# *Prevalence and molecular characterization of* Cryptosporidium *spp. and* Giardia intestinalis *in household dogs and cats from Shiraz, Southwestern Iran*

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**Keywords**
*Cryptosporidium*,
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Dogs,
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Beta-giardin,
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**Summary**
In the present study, a total of 615 fecal samples from veterinary clinics were screened by microscopy for the presence of *Cryptosporidium* and *Giardia* oocysts. Molecular genotyping of *Cryptosporidium* and *Giardia* were carried out using PCR and sequence analysis. Overall, *Cryptosporidium* and *Giardia* oocysts were detected in the 0.6% (2/315) and 1.9% (6/315) of dogs and in the 0.7% (2/300) and 1.3% (4/300) of cats, respectively. Sequencing revealed the presence of *C. canis* (n = 2) in dogs and *C. felis* (n = 2) in cats. Moreover, *G. intestinalis* assemblage D (n = 2), C (n = 3) and A, sub-assemblage AII (n = 1) were identified in dogs; *G. intestinalis* assemblage F (n = 3) and assemblage A, sub-assemblage AI (n = 1) were identified in cats. The highest prevalence of *Giardia* was observed in dogs younger than one year (6/315), and in those with diarrhea ($p < 0.05$). Data of the study suggest that dogs and cats play a minor role in the zoonotic transmission of cryptosporidiosis and giardiasis in Southwestern Iran.

# *Caratterizzazione molecolare e potenziale zoonotico di* Cryptosporidium *spp. e* Giardia intestinalis *in cani e gatti domestici di Shiraz, sud-ovest dell'Iran*

**Parole chiave**
*Cryptosporidium*,
*G. intestinalis*,
Cani,
Gatti,
Genotipizzazione,
Beta-giardin,
SSU rRNA.

**Riassunto**
Nel presente studio 615 campioni fecali provenienti da cliniche veterinarie sono stati esaminati per la presenza di *Cryptosporidium* e *Giardia*. Sono state inoltre effettuate la genotipizzazione molecolare dei due microganismi mediante PCR e l'analisi di sequenza. Complessivamente, sono state rilevate oocisti di *Cryptosporidium* e *Giardia* rispettivamente nello 0,6% (2/315) e nell'1,9% (6/315) dei cani e nello 0,7% (2/300) e 1,3% (4/300) dei gatti. I dati molecolari hanno dimostrato la presenza di *C. canis* (n = 2) nei cani e *C. felis* (n = 2) nei gatti; inoltre nei cani sono stati evidenziati *G. intestinalis* assemblage D (n = 2), C (n = 3) e A, sub-assemblage AII (n = 1); *G. intestinalis* assemblage F (n = 3) e assemblage A, sub-assemblage AI (n = 1) nei gatti. La più alta prevalenza di *Giardia* è stata osservata nei cani di età inferiore a un anno (6/315) e in quelli con diarrea ($p < 0,05$).

## Introduction

Foodborne zoonotic pathogens are a serious public health issue and result in significant global economic losses. *Giardia* and *Cryptosporidium*, genera of common protozoan parasites that infect domestic and wild animals and humans, generally cause diarrhea. As for *Cryptosporidium*, the most common species that causes human infection are the *Cryptosporidium parvum* and *Cryptosporidium hominis*. They are divided into many species/genotype and subtypes using various molecular methods. These molecular tools are necessary for epidemiological purposes and understanding of the transmission of infection to humans and animals. Domestic dogs and cats are frequently infected by *C. canis* and *C. felis* (Itoh *et al.* 2014, Sotiriadou *et al.* 2013, Berahmat *et al.* 2017, Xiao 2010). Furthermore, *C. parvum* and *C. muris* are frequently reported in domestic dogs and cats, respectively (Pavlasek and Ryan 2007, Santín *et al.* 2006). *Giardia* is extremely common and is responsible for ~ 280 million human cases of diarrhoea every year (total giardiasis acquired by all transmission routes) and infects > 40 animal species (Horlock-Roberts *et al.* 2017). Currently eight species of *Giardia* are accepted as valid, including the recently described *Giardia cricetidarum* in hamsters and *Giardia peramelis* in bandicoots (Hillman *et al.* 2016, Lyu *et al.*2018).

*Giardia intestinalis* infects humans and is a species complex consisting of eight assemblages (A-H) (Ryan and Cacciò 2013). Assemblages A and B are the predominant assemblages in humans, but assemblages C, D, E and F have also been identified (Cacciò *et al.* 2017). Within Assemblage A, sub-assemblages AI, AII and AIII have been identified and of these AI and AII are commonly reported in humans and animals with sub-assemblage AIII reported in wild ruminants (Feng and Xiao 2011). Assemblages C-H, are generally the host-specific *Giardia* assemblages. Assemblages C and D are widespread in dogs and assemblage F is the prevalent assemblage in cats (Yang *et al.* 2015). Some researchers suggest that dogs and cats may play a role in zoonotic transmission of cryptosporidiosis and giardiasis (Berrilli *et al.* 2012, Pallant *et al.* 2015), while others reject this hypothesis (De Lucio *et al.* 2017, Rehbein *et al.* 2019). In Iran, like in other developing countries, cryptosporidiosis and giardiasis are a public health concern with socio-economic impact. Prevalence rates of *G. intestinalis* and *Cryptosporidium* have been reported in Iran. *G. intestinalis* was found in the 2.7% (12/450) of children in Behbahan (Kasaei *et al.* 2018) while Taghipour and colleagues (Taghipour *et al.* 2011) reported the 2.4% of *Cryptosporidium* prevalence in humans with diarrhea in Tehran. *C. parvum* (particularly subtype IIaA15G2R1) and *C. hominis* are the most common agents of cryptosporidiosis (Berahmat *et al.* 2017). *G. intestinalis* AII, BIII, and BIV are the most common assemblages identified in humans in Iran (Hooshyar *et al.* 2017, Kasaei *et al.* 2018). The frequency of *G. intestinalis* and *Cryptosporidium* in Iranian dogs and cats has been investigated mostly by microscopic methods. In a study conducted in dogs, in central Iran, *Cryptosporidium* oocysts were found in the 2.1% (3/140) in Isfahan (Ranjbar *et al.* 2017), in the 6% (4/77) in Mashhad (Beiromvand *et al.* 2013), and in the 0.4% (2/450) in Zanjan (Kohansal *et al.* 2017). *Cryptosporidium* was also detected in the 18% (9/50) of cats in Tehran (Mirzaghavami *et al.* 2016). Based on microscopic data, prevalence rates of *G. intestinalis* in dogs and cats range from 0.6% (1/147) to 1.6% (6/450) in dogs (Jafari Shoorijeh *et al.* 2008, Kohansal *et al.* 2017), and 10.7%, (15/140) to 18.9% (7/37) in cats (Bahrami *et al.* 2011, Khademvatan *et al.* 2014). Data upon molecular epidemiology and genetic diversity of *G. intestinalis* and *Cryptosporidium* in Iranian dogs and cats are scarce and poorly understood. Accordingly, the aim of this study was to evaluate the prevalence and molecular diversity of *Cryptosporidium* and *G. intestinalis* in household dogs and cats from Shiraz, Southwestern Iran.

## Material and methods

### Samples collection

The study was carried out from July 2017 to March 2018. Fecal samples were collected from 615 household dogs (n = 315) and cats (n = 300) referred to three veterinary clinics in Shiraz, the capital of Fars province, Iran. The majority of dogs (304/315) and cats (202/300) were asymptomatic. Metadata such as gender, breed, age, keeping conditions (indoor/outdoor), fecal consistency (diarrheic/non-diarrheic), and diet were recorded. A safe diet was defined when clean food (e.g. canned and/or packed and cooked food) and water were provided to the animal.

### Microscopic examination and sucrose flotation

All stool samples were screened for *Cryptosporidium* oocysts using modified acid-fast staining method. Furthermore, wet smears with saline and Lugol's iodine were prepared for all fecal samples for detection of *G. intestinalis* oocysts. As for purification of *Giardia* and *Cryptosporidium* oocysts, sucrose gradient flotation technique was performed as previously described (Lagapa *et al.* 2009, Asadpour *et al.* 2018). Then, the recovered

oocysts were washed 3 times (1,500 rpm for 15 min) with phosphate buffer saline (PBS) (1 M, pH = 7.4), kept in 2.5% potassium dichromate and stored at 4 °C until further use.

## DNA extraction

Total DNA was extracted using Stool Genomic DNA Extraction commercial kit (Bioneer, cat. no. K-3036, Daejeon, Korea) based on the manufacturer's procedure with some modifications as described earlier (Asadpour *et al.* 2018). Briefly, the recovered oocyst were washed three times with tap water (2,000 rpm, 12 min), the supernatant was removed, oocyst were supplemented with 400 µl of lysis buffer and 40 µl of proteinase K (Bioneer, cat. no. KB-0111, South Korea), mixed gently, and incubated at 65 °C overnight. Then, the supernatant was transferred to a fresh tube and centrifugated (6,000 rpm, 5 min). The remaining steps were accomplished according to the kit procedure. Extracted DNA (~ 150 µl) was kept at - 20 °C until further use.

## PCR for *Cryptosporidium*

*Cryptosporidium* -positive samples were genotyped by nested-PCR amplification of a 830-bp fragment of the DNA sequence coding for the *SSU* rRNA as described previously (Xiao *et al.* 2006). CryptF1 (5'-TTCTAGAGCTAATACATGCG-3') and CryptR1 (5'-CCCATTTCCTTCGAAACAGGA-3') in the first PCR, and CryptF2 (5'-GGAAGGGTTGTATTTATTAGATA-3') and CryptR2 (5'- CTCATAAGGTGCTGAAGGAGTA-3') for the second PCR reaction were used. In the present study we used a ready to use master mix (Taq DNA Polymerase Master Mix RED, Ampliqon, Denmark). Each 50 µl PCR tube contained 25 µl of master mix, 2 µl (20 pmol) of each forward and reverse primer (Bioneer, Daejeon, South Korea), 8 µl of DNA (~ 40 ng) was extracted as template, filled to 50 µl with distilled water. For amplification, a Bio-Rad thermocycler machine (Bio-Rad, CA, USA) was used with an initial denaturation at 95 °C for 4 min, followed by 30 cycles, consisting of denaturation at 94 °C for 40 s, annealing at 55 °C (for primary PCR) and 58 °C (for secondary PCR) for 45 s, extension at 72 °C for 60 s. A final extension at 72 °C for 7 min was included at the end of the amplification cycles. For positive and negative controls, a *C. parvum* isolate (https://www.ncbi.nlm.nih.gov/nuccore/KY410237) and sterile water were included in each reaction, respectively. In order to confirm the genotype, all secondary PCR-products were sequenced.

## PCR for *Giardia*

Two target genes were used for molecular detection and genotyping of *Giardia*. A 292-bp fragment of the DNA sequence coding for the *SSU* rRNA was amplified using RH4 (5'- AGTCGAACCCTGATTCTCCGCCAGG-3') and RH11 (5'-CATCCGGTCGATCCTGCC-3') (Hopkins *et al.* 1997). PCR amplification was performed with the following conditions: 25 µl of master mix, supplemented with 2µl (20 pmol) of each forward and reverse primer, 8 µl of DNA (~ 40 ng) and filled to 50 µl with distilled water. PCR program was set as follows: after a primary denaturation step at 96 °C for 10 min, 35 cycles were performed consisting of denaturation at 95 °C for 30 s, annealing at 65 °C for 35 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min. Moreover, a 753-bp fragment of the Beta-giardin (*bg*) locus was amplified using G7 (5'-AAGCCCGACGACCTCACCCGCAGTGC-3') and G759 (5'-GAGGCCGCCCTGGATCTTCGAGACG AC-3') primers as described previously (Caccio *et al.* 2002). PCR was performed in a reaction mixture containing 25 µl of master mix, supplemented with 2 µl (20 pmol) of each primer, 8 µl of DNA (~ 40 ng) as template and filled to 50 µl with distilled water. PCR program consisted of an initial denaturation at 96 °C for 5 min, followed by 30 cycles consisting of denaturation at 94 °C for 35 s, annealing at 58 °C for 40 s, extension at 72 °C for 60 s, and a final extension at 72 °C for 7 min. For positive and negative controls, consisting of a *G. intestinalis* assemblage A (isolated from humans) and sterile water were included in all amplifications, respectively. Analysis of PCR products was conducted by 1.5% agarose gel electrophoresis (Fermentas, USA) and visualization using a UV transilluminator.

## Purification of PCR products and sequencing analysis

PCR products were purified by gel excision (Vivantis Technologies, Selangor, Malaysia). The recovered products were sequenced by Sanger dideoxy sequencing technology. Obtained sequences data were aligned by CLC Main Workbench 6.0 software (CLC bio, Denmark) and Clustal W MEGA 6 software. Neighbor-joining method and bootstrap analysis over 1,500 replicates were used for reconstructing phylogenetic trees (Tamura *et al.* 2013).

## Statistical analysis

Statistical analysis was performed using SPSS version 21 software (SPSS Inc. Chicago, IL, USA). Pearson's chi-squared ($\chi^2$) for independence and Fisher's exact two-sided tests were conducted to evaluate association between infections and host factors including gender, breed, age, fecal consistency, keeping conditions, and diet. $p < 0.05$ was considered significant.

**Table I.** *Potential host factors associated with* Cryptosporidium *and* Giardia *infections in household dogs.*

| Potential host factors | | No. of screened dogs (%) (n = 315) | Positive for *Cryptosporidium* (*n* = 2) (%) | *P* value[#] | Positive for *Giardia* (*n* = 6) (%) | *P* value# |
|---|---|---|---|---|---|---|
| Age (years) | ≥ 1 | 143 (45.4) | 2 (100) | | 0 (0.00) | |
| | ≤ 1 | 172 (54.6) | 0 (0.00) | 0.503 | 6 (100) | 0.034[*] |
| Gender | Male | 157 (49.8) | 2 (100) | 0.248 | 3 (50) | |
| | Female | 158 (50.2) | 0 (0.00) | | 3 (50) | 0.655 |
| Breed | Terrier | 63 (20.0) | 1 (16.66) | | 2 (33.33) | |
| | Great Dane | 10 (3.2) | 0 (0.00) | | 0 (0.00) | |
| | Husky | 53 (16.8) | 0 (0.00) | | 1 (16.66) | |
| | German Shepherd | 62 (19.7) | 1 (16.66) | | 0 (0.00) | |
| | Dobermann | 85 (27.0) | 0 (0.00) | 0.801 | 2 (33.33) | 0.277 |
| | Shih Tzu | 34 (10.8) | 0 (0.00) | | 0 (0.00) | |
| | Pomeranian | 8 (2.5) | 0 (0.00) | | 1 (16.66) | |
| Fecal consistency | Diarrheic | 11(3.49) | 2 (100) | 0.069 | 6 (100) | < 0.001[*] |
| | Non-diarrheic | 304 (96.50) | 0 (0.00) | | 0 (0.00) | |
| Keeping condition | Indoor | 65 (20.63) | 1 (50) | | 1 (16.66) | |
| | Outdoor | 250 (79.36) | 1 (50) | 0.371 | 5 (83.33) | 0.105 |
| Unsafe Diet | Yes | 145 (46.03) | 2 (100) | 0.801 | 6 (100) | 0.127 |
| | No | 170 (53.96) | 0 (0.00) | | 0 (0.00) | |

[#] Statistical significance, $p < 0.05$. [*] The association was evaluated using Fisher exact test.

## Results

### Prevalence rates of *Cryptosporidium* and *G. intestinalis*

As shown in Table I and II, *Cryptosporidium* and *G. intestinalis* were detected in the 0.6% (2 of 315) and 1.9% (6 of 315) of dogs; and in the 0.7% (2 of 300) and 1.3% (4 of 300) of cats, respectively. Statistical analysis revealed a significant higher prevalence of *Giardia* in dogs younger than one year (6/315) ($p < 0.05$). Besides, a significant correlation resulted between *G. intestinalis* infection and diarrhea in dogs ($p = 0.001$). No association was evidenced between gender, breed or diet with the presence of *G. intestinalis* and *Cryptosporidium* in dogs ($p > 0.05$) (Tables I and II). In this study, all examined cats belonged to the same breed (Persian short hair).

## Molecular results

### Genotyping of *Cryptosporidium* isolates

Sequencing analysis revealed the presence of *C. canis* (n = 2) in dogs and *C. felis* (n = 2) in cats, respectively. Nucleotide sequences were deposited in GenBank under accession numbers MG888049.1-MG888051.1 and MG889862.1, as shown in Table III. Figure 1 shows the phylogenetic relatedness of the samples of this study. Phylogenetic analysis showed that *C. canis* and the two *C. felis* isolates grouped in specific clusters.

**Table II.** *Potential host factors associated with* Cryptosporidium *and* Giardia *infections in household cats.*

| Potential host factors | | No. of screened cats (%) (n = 300)[a] | Positive for *Cryptosporidium* (*n* = 2) (%) | *P* value[b] | Positive for *Giardia* (*n* = 4) (%) | *P* value[b] |
|---|---|---|---|---|---|---|
| Age (years) | ≥ 1 | 172 (57.3) | 1 (50.0) | 0.672 | 4 (100.0) | 0.139 |
| | ≤ 1 | 128 (42.7) | 1 (50.0) | | 0.0 (0.00) | |
| Gender | Male | 174 (58.0) | 1 (50.0) | 0.664 | 2 (50.0) | 0.142 |
| | Female | 126 (42.0) | 1 (50.0) | | 2 (50.0) | |
| Fecal consistency | Diarrheic | 98 (32.7) | 2 (100.0) | | 2 (50.0) | |
| | Non-diarrheic | 202 (67.3) | 0.0 (0.00) | 0.106 | 2 (50.0) | 0.599 |
| Keeping condition | Indoor | 55 (18.3) | 0.00 (0.00) | | 2 (50.0) | |
| | Outdoor | 245 (81.7) | 2 (100.0) | 0.666 | 2 (50.0) | 0.155 |
| Unsafe Diet | Yes | 258 (86.0) | 2 (100.0) | 0.843 | 4 (100.0) | 0.099 |
| | No | 42 (14.0) | 0.0 (0.00) | | 0.0 (0.00) | |

[a] All screened cats were Persian short hire breed. Statistical significance, $p < 0.05$. [b] The association was evaluated using Fisher exact test.

**Table III.** Cryptosporidium *positive samples identified in household dogs and cats by nested-PCR of the SSUrRNA coding gene.*

| Sample name | Host* | Species | GenBank Accession No. |
|---|---|---|---|
| 24 | Cat | *C. felis* | MG888051.1 |
| 111 | Dog | *C. canis* | MG888050.1 |
| 216 | Dog | *C. canis* | MG888049.1 |
| 218 | Cat | *C. felis* | MG889862.1 |

**Cryptosporidium* were detected in 2 dogs and 2 cats.

## Genotyping of *Giardia* isolates

DNA sequence analysis revealed the presence of *G. intestinalis* assemblages C (3/6), D (2/6) and sub-assemblage AII (1/6) in dogs. *G. intestinalis* assemblage F (3/4), and sub-assemblage AI (1/4) were identified in cats. Nucleotide sequence data were deposited in GenBank (Table IV). Phylogeny is depicted in Figure 2.

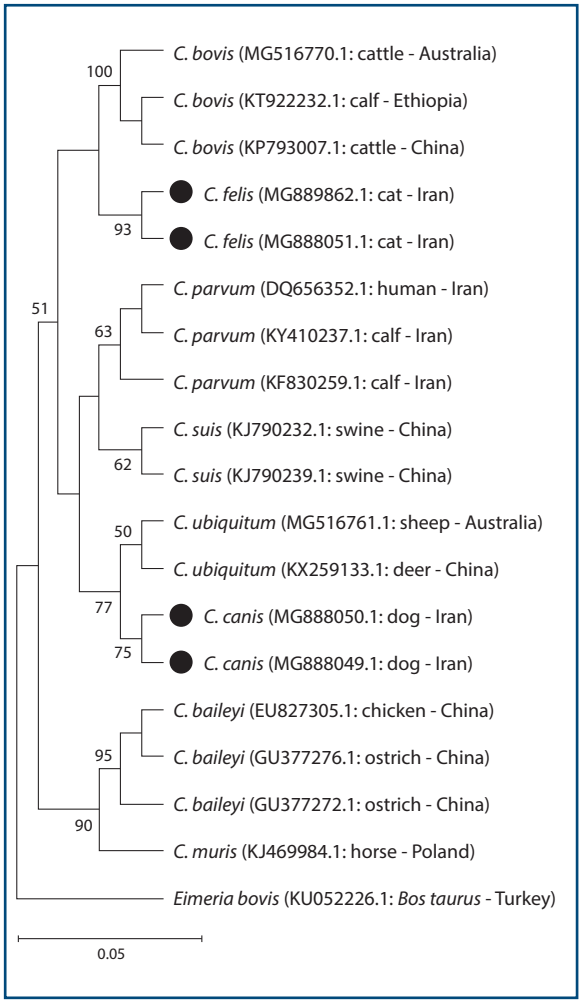


**Figure 1.** *Neighbor-joining (NJ) tree based on the* SSU *rRNA coding sequences.* Black dots represent the *Cryptosporidium* species detected in household dogs and cats of this study (Shiraz, Southwestern Iran).

## *Discussion*

In this study, a total of 615 stool samples were collected from household dogs and cats and screened for detection of *Cryptosporidium* and *Giardia* (oo) cysts. Moreover, PCR methods using different targets genes were used to determine the species/genotype/assemblage of *Cryptosporidium* and *G. intestinalis* detected in this study. The prevalence rates of *Cryptosporidium* in household dogs and cats in this study was lower compared to those reported previously (Mirzaghavami *et al.* 2016,

**Table IV.** Giardia intestinalis *identified in dogs and cats by PCR of the 16srRNA gene.*

| Sample name | Host* | Assemblage | GenBank Accession No. |
|---|---|---|---|
| 1 | Dog | D | MG851692.1 |
| 2 | Cat | F | MG832845.1 |
| 3 | Cat | A | MG832842.1 |
| 4 | Cat | F | MG832844.1 |
| 11 | Cat | F | MG832843.1 |
| 36 | Dog | C | MG851690.1 |
| 113 | Dog | D | MG851691.1 |
| 192 | Dog | C | MG851695.1 |
| 226 | Dog | C | MG851693.1 |
| 306 | Dog | A | MG851694.1 |

**Giardia* was detected in 6 dogs and 4 cats.

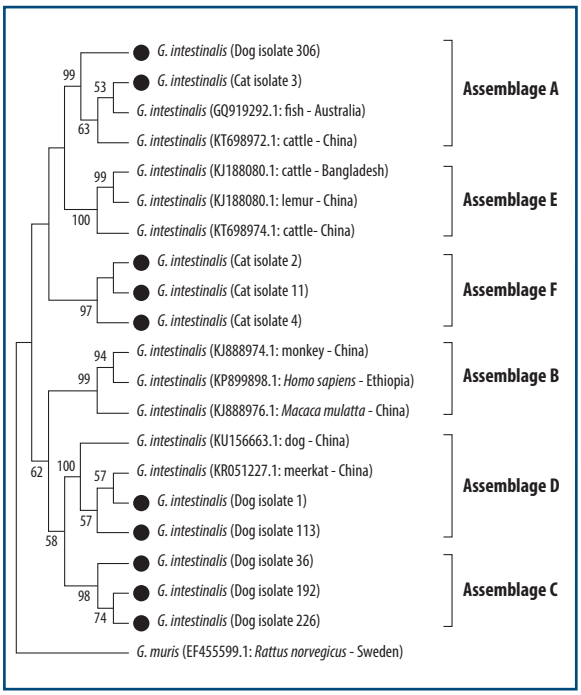


**Figure 2.** *Neighbor-joining (NJ) tree based on b-giardin coding sequences of* G. intestinalis *assemblages.* Black dots represent *G. intestinalis* assemblages from dogs and cats of this study (Shiraz, Southwestern Iran).

Ranjbar *et al.* 2017, Beiromvand *et al.* 2013). This study indicates *C. canis* and *C. felis* as the prevalent species detected in dogs and cats, respectively, in this area of Iran. This finding is in line with other studies (Ranjbar *et al.* 2017, Neves *et al.* 2014, Pallant *et al.* 2015, Yang *et al.* 2015). The overall prevalence of *G. intestinalis* in dogs (1.9%) was higher than that of previous studies (0/6%) (Shoorijeh *et al.* 2008). *G. intestinalis* was detected in the 1.3% of sampled cats. These rates were also lower compared to previous studies (Bahrami *et al.* 2011, Khademvatan *et al.* 2017). Molecular data revealed that household dogs were mostly infected with *G. intestinalis* host-specific assemblages C and D. It is well established that *G. intestinalis* assemblages C and D are the most prevalent assemblages in dogs (Ryan and Cacciò 2013, Sotiriadou *et al.* 2013, Uehlinger *et al.* 2013) while several studies, including this one, indicates also assemblage F capable of infecting cats (Cacciò *et al.* 2005, Santín *et al.* 2006, Yang *et al.* 2015). Overall, data obtained in the present study showed that household dogs and cats likely play a minor role in zoonotic transmission of these parasites, at least in the areas under investigation.

## Conclusions

The overall prevalence of *Cryptosporidium* and *Giardia* in household dogs and cats was low. Further studies using multiple target genes are recommended in different geographical areas of Iran to provide a better understanding of the epidemiology of these two parasites.

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