ORIGINAL ARTICLE

Veterinary Research Forum. 2023; 14 (6) 335 - 340
doi: 10.30466/vrf.2022.545726.3336
Journal Homepage: vrf.iranjournals.ir

Veterinary Research Forum

Frequency and genotyping of *Giardia duodenalis* in dogs of Urmia, northwest of Iran

Reza Esmailzadeh[1], Farnaz Malekifard[1]*, Alaleh Rakhshanpour[2], Mousa Tavassoli[1]

[1] Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran; [2] Department of Internal Medicine and Clinical Pathology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran.

Article Info

Article history:

Received: 28 December 2021
Accepted: 13 March 2022
Available online: 15 June 2023

Keywords:

Dogs
Frequency
Genotyping
Giardia duodenalis
Iran

Abstract

Giardia duodenalis is a zoonotic protozoan infecting various vertebrates such as humans and domestic animals. The aim of this study was to determine the frequency and genotypes of *G. duodenalis* using polymerase chain reaction-restriction fragment length polymorphism (PCR-RFLP) in dogs of Urmia, Iran. Overall, 246 stool specimens were collected from 100 pet, 49 stray, and 97 shelter dogs in the Urmia, Iran. Totally, seven samples (2.48%) were microscopically positive in terms of *Giardia* cyst. The PCR-RFLP analysis revealed that three (1.21%) and two (0.83%) samples have the C and D genotypes, respectively. In addition, two samples (0.83%) were belonged to the AI sub-group. A significant association was determined between the frequency of *Giardia* infection and life style, age, and stool form of dogs. The findings of the study showed the high frequency of *Giardia* infection in stray dogs and the dogs under one-year-old. Furthermore, the C and D genotypes of *G. duodenalis* were predominant in dogs of Urmia, Iran.

Introduction

Giardia duodenalis (also known as *G. intestinalis* or *G. lamblia*) is a protozoan parasite infecting human, and domestic and wild animals.[1] Giardiosis results from ingesting cysts, which are transmitted through various sources such as contaminated water and food.[2] There are reports regarding more than 280 million human cases of diarrhea caused by *Giardia* every year, and the *Giardia* infection has been noticed in more than 40 animal species all over the world.[3] A former study has showed the significant role of dogs in *Giardia* transmission to human beings;[4] however, this result was rejected by another study.[5]

The molecular genetic analysis of the conserved genes loci has shown that *G. duodenalis* parasite has at least seven major genotypes (A-H).[6] Two genotypes of A and B are detected in different mammalian hosts and human beings; therefore, they are considered as genotypes with the highest zoonotic potential for public health.[7] Genotypes of C and D have found only in dog isolates, E genotype infects hoofed livestock, F genotype infects cats, G genotype has the potential to infect rats, and H genotype has been found in marine mammals.[8,9] The A genotype consists of isolates being classified into two clusters with distinct features. The AI includes a combination of closely related human and animal isolates being widespread in various parts of the world; most attention conducted on the zoonotic potential of the AI sub-group.[9,10] However, the second sub-group (AII) is composed of human isolates. Furthermore, B genotype has two sub-groups named III and IV; IV sub-group seems to be specific for the humans.[1,6]

Direct wet-mount, staining, and concentration are parasitological procedures used for detecting *Giardia.*[11,12] Furthermore, different polymerase chain reaction (PCR)-based molecular genetics methods have been used to detect *G. duodenalis* cysts in the feces of isolates originating from humans and animals. Several researchers have used the PCR- restriction fragment length polymorphism (RFLP) method to distinguish *Giardia* genotypes related to human and animals.[1,13-15] Genotypes of *G. duodenalis* are based on the analysis of various parameters including small sub-unit ribosomal RNA, triosephosphate isomerise, glutamate dehydrogenase (*gdh*), elongation factor 1-alpha, β-giardin, and variant surface protein genes.[16,17] The *gdh* gene is proven to be useful for determining the genotype of *Giardia* isolated from mammalian species.[14]

***Correspondence:**

Farnaz Malekifard. DVM, PhD
Department of Pathobiology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran
E-mail: f.malekifard@urmia.ac.ir

Several epidemiological studies in Iran have investigated the zoonotic intestinal parasites in canines.[2,18,19] Recently, in a study by Homayouni *et al*.,[20] D, C and A assemblages of *G. intestinalis* having sub-assemblage of AII were identified in dogs of Shiraz, southwestern Iran. However, there are limited data about the *G. duodenalis* assemblages distributions in dogs residing in Urmia, Iran. Hence, this study was designed to determine the frequency and genotypes of *G. duodenalis* isolates in dogs in Urmia, northwestern Iran, to evaluate these pathogens zoonotic potential.

Materials and Methods

Chemicals. All chemical compounds used in the present study were obtained from Sigma-Aldrich Company (St. Louis, USA).

Study area. The study was carried out from July 2018 to March 2019 in Urmia, West Azerbaijan province, northwestern Iran. The region of the study is an agriculturally fertile area being located between 37° 32′ N and 45° 04′ E, and the area is estimated to be around 8,000 km^2. The temperature of the study area varies from – 3.80 ˚C to 23.40 ˚C during different seasons. The area is bordered with Iraq and Türkiye.[21]

Collection of Samples. For this study, a total of 246 dogs including 49 strays, 97 shelter, and 100 pet dogs were selected randomly. The majority of sampled dogs (228/246) had no symptoms. Various parameters including age, gender, keeping conditions (indoor/outdoor), and fecal consistency (diarrheic/non-diarrheic) of all selected dogs were recorded. An individual disposable latex glove was used to collect fecal samples from the rectum of all dogs. The collected samples were placed in plastic specimen cups equipped with a screw-on lid, labeled and sent to the Parasitology Laboratory of Faculty of Veterinary Medicine, Urmia University, Urmia, Iran, within 2 hr after collection. The samples were stored at 4.00 ˚C and examined within 24 hr. Sucrose flotation was utilized to isolate and partially purify the *Giardia* cysts.[22,23] The semi-filtered and concentrated cysts were kept in sterile distilled water without adding any preservatives for up to two weeks at – 20.00 ˚C. Ethical approval for this study was obtained from Veterinary Ethic Committee of Urmia University, Urmia, Iran (Approval Number: IRR-UU-AEC-3-89-AD).

DNA extraction. The cyst wall was disrupted in fecal samples with freeze-thaw or glass beads. The following procedure was used to extract DNA by utilizing phenol-chloroform-isoamylalcohol (PCI) on purified and concentrated fecal samples by sucrose gradient and sedimentation of ethyl acetate samples. In the first step, 200 µL of sucrose gradient or sediment concentrated cysts sample was mixed with 200 µL 3.00% Triton X100 and incubated in a water bath for 1 hr at 75.00 ˚C. Next, 200 µL of lysis buffer and 10.00 µL of proteinase K were mixed with 200 µL of homogenate and incubated overnight at 37.00 ˚C. Genomic DNA was extracted from the cysts by PCI method. Then, PCI was mixed with the solution prior to centrifugation (Eppendorf, Hamburg, Germany) at 15,000 rpm for 10 min at 25.00 ˚C. Ethanol absolute (two or four equals) was used to precipitate the supernatant in a new tube for 24 hr at – 20.00 ˚C. The resulting solution was centrifuged at 15,000 rpm for 10 min at a temperature of 4.00 ˚C. The sediment was air-dried, and 100 mL deionized distilled water was added to it before storing at – 20.00 ˚C until PCR analysis.[24]

Polymerase chain reaction amplification. The *gdh* genes were amplified by a single PCR. Amplification of the 432 bp fragment during the PCR was done using the forward primer (GDHiF), 5′-CAGTACAACTCTGCTCTCGG-3' and the reverse primer (GDHiR), 5′-GTTGTCCTTGCA CATCTCC-3'.[24] For this study, the amplification reaction was modified as follows: PCR mix had 1X buffer containing 1.50 mM $MgCl_2$, each deoxynucleotide triphosphate at the concentration of 100 µM, each primer at a concentration of 0.50 µM, 10.00 ng of DNA, and 2.50 U of Taq DNA polymerase (Sinaclon, Tehran, Iran). Cycling parameters were 10 min at 94.00 ˚C (initial heat activation step), followed by 30 cycles of 35 sec at 94.00 ˚C, 35 sec at 61.00 ˚C, and 50 sec at 72.00 ˚C, with a final extension of 7 min at 72.00 ˚C.[24] To validate the results, negative and positive controls were included in the PCR analysis. Cysts and distilled water were used as templates for positive and negative controls, respectively.

Restriction fragment length polymorphism of the *gdh* gene. The RFLP was conducted on all specimens being PCR-positive. Furthermore, RFLP was used for genotyping *G. duodenalis* according to the method described previously.[1] The RFLP analysis was conducted by digesting 8.00 µL of PCR products with 1.50 U 0.80 U of *NIaIV* enzyme (Vivantis, Shah Alam, Malaysia) in 2.00 µL of 10X enzyme buffer in a final volume of 20.00 µL for 3 hr at 37.00 ˚C.[1] Horizontal electrophoresis in 1.50 and 2.00% agarose gels and Ethidium Bromide were respectively used for the separation of PCR products and restriction fragments. Table 1 shows the expected fragments following digestion by specific restriction *NlaIV* enzymes.

Statistical analysis. The SPSS software (version 26.0; IBM Corp., Armonk, USA) was used to analyze the results. The association between the frequency of *Giardia* infection and various variables such as age, sex, and fecal consistency was analyzed by Chi-square test; values less than 0.05 were considered significant ($p < 0.05$).

Table 1. Restriction fragment length polymorphism profiles of *Giardia duodenalis* assemblages after digesting with *Nla*IV.

Assemblages of *G. duodenalis*	Diagnostic genotyping profile
AI	90, 120, 150
AII	70, 80, 90, 120
C	120, 70, 190
D	120, 250

Results

Of 246 fecal samples, the *Giardia* cysts were microscopically diagnosed in seven samples (2.84%), and GDHiF and GDHiR primers were used to amplify the *gdh* gene in the PCR as shown in Figure 1A. At the Table 2, the frequency of *G. duodenalis* in association with age, sex, fecal consistency, and keeping conditions is shown. As shown in Table 2, the frequency of *Giardia* infection was significantly higher in dogs less than one-year-old and stray and diarrheic dogs than other dogs ($p < 0.05$). The PCR-RFLP analysis showed that the AI, C and D genotypes were detected in PCR products samples (Fig. 1B).

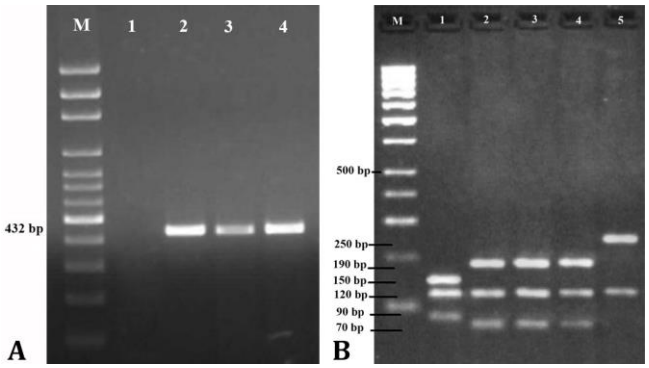

Fig. 1. A) Electrophoretic separation of polymerase chain reaction (PCR) product from DNA amplified at the *gdh* locus of *Giardia duodenalis* on an Ethidium Bromide stained 1.50% agarose gel. Lane M: 100 bp gene ruler (fermentase); Lane 1: Negative control; Lane 2: Positive control; Lanes 3 and 4: PCR products from examined samples (432 bp fragment). **B)** The *NlaIV* digestion of polymerase chain reaction products on an Ethidium Bromide stained 2.00% high resolution agarose gel. Lane M: 100 bp gene ruler (fermentase); Lane1: AI assemblage of *Giardia duodenalis*; Lanes 2-4: C assemblage of *G. duodenalis*; Lane 5: D assemblage of *G. duodenalis*.

Discussion

The current study identified *G. duodenalis* cyst in 2.84% of the fecal samples using microscopic evaluation. Some other studies conducted in Iran have used microscopical examination; but, they have reported a lower prevalence of *G. duodenalis*. These studies found 0.07% *G. duodenalis* in 98 stray dogs and 0.09% *G. duodenalis* infection in 100 samples.[25,26] Fazaeli *et al*. have conducted a study in Zanjan, Iran, and reported an infection rate of 1.60% in stray dogs.[2] The *G. duodenalis* infection rate in dogs reported in different studies in various geographical locations after fecal examination ranged from 0.09 to 28.00%.[27-30] The different results may be due to several factors including the type of climate, life style of dogs, and applied diagnostic methods.[10,30] Diarrhea or loose stool is a significant symptom of giardiosis.[31] Our study results showed that the dogs suffering from diarrhea had a higher prevalence of *G. duodenalis* compared to healthy ones, and this result was consistent with that of Shin *et al*.[32] Uiterwijk *et al*.[31] have showed that dogs diagnosed as positives for *Giardia* have loose stool and shed more cysts. Our study also added the young age as a risk factor for the *G. duodenalis* transmission in dogs, being consistent with the results obtained by other studies in different geographical areas. Gender did not have a significant role in the prevalence of *Giardia* infection in the present study, and this result is in line with other studies conducted in Korea, Brazil, and Australia.[32-34]

Giardiosis is considered as an infectious zoonotic disease that can be transmitted from infected dogs, cats, sheep, and cattle to human. Although A and B assemblages are the genotypes known to be zoonotic, more rarely human cases have been identified with C, D, and F assemblages.[35-38] The zoonotic genotypes (A and B) and the host-related, non-zoonotic genotypes (C and D) lead to the dogs infection.[39]

Determination of *G. duodenalis* genotype is a way to determine the infection epidemiology and implement preventive measures.[14] It is noteworthy to mention that *gdh* locus is stable in the isolates taken from various species and identified in different geographical locations. It is the reason of its application to differentiate *G. duodenalis* assemblages and their genetic diversity.[16,40-43] Genotyping of *Giardia* is mainly done by PCR-RFLP due to its simple, rapid, and sensitive nature.[25,44,45] It is also the best tool available for researchers to detect mixed genotypes.[1,15]

Most of samples in this study were C and D assemblages of *G. duodenalis*. Various other studies in different geographical areas including Hungary, Brazil, China, and Japan have showed that C and D genotypes have the highest prevalence.[46-48] It should also be noted that the fecal origin can influence the outcome of genotyping. The results obtained from this study indicated that stray dogs had a higher infection rate compared to the pet and shelter dogs. The results of a previous study being conducted on fecal samples from stray dogs have showed that the close contact between stray dogs living together is a reason for the transmission of dog-specific genotypes and the higher prevalence of *G. duodenalis* infection in stray dogs.[2]

Table 2. Frequency of *Giardia duodenalis* infection in dogs of Urmia, Iran.

Results	lifestyle			Age		Gender		Fecal *consistency*	
	Stray	Shelter	Pet	<1 yr	≥1 yr	Female	Male	Diarrheic	Non-diarrheic
Negative	45	95	99	82	157	102	137	13	226
Positive (%)	4(8.16)	2(2.06)	1(1.00)	5(5.74)	2(1.25)	4(3.77)	3(2.14)	5(27.77)	2(0.87)
Total number of dogs	49	97	100	87	159	106	140	18	228
***p* value**	$X^2 = 6.46$; $p = 0.040$			$X^2 = 4.09$; $p = 0.043$		$X^2 = 0.58$; $p = 0.446$		$X^2 = 43.66$; $p = 0.000$	

In this study, AI zoonotic genotype was detected in positive animals. Previous studies have showed the prevalence of zoonotic genotypes in Australia, Thailand, and Belgium.[49] A study in China conducted by Zheng *et al*.[47] has showed that most dogs in this region are infected with the A zoonotic assemblage. A study done in Japan has also showed that out of 24 fecal samples analyzed, 14 are belonged to the A assemblage and three of the samples have the sequences related to both A and D assemblages.[15] Transmission of zoonotic assemblages was studied by Adell-Aledón *et al*.[50] in Spain, and the results reported A and B zoonotic assemblages as well as C and D host-specific assemblages. The results of a study conducted by Shin *et al*.[32] have showed an assemblage of *G. intestinalis* in fecal samples collected from dogs in Korea. They have also noted the potential of *Giardia* transmission from dogs to humans and vice versa.

In conclusion, as far as we are concerned, this is the first molecular study regarding *Giardia* genotyping of dogs conducted in this region. The results of this analysis showed that C and D genotypes of *G. duodenalis* had the highest prevalence in this region. It should also be noted that A zoonotic genotype was reported in some cases. More studies are needed in other endemic regions of Iran to assess the effects of *Giardia* infection in dogs on public health.

Acknowledgments

The present work was extracted from the Doctor of Veterinary Medicine thesis of Dr. Reza Esmailzadeh conducted at Urmia University, Urmia, Iran. The authors would like to sincerely thank the members of the Faculty of Veterinary Medicine and Urmia University Research Council for the approval and support of this research.

Conflict of interest

None of the authors have any conflict of interest to declare.

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