosporidium* infections in dogs and cats were 7.6% (95% CI: 4.6–10.6) and 12.1% (95% CI: 4.2–20.0), respectively. Detection of a single parasite, as opposed to multiple parasites, was most commonly encountered in both dogs and cats (Tables 1 and 2).

The univariable analyses of risk factors associated with positive test

Table 2
Samples testing positive for intestinal parasites in cats in Chiang Mai, Thailand (n = 66).

Parasite	No. positive (%)
At least one infection	30 (45.5)
Single infection	
Giardia duodenalis	**9 (13.6)**
Cryptosporidium spp.	2 (3.0)
Cystoisospora spp.	3 (4.5)
Hookworm	2 (3.0)
Spirometra spp.	2 (3.0)
Dual infection	
***G. duodenalis* - *Cryptosporidium* spp.**	**6 (9.1)**
G. duodenalis - *Cystoisospora* spp.	2 (3.0)
G. duodenalis - *Spirometra* spp.	1 (1.5)
Hookworm - *Cystoisospora* spp.	1 (1.5)
T. cati - *Cystoisospora* spp.	1 (1.5)
T. cati - *T. leonina*	1 (1.5)

Table 3
Univariable logistic regression analysis for variables associated with *Giardia* and *Cryptosporidium* infections in dogs in Chiang Mai, Thailand. (n = 301).

Factor	n[3]	*G. duodenalis* % positive (n)	OR (95%CI)*	*P* value	*Cryptosporidium* spp. % positive (n)	OR (95%CI)*	*P* value
Season							
Rainy	167	31.7 (53)	2.24 (1.29-3.91)	< **0.01**	6.0 (10)	0.59 (0.25-1.40)	0.23
Winter	134	17.2 (23)	*Reference*		9.7 (13)	*Reference*	
Age							
< 1 year	67	37.3 (25)	1.80 (0.98-3.31)	0.06	17.9 (12)	4.17 (1.62-10.74)	**0.02**
1 – 7 years	161	24.8 (40)	*Reference*		4.9 (8)	*Reference*	
> 7 years	73	15.0 (11)	0.54 (0.26-1.12)	0.10	4.1 (3)	0.82 (0.21-3.18)	0.77
Sex							
Male	146	21.9 (32)	0.71 (0.42-1.20)	0.20	8.2 (12)	1.17 (0.50-2.75)	0.71
Female	155	28.4 (44)	*Reference*		7.1 (11)	*Reference*	
Breed							
Purebred	62	32.3 (20)	*Reference*		3.2 (2)	*Reference*	
Mixed	239	23.4 (56)	0.64 (0.35-1.18)	0.16	8.8 (21)	2.89 (0.66-12.67)	0.16
Diarrhea status							
Yes	67	40.3 (27)	2.55 (1.43-4.56)	< **0.01**	14.93 (10)	2.98 (1.24-7.15)	**0.01**
No	234	20.9 (49)	*Reference*		5.56 (13)	*Reference*	
Hookworm eggs present							
Yes	36	22.2 (8)	0.83 (0.36-1.90)	0.66	8.3 (3)	1.11 (0.31-3.95)	0.87
No	265	25.7 (68)	*Reference*		7.6 (20)	*Reference*	
Toxocara canis eggs present							
Yes	8	50.0 (4)	3.07 (0.75-12.59)	0.12	0 (0)		N/A
No	293	24.6 (72)	*Reference*		7.7 (23)		
Toxascaris leonina eggs present							
Yes	2	50.0 (1)	2.99 (0.18-48.34)	0.44	0 (0)		N/A
No	299	25.1 (75)	*Reference*		7.8 (23)		
Cystoisospora oocysts present							
Yes	18	50.0 (9)	3.22 (1.23-8.45)	**0.02**	5.6 (1)	0.70 (0.09-5.49)	0.73
No	283	23.7 (67)	*Reference*		7.7 (22)	*Reference*	
Cryptosporidium oocysts present							
Yes	23	95.7 (22)	91.26 (12.03-692.03)	< **0.01**			
No	278	19.4 (54)	*Reference*				
Giardia cysts present							
Yes	76				28.9 (22)	91.26 (12.03-692.03)	< **0.01**
No	225				0.4 (1)	*Reference*	
Type of Housing							
Household	281	23.1 (65)	*Reference*		6.4 (18)	*Reference*	
Shelter/breeder/Temple	20	55.0 (11)	4.03 (1.59-10.17)	< **0.01**	25.0 (5)	5.13 (1.65-15.89)	< **0.01**
Multi-dog household							
Yes	219	24.2 (53)	0.74 (0.40-1.40)	0.36	7.76 (17)	1.18 (0.38-3.64)	0.78
No	60	30.0 (18)	*Reference*		6.67 (4)	*Reference*	
No of animals							
1	60	30.0 (18)	*Reference*		6.7 (4)	*Reference*	
2-4	106	24.5 (26)	0.76 (0.37-1.54)	0.44	9.4 (10)	1.46 (0.44-4.87)	0.54
5-10	70	15.7 (11)	0.44 (0.19-1.02)	0.05	1.4 (1)	0.20 (0.02-1.87)	0.16
> 10	43	37.2 (16)	1.38 (0.60-3.17)	0.44	14.0 (6)	2.27 (0.60-8.60)	0.23
Cat in the same house							
Yes	44	20.5 (9)	0.70 (0.32-1.54)	0.38	6.8 (3)	0.87 (0.24-3.07)	0.82
No	231	26.8 (62)	*Reference*		7.8 (18)	*Reference*	
Free roaming							
Yes	48	22.9 (11)	0.84 (0.40-1.76)	0.65	0 (0)		N/A
No	234	26.1 (61)	*Reference*		9.0 (21)		
Defecating area							
Inside household area	199	25.6 (51)	*Reference*		9.6 (19)	*Reference*	
Outside household area	37	8.1 (3)	0.26 (0.08-0.87)	**0.03**	2.7 (1)	0.26 (0.03-2.03)	0.20
Feces picked up immediately							
Yes	86	20.9 (18)	*Reference*		8.1 (7)	*Reference*	
No	143	23.1 (33)	1.13 (0.59-2.17)	0.71	7.7 (11)	0.94 (0.35-2.53)	0.90
Drinking water source							
Bottled/Filtered water	55	30.9 (17)	*Reference*		12.7 (7)	*Reference*	
Tap water	180	20.6 (37)	0.58 (0.29-1.14)	0.11	4.4 (8)	0.32 (0.11-0.92)	**0.04**
Untreated water[a]	44	38.6 (17)	1.40 (0.61-3.24)	0.42	13.6 (6)	1.08 (0.35-3.49)	0.70
Food type							
Commercial	65	32.3 (21)	*Reference*		9.2 (6)	*Reference*	
Homemade	35	25.7 (9)	0.73 (0.29-1.82)	0.49	11.4 (4)	1.27 (0.33-4.83)	0.73
Mix	179	22.9 (41)	0.62 (0.33-1.16)	0.14	6.15 (11)	0.64 (0.23-1.82)	0.41
Ever eaten raw meat							
Yes	21	38.1 (8)	1.92 (0.76-4.83)	0.17	14.3 (3)	2.19 (0.59-8.16)	0.24
No	255	24.3 (62)	*Reference*		7.1 (18)	*Reference*	
Regular deworming							
Yes	142	24.0 (34)	*Reference*		7.8 (11)	*Reference*	
No	130	26.2 (34)	1.13 (0.65-1.95)	0.67	6.2 (8)	0.78 (0.30-2.00)	0.61
Household ever been flooded							
Yes	54	22.2 (12)	0.77 (0.38-1.57)	0.48	5.6 (3)	0.69 (0.19-2.43)	0.56

(*continued on next page*)

Table 3 (*continued*)

Factor	n[§]	*G. duodenalis* % positive (n)	OR (95%CI)*	*P* value	*Cryptosporidium* spp. % positive (n)	OR (95%CI)*	*P* value
No	215	27.0 (58)	*Reference*		7.9 (17)	*Reference*	
Chronic diarrhea reported by owner							
Yes	17	58.8 (10)	4.61 (1.68-12.64)	< **0.01**	5.9 (1)	0.74 (0.09-5.90)	0.78
No	258	23.6 (61)	*Reference*		7.8 (20)	*Reference*	
Owners/Care givers Educational level							
Higher school and lower	45	31.1 (14)	2.11 (0.81-5.49)	0.13	11.1 (5)	2.00 (0.45-8.89)	0.36
Bachelor	139	28.8 (40)	1.89 (0.84-4.23)	0.12	8.6 (12)	1.51 (0.41-5.59)	0.54
Diploma	33	18.2 (6)	1.04 (0.33-3.24)	0.91	0 (0)	N/A	
Master&PhD	51	31.1 (14)	*Reference*		5.9 (3)	*Reference*	
Household income (Baht)							
< 10,000	44	25.0 (11)	0.98 (0.41-2.34)	0.97	9.1 (4)	1.08 (0.29-4.08)	0.91
10,000 – 25,000	64	25.0 (16)	0.98 (0.45-2.13)	0.96	3.1 (2)	0.35 (0.07-1.80)	0.21
25,001 – 50,000	79	21.5 (17)	0.81 (0.38-1.72)	0.58	7.6 (6)	0.89 (0.27-2.90)	0.85
> 50,000	71	25.4 (18)	*Reference*		8.5 (6)	*Reference*	

N/A = not applicable.

[§] In some factors, the total number is less than 301, due to lack of response on the questionnaire.

* OR (95% CI) = Odds ratio (95% confidence interval).

[a] Untreated water includes river, underground and well water.

results for *Giardia* and *Cryptosporidium* in dogs and cats are shown in Table 3 and Table 4, respectively. Variables with a p value of < 0.05 were bolded and regarded as associated with the infection. From the univariable analyses for dogs (Table 3), the candidate variables ($p \leq 0.1$) which were included in the multivariable analysis for *Giardia* positive test results in dogs were season, age, diarrhea status, presence of *Cystoisospora* oocysts, presence of *Cryptosporidium* oocysts, type of housing, number of animals in the household, defecating outside the immediate household area, reporting chronic diarrhea, and the source of drinking water. For the multivariable analysis of *Cryptosporidium* positive test results in dogs, age, diarrhea status, presence of *Giardia* cysts, source of drinking water, and type of housing were included. The variables remaining in the multivariable logistic regression model for *Giardia* positive test results were presence of *Cryptosporidium* oocysts, residing in shelter/breeder/temple, reporting chronic diarrhea, feces collected in rainy season, drinking untreated water, and having acute diarrhea (Table 5). The variables remaining in the multivariable logistic regression model for *Cryptosporidium* positive test results were presence of *Giardia* cysts and dogs age less than one year (Table 6). For cats, because there was a small sample size and there was collinearity between risk factors, multivariable logistic regression was not performed.

Seventy-six dog and 18 cat samples containing *Giardia* cysts were assayed in the *gdh*, *bg*, and *tpi* PCR assays. *Giardia* DNA were successfully amplified from 19 dog samples and 6 cat samples (Table 7). For *Cryptosporidium* genotyping, 23 dog and 8 cat samples were analyzed. Using 3 PCR assays, *hsp70*, one-step and nested for *SSU-rRNA* genes, PCR products were amplified from 10 dog and 3 cat samples (Table 8).

4. Discussion

This study was conducted to determine the prevalence of *Giardia* and *Cryptosporidium* infection, the risk factors associated with the infections as well as to determine the predominant assemblages/genotypes in dogs and cats visiting Chiang Mai University Small Animal Veterinary Teaching Hospital. Overall, *Giardia* cysts were commonly detected in both dogs and cats in the region. The prevalence of *Giardia* and *Cryptosporidium* detected in fecal samples can vary with the population sampled, season, and diagnostic techniques.

In our previous study (Tangtrongsup et al., 2017), the prevalence rates of *G. duodenalis* and *Cryptosporidium* spp. infection in dogs were 45.9% and 31.2%, respectively. In contrast, in the current study, performed approximately one year later, the estimated prevalence rates of *Giardia* and *Cryptosporidium* infection in dogs were 25.2% and 7.6%, respectively. The difference in *Giardia* estimated prevalence may be due to the fact that in the previous study, the majority of samples were collected from shelters/breeders (55%), a high-risk environment, whereas in the current study, 93.3% of dog samples and 80% of cat samples were from household pets. Secondly, in the previous study, fecal samples were collected only during the rainy season (July–August) which is now shown to be a period of higher prevalence.

In this study, the risk factors in dogs included a higher estimated prevalence of *Giardia* infection in the rainy season, in high-density settings, in settings where untreated water is consumed, co-infection with *Cryptosporidium*, and having acute or chronic diarrhea. However, having acute or chronic diarrhea most likely represents an outcome of infection, rather than a risk factor for initial infection. The factors associated with *Cryptosporidium* infection in dogs included co-infection with *Giardia* and age of less than one year. Previously reported risk factors associated with the *Giardia* and *Cryptosporidium* infection in dogs and cats have varied depending on the population studied. Similar to the results described here, *Giardia* or *Cryptosporidium* infection is usually associated with young age, having diarrhea or chronic diarrhea, the presence of other intestinal parasites, eating homemade food, living in a crowded setting, visiting dog parks, or being abandoned (i.e., stray animals) (Fontanarrosa et al., 2006; Vasilopulos et al., 2006; Rambozzi et al., 2007; Katagiri and Oliveira-Sequeira, 2008; Ballweber et al., 2009; Itoh et al., 2011; Wang et al., 2012). As mentioned above, the presence of *Cryptosporidium* infection was associated with *Giardia* infection and vice versa. This finding is probably a reflection of the high rate of *Giardia* and *Cryptosporidium* co-infection in both dogs and cats when compared to other types of co-infections (Tables 1 and 2).

The limitations of this study included a lack of sampling during the high rainfall months summer season, the small sample size for cats, and convenience sampling. A one-time fecal sampling instead of three days of sampling collection could lead to an underestimation of intestinal parasite detection. Inclusion of dogs with acute or chronic diarrhea may have led to selection bias and an over-estimation of prevalence. Nonetheless, the estimated prevalence of *Giardia* infection in dogs without diarrhea was 21% which indicates that this protozoan infection is common in Chiang Mai. However, the estimated prevalence rates of *Giardia* and *Cryptosporidium* infection in this hospital-based study, may not reflect that of the general population of dogs and cats in this province.

When using procedures that rely on flotation of intact parasite elements, it is generally recommended that freezing of the sample prior to analysis should be avoided (Zajac and Conboy, 2012), however, in this study, freezing were required because the fecal material needed to be stored for up to 8 months before international shipment. This

Table 4
Univariable logistic regression analysis for variables associated with *Giardia* and *Cryptosporidium* infections in cats in Chiang Mai, Thailand. (n = 66).

Factor	n[§]	*G. duodenalis* % positive (n)	OR (95%CI)*	*P* value	*Cryptosporidium* spp. % positive (n)	OR (95%CI)*	*P* value
Season							
Rainy	24	16.7 (4)	0.40 (0.11-1.40)	0.15	0 (0)		N/A
Winter	42	33.3 (14)	*Reference*		19.1 (8)		
Age							
< 1 year	20	15.0 (3)	0.33 (0.08-1.31)	0.12	15.0 (3)	1.24 (0.26-5.79)	0.79
1 – 7 years	40	35.0 (14)	*Reference*		12.5 (5)	*Reference*	
> 7 years	6	16.7 (1)	0.37 (0.04-3.50)	0.39	0 (0)	N/A	
Sex							
Male	38	23.7 (9)	0.66 (0.22-1.95)	0.45	5.3 (2)	0.20 (0.04-1.10)	0.06
Female	28	32.1 (9)	*Reference*		21.4 (6)	*Reference*	
Breed							
DSH	42	19.1 (8)	*Reference*		2.4 (1)	*Reference*	
Persian	16	62.5 (10)	7.08 (1.99-25.27)	< **0.01**	37.5 (6)	24.6 (2.65-228.09)	< **0.01**
Siamese	8	0 (0)	N/A		12.5 (1)	5.86 (0.33-104.90)	0.23
Diarrhea status							
Yes	11	36.4 (4)	1.67 (0.43-6.59)	0.46	9.1 (1)	0.69 (0.08-6.21)	0.74
No	55	25.5 (14)	*Reference*		12.7 (7)	*Reference*	
Spirometra eggs present							
Yes	3	33.3 (1)	1.35 (0.12-15.90)	0.81	0 (0)		N/A
No	63	27.0 (17)	*Reference*		12.7 (8)		
Cystoisospora oocysts present							
Yes	7	28.6 (2)	1.08 (0.19-6.11)	0.94	0 (0)		N/A
No	59	27.1 (16)	*Reference*		13.6 (8)		
Cryptosporidium oocysts present							
Yes	8	75.0 (6)	11.50 (2.06-64.34)	< **0.01**			
No	58	20.7 (12)	*Reference*				
Giardia cysts present							
Yes	18				33.3 (6)	11.50 (2.06-64.34)	< **0.01**
No	48				4.2 (2)	*Reference*	
Type of Housing							
Household	53	15.1 (8)	*Reference*		5.7 (3)	*Reference*	
Breeder	13	76.9 (10)	18.75 (4.21-83.48)	< **0.01**	38.5 (5)	10.42 (2.07-52.33)	< **0.01**
Multi-cat household							
Yes	37	43.2 (6)	5.33 (0.59-47.84)	0.14	16.22 (6)	0.58 (0.09-3.60)	0.56
No	8	12.5 (1)	*Reference*		25.0 (2)	*Reference*	
No of animals							
1	8	12.5 (1)	*Reference*		25 (2)	*Reference*	
2-4	11	0 (0)	N/A		0 (0)	N/A	
5-10	8	75.0 (6)	21.00 (1.50-293.25)	**0.02**	12.5 (1)	0.43 (0.03-5.98)	0.53
> 10	18	55. 0 (10)	8.75 (0.88-86.60)	0.06	27.8 (5)	1.15 (0.17-7.74)	0.88
Dog in the same house							
Yes	6	16.7 (1)	0.31 (0.03-2.92)	0.31	0 (0)		N/A
No	41	39.0 (16)	*Reference*		19.5 (8)		
Free roaming							
Yes	14	7.1 (1)	0.18 (0.02-1.70)	0.13	0 (0)		N/A
No	20	30.0 (6)	*Reference*		15.0 (3)		
Defecating area							
Inside household area	12	41.7 (5)		N/A	8.3 (1)	*Reference*	
Litter box	10	0 (0)			20.0 (2)	2.75 (0.21-35.8)	0.44
Outside household area	1	0 (0)			0 (0)	N/A	
Feces picked up immediately							
Yes	6	40.0 (4)	Reference		0 (0)		N/A
No	13	7.7 (1)	0.13 (0.01-1.38)	0.09	23.1 (3)		
Drinking water source							
Bottled/Filtered water	7	14.3 (1)	*Reference*		14.3 (1)	*Reference*	
Tap water	26	23.1 (6)	1.80 (0.18-18.05)	0.62	7.7 (2)	0.50 (0.04-6.48)	0.51
Untreated water[a]	1	0 (0)	N/A		0 (0)	N/A	
Food type							
Commercial	19	26.3 (5)	*Reference*		10.5 (2)	*Reference*	
Homemade	1	0 (0)	N/A		0 (0)	N/A	
Mix	14	14.3 (2)	0.47 (0.08-2.86)	0.41	7.14 (1)	0.65 (0.05-8.02)	0.74
Ever eaten raw meat							
Yes	2	50.0 (1)	4.17 (0.23-76.60)	0.34	0 (0)		N/A
No	31	19.4 (6)	*Reference*		9.7 (3)		
Regular deworming							
Yes	15	13.3 (1)	*Reference*		20.0 (3)		N/A
No	19	26.3 (5)	2.32 (0.38-14.12)	0.36	0 (0)		
Chronic diarrhea reported by owner							
Yes	6	33.3 (2)	2.30 (0.33-16.22)	0.43	0 (0)		N/A
No	28	17.9 (5)	*Reference*		10.7 (3)		
Owners/Care givers Educational level							
Higher school and lower	9	11.1 (1)	0.23 (0.02-2.30)	0.21	11.1 (1)	0.94 (0.07-12.00)	0.96
Bachelor	17	35.3 (6)	*Reference*		11.8 (2)	*Reference*	

(*continued on next page*)

Table 4 (*continued*)

Factor	n[§]	*G. duodenalis* % positive (n)	OR (95%CI)*	*P* value	*Cryptosporidium* spp. % positive (n)	OR (95%CI)*	*P* value
Master&PhD	7	0 (0)	N/A		0 (0)	N/A	
Household income (Baht)							
< 10,000	4	0 (0)	N/A		25.0 (1)	1.33 (0.09-20.71)	0.84
10,000 – 25,000	5	0 (0)	N/A		0 (0)	N/A	
25,001 – 50,000	10	60.0 (6)	19.5 (1.77 – 213.95)	**0.02**	20.0 (2)	*Reference*	
> 50,000	14	7.14 (1)	*Reference*		0 (0)	N/A	

N/A = not applicable.
[§] In some factors, the total number is less than 66, due to a lack of response on the questionnaire.
* OR (95%CI) = Odds ratio (95% confidence interval).
[a] Untreated water includes river, underground and well water.

Table 5
Multivariable logistic regression analysis of significant variables associated with *Giardia duodenalis* infection in dogs in Chiang Mai, Thailand (n = 231).

Variables	Odds Ratios	95%CI*	*P* value
Presence of *Cryptosporidium* oocysts	157.39	17.15-1,444.43	< 0.01
Shelter/Breeder/Temple	8.54	1.94-37.63	< 0.01
Reporting chronic diarrhea	5.85	1.52-22.53	0.01
In rainy season	5.16	1.77-15.03	< 0.01
Drinking untreated water[a]	3.22	1.16-8.96	0.03
Having acute diarrhea	2.68	1.15-6.26	0.02

* 95% Confidence interval.
[a] Untreated water includes river, underground and well water.

Table 6
Multivariable logistic regression analysis of significant variables associated with *Cryptosporidium* spp. infection in dogs in Chiang Mai, Thailand (n = 217).

Variables	Odds Ratios	95%CI*	*P* value
Presence of *Giardia* cysts	70.68	9.12-547.88	< 0.01
Age < 1 year	3.20	1.08-9.45	0.04

* 95% Confidence interval.

Table 7
Giardia duodenalis assemblages determined by nucleotide sequence analyses of glutamate dehydrogenase (*gdh*), beta-giardin (*bg*) and triosephosphate isomerase (*tpi*) PCR products from dog and cat samples in Chiang Mai, Thailand.

# of genes amplified	Species (n)	*gdh*	*bg*	*tpi*[a]
	Dog (19)			
1 (16)[b]	15	D	n/a	n/a
	1	n/a	n/a	C
2 (2)[b]	2	D	n/a	D
3 (1)	1	D	D	D
	Cat (6)			
1 (6)[c]	4	C	n/a	n/a
	1	D	n/a	n/a
	1	AI	n/a	n/a

n/a = no amplification.
[a] Includes *tpi*-generic and dog-specific primers.
[b] Number of animals that tested positive for the number of genes tested.

freezing step might have affected the detection of *Giardia* and *Cryptosporidium* in the IFA procedure, which has a concentration step utilizing a gradient sugar flotation. Erlandsen et al. (1990) showed that after three freeze-thaw cycles, a loss of approximately 22–27% of *G. muris* cysts in high-concentration fecal samples ($> 4.6 \times 10^5$/ml) and a 70–80% loss at lower cyst concentrations ($< 9 \times 10^4$/ml) occurred. If freezing disrupts the cyst or oocyst wall, detection could be hindered, particularly in those animals shedding few cysts/oocysts. Despite this shortcoming, the prevalence of *Cryptosporidium* in cats was similar to a previous study using the identical technique on unfrozen samples (Ballweber et al., 2009).

Table 8
Cryptosporidium species determined by nucleotide sequence analyses of heat shock protein 70 (*hsp70*), one-step and nested small subunit ribosomal RNA (*SSU-rRNA*) PCR products from dog and cat samples in Chiang Mai, Thailand.

# of positive PCRs	Species (n)	*hsp70*	One step *SSU-rRNA*	Nested PCR *SSU-rRNA*
	Dog (10)			
1 (6)[a]	5	n/a	*C. canis*	n/a
	1	n/a	*C. parvum*	n/a
2 (2)[a]	1	n/a	*C. canis*	*C. canis*
	1	*C. parvum*	*C. canis*	n/a
3 (2)[a]	2	*C. canis*	*C. canis*	*C. canis*
	Cat (3)			
1 (3)[a]	2	*C. parvum*	n/a	n/a
	1	n/a	n/a	*C. felis*

n/a = no amplification.
[a] Number of animals that tested positive for the number of PCR assays tested.

Giardia cysts and *Cryptosporidium* oocysts can survive for months in high humidity, low temperature, low exposure to sunlight and low salinity environments (Erickson and Ortega, 2006). Most of Thailand has a tropical wet and dry or savanna climate, in which some months of the year have heavier rainfall than others, and this condition could increase the survivability of *Giardia* cysts and *Cryptosporidium* oocysts. Thus, we hypothesized that the prevalence of *Giardia* and *Cryptosporidium* would be higher in the rainy season than in the drier winter months in part due to increased environmental survival of the pathogens. The hypothesis was confirmed in the multivariable analysis, in which the risk of *Giardia* infection was found to be 5.16 times higher in the rainy season compared to the drier winter. This seasonal difference in risk was not seen for *Cryptosporidium* infection. Interestingly, it has been reported that *Cryptosporidium* oocysts are sensitive to temperature (King et al., 2005). They can maintain infectivity for at least 3 months when stored between 4 °C and 15 °C, whereas 100% inactivation rate is reached when stored for 12 weeks and 8 weeks at 20 °C and 25 °C, respectively. This inactivation is due to the exhaustion of oocyst energy reserves at a higher temperature, resulting in the inability of sporozoites to initiate infection. In addition, the effect of low humidity on the survival of *Giardia* cysts and *Cryptosporidium* oocysts has been demonstrated (Olson et al., 1999). *Giardia* cysts stored at 25 °C in dry soil (17% humidity) survived for a shorter amount of time than those stored in water, 1 week compared to 2 weeks, respectively. This was also observed for *Cryptosporidium* oocyst survival. Oocysts stored in dry soil remained viable up to 4 weeks whereas those stored in water were viable up to 10 weeks (Olson et al., 1999). In Chiang Mai, the temperature is close to 25 °C with an average relative humidity of 77.9% in the rainy season and 68.6% during the winter season (Thai Meteorological Department: Northern Meteorological Center, 2018).

Therefore, the effect of environment on *Giardia* cyst and *Cryptosporidium* oocysts survival in Chiang Mai could be more likely due to warm temperature rather than low humidity. However, the failure to find a seasonal association with *Cryptosporidium* infection in dogs may be a result of the lack of the samples from higher rainfall periods (mid-May to July), a reflection of the sample size or assay sensitivity.

The presence of *Giardia* cysts and *Cryptosporidium* oocysts in feces of dogs and cats can be investigated using a light microscope after zinc sulfate centrifugation flotation, antigen detection, IFA, and PCR assays. Although there has no true gold standard for *Giardia* and *Cryptosporidium* detection, the IFA has the highest sensitivity and specificity and is considered as a gold standard test (Scorza and Tangtrongsup, 2010; Tangtrongsup and Scorza, 2010; Gotfred-Rasmussen et al., 2016; Uehlinger et al., 2017). Although conventional PCR is considered to be a highly sensitive method, it has been shown that PCR has lower sensitivity when compared to some other methods (Gotfred-Rasmussen et al., 2016; Uehlinger et al., 2017). Therefore, in this study, the PCR assays were performed only on the IFA positive samples. In a recent report, Uiterwijk et al. (2018) addressed underestimation or overestimation of various diagnostic tests. They employed a Bayesian latent class analysis to estimate the median sensitivity and specificity of a centrifugal sediment flotation (CSF), a direct immunofluorescence assay (DFA; Merifluor® *Cryptosporidium/Giardia* kit), a rapid enzyme immunochromatographic assay (IDEXX SNAP *Giardia*® test), and a quantitative PCR assay for the detection of *Giardia* in canine fecal samples. From this study, qPCR had highest median diagnostic sensitivity (97.0%), followed by DFA (78.6%), SNAP (71.9%), and CSF (48.2%). In contrast, the median diagnostic specificity was highest in SNAP (99.6%), followed by CSF (99.5%), DFA (96.5%), and qPCR (85.6%). The authors suggested that qPCR be used as a screening test for *Giardia* infection in canine fecal samples. However, DFA had the highest overall performance and can detect both *Giardia* and *Cryptosporidium* in a single test.

The genera *Giardia* and *Cryptosporidium* comprise host-adapted and zoonotic genotypes or species (Ballweber, 2015; Ryan and Hijjawi, 2015). *Giardia duodenalis* assemblage A and B have been detected in small numbers of dog and cat fecal samples. However, assemblage A can be further subdivided into 4 types, AI-AIV. AI is found more frequently in animals than humans. AII tends to be found more frequently in humans than animals. AIII tends to be found in wildlife and AIV has been found in sheep in China and humans in Sweden and Western Sahara (Ballweber, 2015). In the current study, *Giardia* isolates from dogs that could be amplified using PCR were the dog-adapted genotype assemblage C and D, whereas in cats, one of six *G. duodenalis* isolates was assemblage AI. The majority of *Cryptosporidium* isolates from dogs were *C. canis* (8/10). *Cryptosporidium parvum* was detected in two dogs; however, in one dog a different genotype of *C. canis* was also identified. Although these data could imply lack of zoonotic risk for these parasites, given that only 25% of *Giardia* isolates of dogs and 33% of cats, and 43.5% of *Cryptosporidium* isolates of dogs and 37.5% of cats were successfully amplified. In this study, an assessment of the potential for PCR inhibition was not performed. Therefore, it is possible that failure of PCR amplification could have occurred, and the role of dogs and cats as a potential source of zoonotic transmission for humans remains undetermined.

Cryptosporidium parvum, a zoonotic species, has been previously identified in household dogs (Giangaspero et al., 2006; Tangtrongsup et al., 2017; Gharieb et al., 2018). This zoonotic species was also identified in both dog and cat fecal samples in the current study. *Cryptosporidium parvum* is more commonly found in cattle or humans (Feng et al., 2018); thus the detection of *C. parvum*, in general, is likely to be from potential environmental contamination with feces of infected agriculture animals or humans. However, the two cats that harbored *C. parvum* one was from a breeding cattery and another one was from a household but has been purchased from a breeding cattery and both are indoor cats. Either environment contamination from agriculture animal or human hosts could not be determined. Although few in numbers, this finding also raises concern for potential zoonotic transmission of *C. parvum* from household pets. However, whether dogs or cats are efficient reservoir hosts for *C. parvum* has not been determined.

Although the current study was conducted one year later than our previous study (Tangtrongsup et al., 2017), young age, residing in high-density environments and having diarrhea were significant risk factors in both studies. Therefore, in a shelter – for example – the transmission of *Giardia* and *Cryptosporidium* in dogs may be reduced by limiting the exposure to diarrheic dogs. In addition, infected dogs should be separated from other animals and should be treated and bathed before moving to a new clean cage. A soiled cage or contaminated floor should be thoroughly cleaned followed by steam cleaning or disinfecting with quaternary ammonium compounds with a minimum contact time of 1 min (Tangtrongsup and Scorza, 2010). In addition, untreated water is not only a risk for these protozoal infections but also for other waterborne pathogens (Smith et al., 2006; O'Reilly et al., 2007; Baldursson and Karanis, 2011). Choosing safe, reliable water sources, and boiling or filtering untreated water (i.e., river water, underground and well water) before giving to animals is recommended.

5. Conclusion

Our study has shown that the *Giardia* infection in dogs and cats in Chiang Mai province is common, consistent with that seen elsewhere in the world. We have confirmed that *Giardia* infections are more prevalent in the rainy season than in the winter in dogs. We also showed that dogs having acute or chronic diarrhea, living in a high-density environment, and shedding *Cryptosporidium* oocysts are more likely to test positive for *Giardia* infection, and dogs less than one year old and dogs shedding *Giardia* cysts are more likely to test positive for *Cryptosporidium* infection. Although suggested by the finding of *C. parvum* in a dog and a cat, we could not confirm the role of the dogs and cats in zoonotic transmission of any of these parasites. Therefore, for more understanding of transmission cycles of these protozoa, molecular analyses of *Giardia* and *Cryptosporidium* isolates from dogs, cats, and their owners could allow us to establish the link or unlink the zoonotic transmission potential from dogs and cats.

Declaration of competing interest

The authors declare that there is no competing interest.

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