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Molecular detection and identification of *Giardia duodenalis* in cattle of Urmia, northwest of Iran

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Abstract

Giardia duodenalis is one of the most prevalent intestinal protozoa infecting humans and domestic animals. The aim of this study was to identify subspecies of *G. duodenalis* by polymerase chain reaction and restriction fragment length polymorphism (PCR-RFLP) method from fecal samples of naturally infected cattle in the Urmia, West Azerbaijan province, Iran. Overall, 246 fecal specimens were collected from the cattle (diarrheic and healthy) and microscopically examined for *G. duodenalis.* The PCR-RFLP analysis of glutamate dehydrogenase *(gdh)* locus was used to identify the genotypes found in cattle. In this method, 432 bp expected size was amplified and then specific restriction *Nla*IV enzyme was used for subspecies detection. Totally, 23 (9.34%) specimens were microscopically positive for giardia cyst out of 246 examined samples. The PCR-RFLP analysis revealed that 19 samples (82.60%) have the genotype E and 4 samples (17.39%) belong to the subgroup AI. Our findings indicated that *G. duodenalis* infection is prevalent in cattle of Urmia and the non-zoonotic genotype E predominates in cattle in this region.

تشخیص مولکولی و شناسایی *ژیاردیا دئودنالیس* در گاوهای شهرستان ارومیه، شمال غرب ایران

چکیده

ژیاردیا دئودنالیس یکی از شایع ترین تک یاخته های روده ای است که موجب آلوده شدن انسان و حیوانات اهلی می شود. هدف از این مطالعه تعیین تحت گونه های *ژیاردیا دئودنالیس* با استفاده از واکنش زنجیره ای پلیمراز چند شکلی طول قطعه محدود (PCR-RFLP) از نمونه های مدفوع گاوهای آلوده در شهرستان ارومیه، استان آذربایجان غربی، ایران بود. در مجموع، ۲۴۶ نمونه مدفوع از گاوهای سالم و مبتلا به اسهال جمع آوری شد و به صورت ریزبینی از جهت *ژیاردیا دئودنالیس* موردبررسی قرار گرفت. بررسی PCR-RFLP ژن گلوتامات دهیدروژنازبرای تشخیص ژنوتیپ های گاو مورد استفاده قرار گرفت. در این روش، قطعه ای به اندازه ۴۳۲ جفت بازتکثیر یافت وسپس برای تشخیص تحت گونه ها آنزیم محدود کننده اختصاصی *Nla*IV مورد استفاده قرار گرفت. در مجموع، ۲۳ (۹/۳۴ درصد) نمونه از ۲۴۶ نمونه در بررسی میکروسکوپی از لحاظ کیست *ژیاردیا* مثبت بودند. بررسی PCR-RFLP نشان داد که ۱۹ نمونه (۸۲/۶۰ درصد) دارای ژنوتیپ E و چهار نمونه (۱۷/۳۹ درصد) متعلق به زیرگروه AI می باشند. یافته های ما نشان داد که آلودگی با *ژیاردیا دئودنالیس* در گاوهای شهرستان ارومیه شایع می باشد و ژنوتیپ E غیر مشترک بین انسان و دام در گاوهای این منطقه غالب است.

واژه های کلیدی: ایران، *ژیاردیا دئودنالیس*، گاو، گلوتامات دهیدروژناز، واکنش زنجیره ای پلیمراز چند شکلی طول قطعه محدود

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Introduction

Giardia duodenalis (syn *Giardia intestinalis*) is an important protozoan parasite infecting a wide range of vertebrates including humans and domestic animals.[1] It has been found in the feces of calves, beefs and dairy cattle worldwide.[2-5] Giardiasis clinical manifestations in cattle are relatively variable, ranging from the absence of symptoms to persistent diarrhea, mucoid and fatty stool, weight loss and growth rate reduction.[6] Cattle have been considered as potential sources of giardiasis in humans through direct contact and/or surface water supplies contamination.[7-10]

G. duodenalis is now considered as a species complex comprising at least seven major genotypes (A–G).[11] The molecular analysis of cattle isolates from different geographical locations has demonstrated that only *G. duodenalis* genotype E and the zoonotic genotypes (A and B) are associated with cattle infections.[9,12] Recently, molecular techniques have been applied for *G. duodenalis* detection and genotyping in animals and humans.[13-15] The use of molecular diagnostic techniques in the genotypic specifying of *G. duodenalis* has led to increased recognition of the diversity of parasites infecting humans and animals and role of animals in the transmission of human giardiasis.[16] Specifying of *G. duodenalis* genotypes has performed based on the characterization of the small subunit ribosomal RNA (SSU-rRNA), β-giardin (*bg*), glutamate dehydrogenase (*gdh*) and triose phosphate isomerise (*tpi*) genes.[17,18] The *gdh* gene is useful for genotypic analysis of *G. duodenalis* parasites from mammals.[19]

In Iran, although giardiasis has been reported in cattle in some regions, but there is not any data about *G. duodenalis* genotypes in cattle. Therefore, the main objective of the current study was to determine the genotypes of *G. duodenalis* isolates from cattle in Urmia, northwest of Iran using polymerase chain reaction and restriction fragment length poly-morphism (PCR-RFLP) assay on the organism's glutamate dehydrogenase (*gdh*) gene.

Materials and Methods

Study area and sample collection. This study was performed from February to September 2015 in Urmia, in northwest of Iran. Cattle fecal samples were collected from the rectum of each animal using an individual disposable latex glove. Each sample was placed in a plastic specimen cup with a screw-on lid, labeled and transported to the to the Laboratory of Parasitology, Faculty of Veterinary Medicine, Urmia University, Urmia, Iran within 2 hr after collection. The age, sex and consistency of the fecal samples were recorded for each animal. The samples were stored at 4 ˚C and processed within 24 hr.

Light microscopy examination. Giardia cysts were identified microscopically in fecal smears and partially concentrated by sucrose flotation.[20,21] The concentrated cysts were stored in sterile distilled water without adding any preservatives, up to two weeks at – 20 ˚C.

DNA extraction. DNA was extracted by phenol-chloroform-isoamyl alcohol (PCI) on concentrated fecal by sucrose gradient samples according to Rayani *et al.* with some modifications.[22] Also, freeze-thaw was used for cyst wall disruption in some samples. Briefly, 200 µL of sediment concentrated cysts sample and 200 µL 3.00% Triton X100 were mixed and incubated in a water bath at 75 ˚C for 1 hr. Then, 200 µL of lysis buffer and 10 µL of proteinase K were added to 200 µL of homogenate and incubated at 37 ˚C overnight. The parasite DNA was extracted with PCI and precipitated with ethanol. The purified DNA pellets were dissolved in 100 µL of double-distilled water and stored at –20 ˚C for subsequent PCR reactions.

PCR amplification. In the PCR reaction, the 432 bp fragment of the *gdh* genes was amplified using the forward primer (*GDHiF*) 5´-CAG TAC AAC TCT GCT CTC GG-3´ and the reverse primer (*GDHiR*), 5´-GTT GTC CTT GCA CAT CTC C-3´.[14] The PCR amplification was done using a programmable thermal cycler (Eppendorf, Hamburg, Germany). Amplification reaction was modified as follows, the PCR mix consisted of 1X buffer containing 1.50 mM MgCl2 (Cinaclon, Tehran, Iran), each deoxynucleotide triphosphate at the concentration of 100 µM, each primer at a concentration of 0.50 µM, 10 ng of DNA and 2.50 U of Taq DNA polymerase (Cinaclon, Tehran, Iran). Cycling parameters were 10 min at 94 ˚C (initial heat activation step), followed by 50 cycles of 35 sec at 94 ˚C, 35 sec at 61 ˚C and 50 sec at 72 ˚C, with a final extension of 7 min at 72 ˚C.[13] Positive and negative controls were included in each PCR to validate results. Cysts were utilized as the templates for the positive controls and distilled water was utilized as the template for negative controls.

The RFLP of the *gdh* gene. All PCR positive specimens were subjected to RFLP analysis. *G. duodenalis* genotypes were determined by RFLP analysis as previously described.[14] The RFLP analysis was carried out by digesting 8 µL of PCR products with 1.50 U of *NlaIV* enzyme (Vivantis, Vilnius, Lithuania) in 2 µL of 10X enzyme buffer in a final volume of 20 µL for 3 hr at 37 ˚C.[19] The PCR products and restriction fragments were separated respectively by horizontal electrophoresis in 1.50 and 2% agarose gels with ethidium bromide staining. A 100-bp DNA ladder (Fermentas, Darmstadt, Germany) was used as a size marker.

Statistical analysis. The prevalence of *G. duodenalis* infection in cattle was compared based on the different age groups, sex and diarrheic or none- diarrheic groups using the chi-square test. Data were analyzed using SPSS (version 17; SPSS Inc., Chicago, USA). A value of $p < 0.05$ was considered as statistically significant.

Results

Giardia duodenalis cysts were detected microscopically in 9.34% (23/246) of fecal samples (Fig. 1). The number of infected cattle based on age, sex and consistency of the fecal samples is summarized in Table 1. Statistically, there was no significant correlation between infection rate and sex factor ($p > 0.05$). The results indicated that infection with *G. duodenalis* is more prevalent in younger animals than older ones ($p < 0.05$), (Table 1). Fecal samples were classified according to the consistency as diarrheic (17/246) and non-diarrheic (229/246). *G. duodenalis* was detected in 52.94% (9/17) of diarrheic cattle and 6.11% (14/229) of non-diarrheic cattle. Thus, the prevalence of *G. duodenalis* in diarrheic cattle was significantly higher than non-diarrheic cattle ($p < 0.05$).

The PCR amplification. The *gdh* gene was successfully amplified from 23 (9.34%) samples. A 432 bp fragment of *gdh* gene was amplified in the PCR using GDHiF and GDHiR primers (Fig. 2A).

The RFLP method. The PCR-RFLP analysis in all *G. duodenalis* positive samples using *Nla*IV enzyme revealed that 19 samples (82.60%) have the genotype E and 4 samples (17.39%) belong to the subgroup AI (Fig. 2B). Genotype B was not detected in this study.

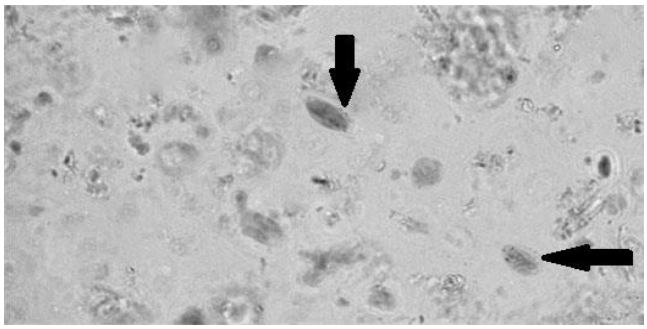

Fig. 1. *Giardia* spp. cysts in the purified fecal samples (Black arrows, 100×).

Discussion

Giardiasis as a zoonotic infection can transmit to humans from infected cattle, sheep, cats and dogs. Zoonotic pathogenic species of giardia found in cattle can infect humans through contact with cattle and their feces and via drinking water contaminated with cattle faeces.[23] There are several reports on the occurrence of *G. duodenalis* infections in cattle in different geographic regions,[7,24-28] but little is known about *G. duodenalis* infection rates and genotypes in cattle in Iran.

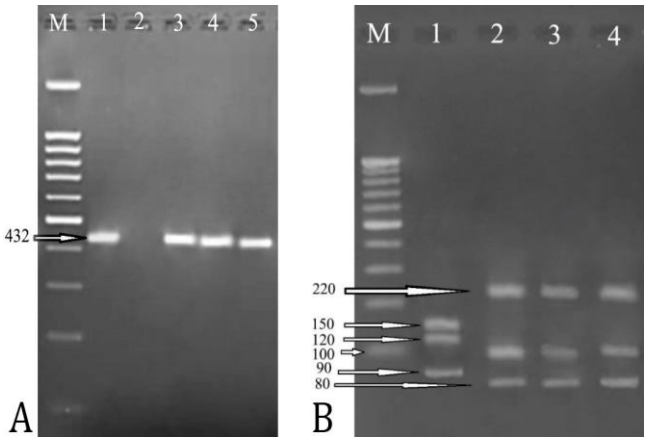

Fig. 2. A) Electrophoretic separation of PCR product from DNA amplified at the *gdh* locus of *G. duodenalis* on an ethidium bromide stained 1.50% agarose gel. Lane M: 100 bp gene ruler (Fermentas); Lane 1: Positive control; Lane 2: Negative control; Lanes 3-5: The PCR products from examined samples (432 bp fragment); **B)** The *NIa*IV digestion of PCR products on an ethidium bromide stained 2% high resolution agarose gel. Lane M: 100 bp gene ruler (Fermentas); Lane1: *G. duodenalis* genotype AI; Lanes 2-4: *G. duodenalis* genotype E.

The present study was performed to detect the *G. duodenalis* from fecal samples of naturally infected cattle in the Urmia, northwest of Iran and determine the genetic characterization of these isolates.

In the present study, the frequency of *G. duodenalis* infection was 9.34% in the cattle. In comparison with studies conducted in other countries, this frequency rate was lower than the infection rates (22.00 to 60.00%) in the dairy cattle.[4,7,25,29-32] Our prevalence was lower than a similar study done in calves in Urmia, Iran.[32] Variations in the prevalence of giardia were probably due to differences in management, climate and study design.[24,33] In the present study, the high prevalence of infection was observed in calves than older ones. The result was along with the previous reports in other countries.[4,29,30] It could be attributed to the development of acquired immunity that may protect animals against disease.[34]

In this study, *G. duodenalis* was detected in 52.94% (9/17) of diarrheic cattle and 6.11% (14/229) of non-diarrheic cattle. Similar to previous study done in this area,[33] a significant association was observed between the presence of giardia cysts and occurrence of diarrhea. It has been shown that 28.00% of diarrheic calves in East Azarbaijan province, Iran were infected with *G. duodenalis*.[35]

Table 1. Prevalence of *Giardia duodenalis* infections by different risk factors in the cattle of Urmia, Iran (n = 246).

Risk factor	Gender		Age		Stool consistency	
	Male	Female	calves(< 1)	cattle (≥1)	diarrheic	non- diarrheic
Total	117	129	92	154	17	229
Infection rate	10(8.54%)	13(10.07%)	16(17.39%)*	7(4.54%)	9(52.94%)*	14(6.11%)

* indicates statistically significant difference compared with the corresponding risk factor at $p < 0.05$.

In the present study, RFLP-PCR with *Nla*IV restriction enzyme was used to identify *G. duodenalis* genotypes. Based on our results, the majority (82.60%) of samples (19/23) were belonged to *G. duodenalis* assemblage E. This finding was in agreement with previous reports in Australia, the United States, Canada, New Zealand and Brazil.[2,4,28,30,32,33]

In this study, zoonotic genotype AI was detected in approximately 17.39% of the positive animals (4/23). Our findings are in agreement with previous studies.[4,7] Besides, our study indicated that the cattle are likely to be potential reservoir of zoonotic *G. duodenalis* in Iran.

It is the first genotypic assessment of *G. duodenalis* in cattle of Iran. Based on our results, the *G. duodenalis* genotype E and A were determined in cattle, in Urmia. The presence of assemblage A indicates that cattle can be a potential source of zoonotic *G. duodenalis* cysts. Further studies in other endemic regions in Iran are required to evaluate the zoonotic importance of giardia in cattle.

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Conflicts of interest

The authors declare that there is no conflict of interest.

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